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CBRN SECURITY MANAGER HANDBOOK

SE-CBRN-URE



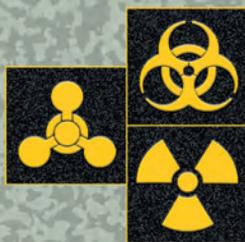
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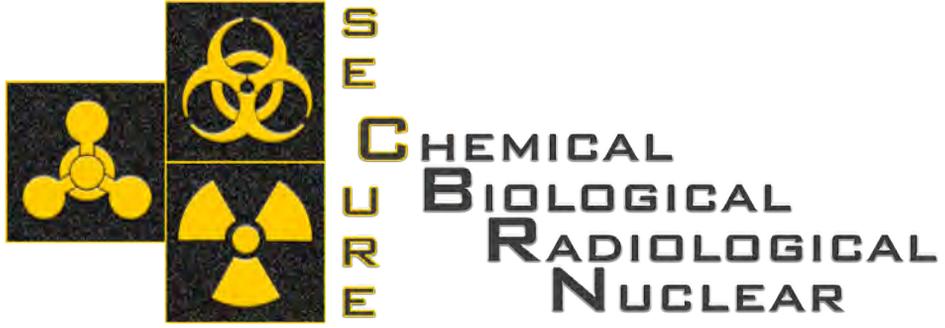
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ABOUT THE PROJECT

Project “Support for European Union action in the field of CBRN security managers education” SE-CBRN-URE is funded by European Commission, The Directorate-General for Migration and Home Affairs (DG HOME) from Internal Security Fund – Police, call “Implementation of the EU CBRN Action Plan, the EU Action Plan on enhancing the security of explosives and the European programme of critical infrastructure protection”. Total project budget – 684 377 Euro (EU contribution – 615 940 Euro). Grant Agreement No. HOME/2015/ISFP/AG/CBRN/4000008459

Acts of chemical, biological, radiological and nuclear (CBRN) terrorism are four new risks that the European public has to take into consideration. With the possibility of these different forms of weapons of mass destruction (WMDs) being used in acts of terrorism, an urgent need has arisen to prevent and combat their use. The use of any of the four types of CBRN weapons in acts of terrorism could open the door to the possibility of destabilization of the European Union, and lead to undermining its economic stability, public security and social integrity. An additional problem is the constant threat of explosives, especially home-made devices, which can be used by themselves or as a release mechanism for CBRN agents.

Consequently, the aim of our project was the creation of CBRN-E Defence postgraduate studies that, upon completion, will entitle graduates as ‘CBRN Security Manager’. Course lasts 12 months and is conducted using a non-stationary model (six meetings of 4–12 days each). After a positive outcome for the basic training, participants are able to choose a specialization, and their choice of specialization will determine the subject of their diploma dissertation. The course culminates in a theoretical and a practical final exam, after which the students receive their diploma certified by the University of Lodz.

Successful implementation of this educational project could significantly improve the anti-terrorist capacity of EU Member States, due to the inclusion of highly specialized personnel (such as incident commanders) on its staff. These professionals will have both the theoretical knowledge and practical skills necessary not only for crisis management in cases of CBRN-E terrorist acts, but also in early detection of CBRN-E risks. Additionally, as graduates, CBRN security managers will strengthen the human capital of the EU at different levels of action response.

PROJECT PARTNERS



The University of Lodz is one of the largest Polish universities located in central Poland, with more than 38,000 students and almost 4,000 personnel across 12 faculties and several interdisciplinary units.

The Faculty of Biology and Environmental Protection of The University of Lodz (Project Leader) is the largest biological faculty in Poland. It has the greatest potential of all the faculties of biological science in Poland, and is a scientific Category A establishment (meaning that it operates at a very good level of scientific activity). Its main scientific disciplines and specialties are: anthropology; evolutionary biology; geobotany; algology; mycology; lichenology; biochemistry; molecular biology; cytobiochemistry; biophysics; thermobiology; radiobiology; cell biology; cytology; cytogenetics; cytochemistry; cytophysiology; plant physiology; animal physiology (neurobiology, neurophysiology); zoology; hydrobiology; ecology; polar biology; environmental protection; microbiology; infectious biology; bacterial immunochemistry and immunobiology; immunology; biotechnology (of microbes, plants); genetics (plants, microbes), and molecular genetics. The intellectual potential of the research teams, laboratory and field equipment of its chairs and centres, as well as the methodical achievements of its staff, enable the Faculty to conduct scientific research at various levels of the organization of organic and inorganic matter. For example, from molecules and cells to organisms, populations, and ecosystems.

Experts from the Faculty are experts in various biological threats, and often work with law enforcements institutions on various CBRN initiatives. The Faculty of Biology and Environmental Protection operates modern laboratory equipment that can analyse pathogens, including through genetic and biochemical means.

The Polish Police (National Police Headquarters) are the uniformed, armed force serving Polish society. Their mandate is to maintain the security of the people and preserve public order. The different departments within the Polish Police force handle various different aspects of law enforcement: criminal, preventive, investigational, aviation, organisational, logistical and technological. The Police also feed the court system in Poland. In the context of CBRN, the Police is a significant element of the national system of identifying, counteracting and responding to terrorist acts. As such, they also comprise units whose remit covers various activities beyond simple maintenance of public order, including:

- Physically combating terrorists—including conduct of negotiations – by the Bureau of Anti-Terrorist Operations of the National Police Headquarters,
- Operational identification of terrorist crimes, by the Central Bureau of Investigation of the National Police Headquarters,
- Safeguarding mass events, supervising the security of critical national infrastructure, by the Chief Police Staff of the National Police Headquarters,
- Social education on counteracting crime, including terrorist threats, by officers of the Preventive Service of police units at the Voivodeship Police Headquarters/Warsaw Police Headquarters level, and at the Municipal/Regional and Poviast Police Headquarters levels by the Prevention Bureau of the National Police Headquarters.

At the strategic and operational levels, there are Polish Police representatives on national and international bodies dealing with the analysis and exchange of information on the threat of terrorist and extremist crimes. In the international context, these bodies mainly include Interpol, Europol, and the Working Group on Terrorism operating within the 3rd Pillar of the EU. At the national level we should mention the Inter-ministerial Team for Terrorist Threats, which has been operating since 2006, and in which the Polish Police is represented by the Police Commander-in-Chief. But regular Police officers have also been working since 2008 in the Anti-Terrorist Centre of the Internal Security Agency. The Antiterrorist Operations Bureau, National Police Headquarters, Poland (BOA) is the central antiterrorist unit in Poland. BOA's main task is combating terrorism, as well as organizing, coordinating and supervising Police activities in this regard.

The Police Academy in Szczytno is part of the Polish police education system. It plays a significant role in training the police executive and commanding staff. The Academy is headed by the Commandant-Rector, who has a representative function and who directly supervises such administrative units as the Rector's Office, Personnel Department, Control Unit and Legal Assistance Unit. The Police Academy offers two fields of studies run by the Department of Administration, and the Department of Internal Security, both of which are headed by a Dean. The Academy has a 60-year history of teaching law enforcement officers in Poland, and is the only Polish educational institution that trains the police executive and high-ranking commissioned-officers. The Police Academy in Szczytno is highly

regarded as a police training centre, both in Poland and abroad. As the only police school in Poland, since 2003 it has been a member of The Association of European Police Colleges (AEPC) and has been actively involved in numerous undertakings as part of the College of European Police (CEPOL). The Academy is also part of the European Police Learning Net (EPLN).

The Military Institute of Chemistry and Radiometry is a Scientific and Research Centre of the Polish Ministry of National Defence, established in 1954. The main scope of its work is scientific research and development projects, and studies in the field of NBC (Nuclear, Biological, Chemical) defence equipment and systems, as well as verification of chemical disarmament processes. The Institute is authorized to issue expertise and opinions as well as certificates for products and technologies falling within their remit, and to carry out equipment testing and personnel training. For scientific-research and implementation purposes, the MICR uses a quality system in accordance with ISO 9001 and NATO standardization documents – the AQAP 2110. Other important parts of the Institute are its testing and calibration laboratories, as well as the Product Certification Body, which is accredited by the Polish Accreditation Centre. The Institute has been appointed by the Minister of Science's Centre for Advanced Technology to coordinate 'The Man, Environment, Anti-terrorism, Protection Against Contamination' programme.

The General Karol Kaczkowski Military Institute of Hygiene and Epidemiology is a state-run body (research institute) of the Polish Ministry of National Defence. Its unique mission is scientific research and detection/identification of agents in terms of CBRN threats. The Biological Threat Identification and Countermeasure Centre of the MIHE, detachment in Puławy with Containment Level 2 and 3 laboratories, is orientated to ward biological threats. As a unique national asset, it serves as a reference bio lab for the Polish Armed Forces (PAF) (listed under NATO AEP-10), and as a biological sampling/identification training facility for civilian and military entities.

The International Security and Emergency Management Institute is a non-profit organisation registered within the Ministry of Interior of the Slovak Republic. Since 1995, the founder and his team have been personally involved in many projects and consulting services in the area of internal security and emergency management, as individual experts. Together, they have recognised many open issues in this area and have identified many gaps that need to be filled.



INTRODUCTION TO “CBRN SECURITY MANAGER”

INTRODUCTION

Contemporary world evolves on many planes, including terrorist threats. Who is a criminal armed with conventional weapons, explosives, attempting to provoke fear, panic and paralysis to manifest their views or achieve other benefits? It is a simplified and common perception of a terrorist seen through the eyes of the shortest century – the 20 century. History of civilisations knows some cases of attempted unconventional warfare, even by using pathogens, as evidenced by the examples listed below:

- 1346 (Theodosia) – the Tatars hurled plague cadavers into the city of Caffa during a siege.
- 15 and 16 century – the conquest of America - Pizarro gives a smallpox-infested gift to the natives.
- 1710 – during the war with Sweden in Peter the First times, Russians hurled plague cadavers into the city of Reval.
- 1767 – a smallpox virus-infested blanket distributed as a gift by the British general, Jeffrey Amherst, to the Native Americans.
- 1797 – an attempt to spread a swamp fever pathogen during Napoleon’s warfare operations at Manuta.

A long list of historical events which involved using naturally occurring potential threats to human organism could be given here. They were made in order to direct their negative impact to the adversary.

The CBRN potential has been and is appreciated by distinguished strategists, which translated into development and classification of a new warfield weapons, namely the Weapons of Mass Destruction (WMD). Used mostly for military purposes, WMD has become well sought-after by terrorist groups. In the era of the so-called “super-terrorism” threats, CBRN is perceived as an asymmetric struggle which does not apply to armed conflicts and is oriented towards destabilising the internal state security system.

The process of preparing for a terrorist attack has gained a new dimension. Apart from the attempt of an illegal acquisition, seizure of hazardous chemical, biological, radiation and nuclear (CBRN) agents, criminal groups put an emphasis on the acquisition of technology, technical knowledge or individuals who make own production of “the CBRN threats” possible.

CBRN refers to hazardous chemical, biological, radiation or nuclear material. While some materials classified to CBRN are used daily for the good of humanity,

its improper use may create hazard for all forms of life and environment, in which we live. On the other hand, a potential size of consequences may be dependent on many parameters (a type of an agent, its quantity, its dispersion method, attack site/place, weather conditions, etc.).

CBRN education was initiated in response to the need to strengthen the internal security potential of the EU states to counteract potential threats that come with use of a hazardous chemical, biological, radiation or nuclear agent for terrorist purposes. A terrorist attack in Tokyo metro may come as an excellent illustration of the need to educate in the CBRN area. On 20 March 1995, Aum Shinrikyō religious sect sprayed a poisonous warfare agent, sarin, in a metro train at Kasumigaseki station. The CBRN terrorist attack caused 12 deaths and affected more than 5 thousand victims, who were injured. The services were not appropriately prepared, lacked knowledge and equipment and, in consequence, the responding rescue services were exposed to a poisonous warfare agent. As a result, 135 members of rescue teams involved in the response operation suffered. Also note that, long after the attack, many persons suffered from illnesses resulting from remaining in sarin-contaminated zone, including depression, breathing difficulties or brain damage.

It is possible that CBRN factors will be used in the aspect of transformation of states, geopolitical conflicts, and religious conflicts as spectacular terrorist attack. Crime may be caused political, religious, economic or national motives. Potential culprits likely to be placed at the heart of a terrorist attack include:

- governmental buildings and complexes;
- politicians;
- public utility buildings;
- critical infrastructure;
- the infrastructure used to transmit chemical compounds classified to CBRN factors;
- complexes/buildings where agents potentially useful for a CBRN attack are manufactured or stored;
- objects of religious cult;
- means of mass transportation;
- (road, rail and water) transports with agents which can be potentially used for a CBRN attack;
- large population concentrations.

One may assume that terrorists will stress the largest possible dispersion of the hazardous CBRN agent, with the use of:

- explosives;
- technical measures such as compressed aerosols, unmanned aircrafts;
- contaminated live organisms.

Potential consequences of a terrorist attack may include:

- human casualties (fatalities, injured, wounded, ill);
- losses in the critical infrastructure;

- interruptions in access to core municipal/communal services such as: environmental pollution, medical/health care, power supply, water supply, ITC, public transport;

- economic losses (costs of decontamination, rebuilding, commercial/trade losses, high costs of treating the ill);

- political consequences (undermining stability of the state security).

In terms of scale, a CBRN incident may be vast and trans-border and, as such, it may require international reaction. In order to understand the essence of the CBRN issue, one must understand their impact on human life, health and the environment.

The first threat described in the CBRN acronym is “C” which refers to chemical compound – they may be used in terrorist attacks due to their toxicity i.e. their chemical properties may cause death, permanent damage to one’s health or temporary incapacity for work. They are divided into chemical warfare agents (CWA) mostly used for the military purposes) and toxic industrial agents (TIA, used mainly in enterprises, households, public and private institutions which ensure satisfaction of community needs). Chemical warfare agents are the basic component of chemical weapons, responsible for the mass nature of paralysis. They are classified on the basis of poisoning symptoms and the goal to be reached.

CWA classification based on the symptom-related criteria:

- nerve agents;
- blister agents;
- pulmonary agents;
- blood agents;
- psychotic agents;
- irritating agents.

In the tactical application context, the CWA may be divided into:

- lethal warfare agents;
- paralyzing warfare agents;
- exercise warfare agents.

Due to high toxicity and long field life, nerve agents are predominantly used in the form of organophosphorous compound such as sarin, soman, VX and blister agents such as sulphur mustard (mustard gas) or lewisite.

The scale and impact of using such poisonous warfare agents during WWI, Iraqi-Iranian war or quite recently (on 4 April 2017) in Chan Schaychun in Syria, chemical weapons may be also used in terrorist attack and Tokyo metro terrorist attack is a good example of such use. However, the majority of the states decided to fully eliminate a potential use of chemical weapons by introducing provisions of the Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction of 13 January 1993.

CWA’s may be also used in terrorist attack and Tokyo metro terrorist attack is a good example of such use. CWA chemical compounds are illegally produced

for such purposes and, in spite of legal restrictions and intensified control; the EU states cannot fully guarantee that they are not acquired by potential terrorists. A potential attempt to smuggle precursor for synthesising CWA into a country which is a potential target of a terrorist CWA attack initiates prevention and deterrence measures in countries. They involve launching a process of modernising the technical equipment used for monitoring and detecting hazardous chemical compounds on border points. The Border Guard is responsible for preventing any illicit traffic of any psychotropic agents, weapons, munitions, explosives, radiation material and hazardous chemical substances across the state border. Anti-terrorist measures taken by states involve not allowing any persons suspected of any terrorist activity into their territory as well as preventing such persons from exiting the country. In Schengen zone, prevention measures are largely based on international collaboration also in the area of exchange of information and joint operations as well as on ensuring tightness of external borders by each Schengen state. The above arises from the system of borders open to all EU members. Such a significant protection-related challenge is also exponentiated by free movement of persons inside Schengen zone, which does not only apply to the states – signatories but also to any nationalities and any citizenship who cross internal borders within the area to which Schengen treaty applies.

A threat of smuggling CWA may arise from the geopolitical situation but also from engagement of some terrorist organisation in an attempt to manufacture such weapons. It is likely that the so-called Islamic States has the technology, know-how and access to the materials which can be used to produce the CWA.

It is easier to initiate a terrorist attack with a use of Toxic Industrial Agents and, while such attack may seem less spectacular, its consequences for human life, health, the environment and the economy are serious.

Such threat may be come from a wilfully caused failure or a catastrophe in industrial complexes (which manufacture or process chemicals). Each industrialised country has many complexes of such kind.

One should be aware that it is possible to cause a transportation (rail, road, sea or air) catastrophe in which terrorist will be interested in unsealing transported toxic industrial agents. The pipeline infrastructure used for transporting TIA may be also the target of attacks classified as CBRN.

To comprehend the scale of the TIA threat in terms of their area coverage, according to the EMERGENCY RESPONSE GUIDEBOOK-2016 issued by the US Department of Transportation, in case of a large leak of chlorine one should assume that contamination will spread within the radius of 275 m from the epicentre of the agent release and the range of the warning zone in the wind direction should be 2.7 km during the day and 6.8 km at night, respectively. For this reason, the issue of a TIA or CWA threat to the population as a result of a terrorist attack applies not only to the epicentre, the potential contamination area (e.g. the site where the railway infrastructure or a power plant is located)

as well as the alarm area i.e. an adjacent area whose population is exposed to the potential risk of the toxic agent.

The second threat included in the CBRN acronym is “B” which applies to potentially hazardous pathogens e.g. micro-organisms, bacteria, fungi, toxins produced by micro-organisms or plants and newly appearing pathogens which may be created through genetic engineering manipulation to enhance their spreading.

The US federal government agency which operating under the Department of Health and Human Services – CDC (Centres for Disease Control and Prevention) introduced 3 categories of the hazardous biological agents:

- Category A – pathogens which are easy to disseminate and therefore result in high mortality rates e.g. anthrax, botulism, and tularaemia.

- Category B – pathogens which are moderately and easy to disseminate, of moderate morbidity and mortality, e.g. Bang’s disease, Melioidosis (*Burkholderia pseudomallei*), Q fever, Glanders (*Burkholderia mallei*).

- Category C – pathogens which are easily accessible and easy to disseminate and, therefore, may cause high morbidity and mortality rate e.g. newly emerging pathogens which may be subject to genetic engineering manipulation for the purpose of more effective dissemination.

Some of potentially hazardous pathogens which may be used in bioterrorist attacks:

- Gram-positive *Bacillus anthracis*;
- Gram-negative *Yersinia pestis*;
- *Vibrio cholera*;
- Gram-negative *Francisella tularensis*;
- Gram-negative *Burkholderia pseudomallei*;
- Gram-negative *Coxiella burnetti* bacteria;
- Gram-negative *Brucella pestis*;
- Gram-negative *Chlamydophila pneumonia*;
- Gram-negative *Neisseria meningitides*;
- Drug-resistant gram-positive *Streptococcus pneumoniae* and *Staphylococcus aureus* bacteria strains;
- Poxvirus variolae smallpox Virus;
- Bunyaviridae family Virus;
- Junin Virus;
- Machupo Virus;
- Marburg Virus;
- Ebola Virus;
- Eastern equine encephalitis Virus;
- Lassa Virus.

Pathogens were used for terrorist purposes for example in the case of salmonella-poisoning salad bars in the Dallas, Oregon, USA, in 1984 by Rajneeshee Cult organisation, resulting in mass poisoning of 751 people. The purpose of the attack

i.e. preventing the local community from voting in the elections, was disclosed one year after the incident. Other examples could be the terrorist attacks with the anthrax-contaminated correspondents on the territory of the USA. They began on 18 September 2001 and lasted for several weeks. Letters containing anthrax spores were posted to several offices of news agencies and to two Democratic senators. They resulted in 5 fatalities and approximately 17 persons becoming ill.

How serious may a terrorist attack with a pathogen be? It took more than 2 years to decontaminate Brentwood post site while the Hamilton post in New York was closed until March 2005. Unofficially, all losses in fixed assets caused by the CBRN terrorist attack are estimated at 1 billion USD or more.

Note that states do notice a potential threat represented by the biological weapons e.g. because of more difficult control. On 10 April 1972, in London, Moscow and Washington the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction, Biological Weapons Convention, BWC) was signed. At present, 178 states are bound by the BWC.

The next type of hazard represented in the CBRN acronym is the RN which refers to a terrorist attack involving radioactive isotopes. WMD nuclear threats may be analysed as special nuclear material and other substances capable of nuclear reaction as well as other radioactive isotopes which may be used in a device designated for dispersion of radioactive contamination. The importance of the issue is illustrated by the Activity of nuclear material intercepted by law and order agencies, which can be potentially used for production of the weapons, in the past 10 to 15 years. According to the International Atomic Energy Agency, since 1993, more than 300 bans on radioactive substance trading have been introduced, including 17 bans on special nuclear material. Special nuclear material is a category of radioactive substances used for production of nuclear weapons, including plutonium, enriched uranium 235 and uranium 233. Huge efforts are taken to ensure the proper control of their transport and storage. In spite of that, cases of intercepting illegally traded nuclear material have been reported. More than 15 kg of highly enriched uranium and 400 g of plutonium was confiscated. Europol estimates that currently, additional 10 to 30 kg of enriched uranium is now being traded on the black market.

Other radioactive isotopes which could be used in the form of dispersion contain some nuclear material but it does not apply to fissionable reactions. Dispersion devices may be built from conventional explosives and a radioactive isotope and their purpose is to disperse radioactive substance causing contamination of the population and its environment. These may be radioactive substances used in medicine and in the industry, orphaned, abandoned or illegally acquired. Hazard in such form may lead to disruption of the public order. Such actions generate enormous costs of decontamination and social panic. The society is governed by stereotypical image of radiation causing fear and a mental block.

After a terrorist incident involving RN, the local community may disapprove using decontaminated sites/ location in spite their successful decontamination. There are millions of radiation sources used on our globe. Many of these sources are weak which translates into an insignificant threat. The International Atomic Energy Agency has a global list of more than 20,000 entities which hold or own radioactive sources i.e.:

- more than 10,000 sources used in medicine;
- more than 12,000 sources used in the industry.

Note that there are countries and regions which leave a lot to be desired for when it comes to the rules for keeping inventory of their radioactive materials. The above-presented numbers illustrate the scale at which radioactive isotopes are used and the potential risk of their sealing or interception for terrorist purposes consequent upon the number. There are several reasons for stealing radioactive isotopes:

- radioactive isotopes stolen for the purpose of illicit trading (for profit);
- theft of some assets (a car, material bringing a high price when sold as scrap metal) containing a radioactive isotope (the perpetrator unaware of a radioactive isotope existing in the stolen goods);
- radioactive isotope stolen to be used for terrorist attacks.

Customs officers, border guard officers and the police reveal many attempts at smuggling and selling stolen sources. State security may be undermined by the radioactive sources which:

- has never been recorded and are unaccounted for (lack of relevant legal regulations in the country or non-compliance);
- had been controlled but their chain of custody was broken due to lack of appropriate supervision (have been abandoned, lost or disappeared);
- have been stolen or removed without an appropriate permit.

For the above-given reasons, the number of orphaned sources globally remains unknown but it is estimated at thousands of pieces.

For security reasons, radioactive sources are transported and stored in special containers which guarantee the minimum exposure to radiation. Such form of protection may attract scrap collectors. From the outside, the container may look as if it were made from high-cost materials, in particular, when its warning labels wore off or are gone. There were cases of persons who, unaware of the risk, in some cases innocent by-standards, tampered with the sources. Such tampering may often cause injuries or even death. In addition, there is a lot of radioactive waste around the world (typically generated by nuclear power plants or by production of weapons), spent atomic fuel and forms of radioactive substances not contained in shielding containers.

At the global scale, there are different programmes supporting countries in their operations in support for counteracting dissemination of radioactive materials. One of them is the Second Line of Defence – SLD Programme of the Department

of Energy (DoE) of the USA. The objective of the Programme is to detect and deter any illicit transport and transfer of nuclear and radioactive material using special RN-detecting instruments. The initiative has the following assets:

- Supporting research, development, production and logistics of special tools for RN detection;
- Organisation of free training and workshop events;
- Handing over, free of charge, of RN-detecting instruments and equipment.

Target users of the SLD Programme are:

- the Border Guard;
- the Customs;
- the Police;
- National Atomic Agencies.

At present, the programme is implemented in 24 countries: Afghanistan, Azerbaijan, Bulgaria, Croatia, the Czech Republic, Djibouti, Estonia, Germany, Georgia, Hungary, Israel, Iraq, Jordan, Lithuania, Mexico, Moldova, the Philippines, Poland, Romania, Slovakia, Slovenia, Tajikistan and the USA.

In the framework of the effort initiated by the Ministry of Internal Affairs and Administration of the Republic of Poland, a Task Force was formed for streamlining the rules for collaboration and managing operations on site of a terrorist incident in case of a CBRN agent involvement, by the virtue of the Decision No. 25 of the Chairman of the Interministerial Terrorist Threat Team of 26 March 2015. Its tasks included:

- revising the existing legal and organisational solutions related to the collaboration and managing terrorist events on site in case when CBRN agents are used and drafting proposed changes/ amendments in this respect;
- preparing recommendations on intensifying collaboration between competent agencies and institutions further to a terrorist incident in case of using a CBRN agent;
- drafting response procedures for competent agencies and institutions further to a terrorist incident in case of using a CBRN agent, taking into account their joint actions and optional scope of their operations depending on their risk assessment.

In view of potential terrorist threats with the use of chemical, biological, radiation or nuclear agents, states have or create their internal security system to counteract these threats or intervene when they take place. Anti-terrorist CBRN operations may be divided into:

- legislative and procedural operations;
- training;
- coordinating operations;
- acquisition of information;
- intervention;
- investigatory operations.

It is necessary to create or revise legislature translating into the organisation of the internal CBRN security system. Lawfully, the state should have a clear and structured division of competence and the algorithm for collaboration of entities and agencies responsible for CBRN anti-terrorist response. Imagine puzzles - with one element not fitting in, the whole image is spoilt. This comparison works here since any legal and procedural non-compliances may eventually result in an operational failure or its incomplete success.

The entire CBRN response system should be coordinated at the inter-ministerial level in order to monitor the system for potential gaps. A model solution is having the National CBRN Coordination Unit delivering the internal policy of the state also in terms of:

- coordinating all in-country CBRN operations;
- setting out the directions for preparing CBRN-response agencies and entities;
- analysing global CBRN incidents, taking into account response tactics used by the intervening agencies;
- supporting training of agencies and entities in CBRN threats and tactics of responding to CBRN incidents;
- collaborating with the academia and science also in the field of R & D programmes and EU projects in support for improving CBRN security;
- collaborating at the international level with institutions, organisations, states in the area of broadly understood CBRN.

An efficiently working CBRN response system also involves knowledge and skills of persons defined as CBRN forces. A broad-range, inter-ministerial training policy is required, unified for the purpose of joint operations of the agencies. The training programme should be a multi-module programme, taking into account groups responding to CBRN incidents i.e. the strategic, tactical and operating level. At each level, they should break down by the specialisation specified for an agency/entity in the national CBRN response system. Acquisition of information/intelligence operations are the necessary element that comes before an effective intervention or makes it possible with a potential prospect of preventing consequences of a CBRN terrorist attack. They are based on own or international sources of information.

An intervention is defined as taking physical actions oriented to the target location of an attack to prevent an actual CBRN crime. It includes: physical detention of the perpetrator, hazard detection, decontamination of detained persons and intervening officers/soldiers, isolation of the hazardous zone and evaluation operations.

In consideration of the most recent terrorist attacks or their failed attempts, with particular focus on mass events, etc., detailed procedures are required to achieve full CBRN security for large groups of people. It is impossible not to mention the importance of stressing security of mass events, important international meetings, conferences and religious events in the context of preventing terrorist CBRN

threats. Correct coordination and collaboration in ensuring anti-terrorist security is the key element of counteracting potential threats coming in the form of physical violence, weapons, CBRN factors and explosives used against people or property with violation of law. Improvement of the rules for coordinating and joint operations in providing anti-terrorist security of important international meetings and conferences and mass events, including improved response to CBRN hazards, is being implemented by Poland under the priorities of the 2015–2019 National Anti-Terrorist Programme.

When providing anti-terrorist CBRN security of important international meetings and conferences as well as mass events, it is necessary to:

- analyse risks, also on the basis of the data acquired by the Special Services, the Police and, potentially, any other competent agency in possession of information important due to the nature of an event;
 - assess risks;
 - indicate competent entity/agency, leading body or form a Task Force;
 - define tasks and their completion dates.

If an entity, the leading body or forming a ministerial or inter-ministerial team competent for coordinating security of an event is appointed or formed, it is expected to perform the following:

- initiating collaboration with the organiser of an event, conference, meeting;
 - analysing risk, assessing hazards;
 - initiating collaboration with competent services according to the division of competence;
 - analysing manpower and equipment necessary to deliver the task;
 - preparing the schedule of works aim at ensuring CBRN security;
 - developing a concept of operations (ConOps) containing, in particular:
 - assessment of the situation and an initial analysis of risks,
 - anticipated scenarios,
 - a threat assessment scheme,
 - operational goal/s and the method to achieve it/them,
 - manpower and equipment of all the services engaged to ensure CBRN anti-terrorist security,
 - tasks to persons responsible for specific area,
 - command and collaboration structure for the entities involved in the ConOps,
 - operation variants – defining the methods to achieve the planned goal of the operation,
 - organisation of connection,
 - organisation of the operational logistics, including equipment and operational technique, uniforms, transportation, medical service/care, providing for needs;

- arranging and structuring measures required to secure an event, preparing and introducing uniform response procedures for the case of a terrorist attack/threat;
- coordinates actions and flow of information related to the tasks to be completed when taking anti-terrorist CBRN operations;
- analysing and assessing own actions in terms of their effectiveness.

On the example of the Polish model of ensuring CBRN security of important international meetings, conferences and mass events by anti-terrorist measures, the role of the Police is to coordinate actions aimed at counteracting terrorist threats with the use of hazardous chemical compounds, biological material (bacterial and viral pathogens), radiation and nuclear material.

CBRN measures also include the following threat detection actions:

- detection of chemical (C) threats, with tactical operations led by the National Fire fighting Brigade, if necessary supported by the National Centre for Contamination Analysis (COAS);
- detection of biological (B) threats where the leading agency for tactical operations is the State Sanitary Inspection, if necessary, supported by the State Sanitary Inspection of the Ministry of Interior, the Epidemiological Response Centre of the Military Forces of the Republic of Poland and the National Fire Brigade;
- radiation and nuclear threats (RN) where the leading entity for tactical operations is the National Atomic Agency, if necessary supported by the National Fire Brigade, the Main Centre for Analysis and Contamination Centre (COAS), the Anti-Terrorist Operations Bureau of the Police HQs (BOA KGP).

The decontamination process is carried out by the National Fire Brigade with the support of the local governor who secures the quarantine site and any potential medical needs.

In addition, in the anti-terrorist security CBRN model, an important role is played by:

1. Analytical and information operations and operational and reconnaissance actions (the lead – the Internal Security Agency). The legal grounds for the Internal Security Agency taking the lead of the analytical and information operations and operational and reconnaissance actions to prevent and counteract terrorism stem from the provisions of art. 21 further to the provisions of art. 5 of the Internal Security Agency and the Intelligence Agency Law of 24 May 2002 (Dz.U. 2016 item 1897). Furthermore, art. 3 of the Counter-Terrorist Operations Law of 10 June 2016 (Dz.U. 2016, item 904) provides that the main responsibility of the Head of the Internal Security Agency is prevention of terrorist incidents. To this end, according to the provisions of art. 5 of the above-mentioned law, the Head of the Internal Security Agency coordinates analytical and information operations taken by institutions and agencies participating in the anti-terrorist security system for Poland who, according to the provisions of par. 3 of the above-

mentioned art. 5 are obligated to forward to the Internal Security Agency, without a delay, any information that serve conducting anti-terrorist operations classified according to the catalogue of terrorist incidents described in the regulation of the Minister of Internal Affairs and Administration of 22 July 2016 on the Catalogue of Terrorist Incidents (Dz.U. of 22 July 2016, item 1092). In addition, on the basis of art. 8 of the same law, the Head of the Internal Security Agency coordinates operational and reconnaissance actions taken by the security intelligence agencies, the Police, the Border Guard, the National Revenue Administration and the Military Police.

2. Warfare operations (the lead – the Police) – a set of operations delivered in a team by armed policemen, officers and soldiers equipped with specialist equipment, using anti-terrorist tactics with elements with the EOD tactics components such as defeating field obstacles and construction locks or neutralising explosive devices. These operations are oriented towards physical fighting terrorist attacks, in particular operations of a considerable degree of complexity as well as performed in the environment exposed to the impact of a chemical, biological agents, ionising and nuclear radiation and explosives.

3. VIP security ops (the lead – the Government Protection Bureau) to ensure security and protection of persons and objects of importance for the good and interest of the state, also in the context of the CBRN hazards.

4. EOD ops. (the lead – the Police) involving, in particular, locating, recognising, identifying, neutralising, removing, transporting and destroying improvised or plant- manufactured explosive materials and devices, taking into account the CBRN factors, which represent a threat for life, health and assets as well as the public security and public order and defeating construction locks and other obstacles by using explosives.

5. Information and press operations (delivered by the lead of the ops) aimed at running information policy, updating the public opinion on an on-going basis on methods of behaving in case of a potential CBRN terrorist incident (starting from notifying competent agencies about such suspicious incident and ending with the general guidelines on how to behave and proceed in specific cases). Through an appropriately run media policy resulting from collaboration of the public entities and the media, the general public may become a partner in recognising and identifying CBRN terrorist threats.

6. Rescue operations (the lead – the National Fire Brigade) understood as taking actions in order to protect life, health, property or the environment as well as liquidation of reasons for fire, natural disasters or another local threat.

7. Operations on the borders of the Republic of Poland (the lead – the Border Guard) by protecting the state border, organising and controlling the border traffic, issuing permits to cross the state border, preventing and detecting CBRN terrorist crimes and prosecuting their perpetrators, ensuring security on board of aircrafts carrying passengers.

The above-presented model solution for CBRN anti-terrorist protection of important international meetings and conferences as well as mass events was worked out on the basis of the experience from securing 2016 NATO Summi and the World Youth Days 2016 in Poland. A full set of innovative solutions has been developed as recommendations by “the Task Force for improving the rules for coordinating and joint operations to ensure anti-terrorist security of important international meetings and conferences and of mass investments, including in relation to the CBRN hazards” formed by the Chairman of the Inter-Ministerial Team for Terrorist Threats (Mr Mariusz Bałaszczak, Minister of Internal Affairs and Administration of the Republic of Poland) with a decision No. 29 of 23 September 2016. Owing to the commitment of the Polish Ministry of Internal Affairs, Poland succeeded in developing and introducing such an invaluable model of organisational and formal arrangements which has a huge impact on the CBRN security.

Many international CBRN agencies which specialise in different hazards work in support for international CBRN security, such as the International Atomic Energy Agency, the OPCW (the Organisation for Prevention of the Chemical Weapons), the international prosecution agencies (EUROPOL, INTERPOL) as well as units and work groups operating at the European Commission. In addition, there are also international initiatives for states, such as the GICNT – the Global Initiative for Combating Nuclear Terrorism. The nuclear terrorist threat is a global issue and an essential element affecting the process of creating internal and external policy of a state. Different states, depending on their potential and commitment, affect the global architecture of the protection system required for combating nuclear terrorism. Further to disproportionate capacities of states to counteract nuclear terrorism, manifesting themselves in the economic development and own expert and technical support, the idea of creating an alliance of countries to combat nuclear terrorism, called the Global Initiative. The Global Initiative was initiated by George W. Bush and Vladimir Putin on 15 July 2006 and consists in voluntary activity of states for the purpose of international cooperation oriented towards combating and counteracting the threat of global nuclear terrorism through:

- forming an union of countries engaged to deliver on the 8 key objectives enabling strengthening each of these countries in the field;
- voluntary actions in support for international exercise and exchange of information on best practices, full integration of independent efforts in combating nuclear terrorism;
- projects oriented towards improving collaboration of field and national units and the private sector with identification of nuclear terrorism combating tasks for each sector.

Appreciating the need for political commitment and systematic work, partner nations listed the objectives accepted by the engaged countries at the first Global Initiative meeting in September 2006, i.e.:

1. When necessary, management, control and physical protection related to the nuclear system and other radioactive materials should be improved.
2. Caring about ensuring security in case of nuclear applications for civil purposes.
3. Detection of nuclear and other radioactive materials by state institutions and entities which are links in the atomic chain to prevent illegal trading of such materials.
4. Preparing specialised institutions for finding, confiscating and controlled interception of nuclear materials and other radioactive materials as well as devices used for their production.
5. Counteracting sublimation of the environment related to the nuclear terrorism support.
6. Creating laws which allow for pressing charges against persons engaged in nuclear terrorism and holding them liable.
7. The ability to conduct investigations in case of crimes related to nuclear terrorism.
8. Observing protection of information related to the Global Terrorism in order to create a hermetic system for transferring knowledge which is essential in combating nuclear terrorism.

Creating and supporting international initiatives is a global pillar in support for actions taken by states on the local area where CBRN terrorism is combated.

CBRN terrorist threat may occur at any time; therefore, the services and agencies responsible for counteracting such attacks must remain vigilant and ready to respond. The above requires an efficient anti-terrorist CBRN system. Legal regulations providing for such incidents, international collaboration, some pre-defined manpower and equipment, including agencies and entities with appropriate technical background come as the core guarantor of CBRN security of the EU. Also note that, in case of such attack, one of the main objectives will be destabilising normal day-to-day life of the society. Building social awareness is of key importance in minimising potential consequences of such incident. It influences the process of overthrowing the stereotypes arising from lack of such awareness, which also translates into minimising symptoms of social destabilisation.

Read the message carried by a quote from the Pope John Paul II: “You must be self-demanding even if others are not demanding toward you”.

Special dedication for M.A.M.

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MODULE I

**MANAGEMENT
AND LEGAL ASPECTS
OF CBRN-E**

1. SELECTED LEGAL ISSUES IN CBRN-E

International terrorism as a social phenomenon is one of the most serious contemporary threats to national and global security. The targets of terrorist and extremist groups might include industrial plants and research institutes (located in cities), that use dangerous substances. A terrorist attack on such facilities might lead to an industrial failure, with effects going beyond their grounds and biohazards causing epidemics.

The essence of terrorism is lawlessness and illegal use of violence in attacks using combat assets, with the aim of enforcing an activity, or intimidating a given community or government in order to achieve political, social, religious or personal goals. In order to maximise the number of victims, terrorists might also use unconventional weapons: weapons of mass destruction (WMDs). In the literature, this is referred to as 'superterrorism'.

Terrorists can obtain weapons of mass destruction and the technology for their production thanks to, for example, funds received from domestic or foreign states, e.g. Iran, North Korea, and Syria. What has also substantially contributed to this is the lack of any control over the weapons of mass destruction stockpiled in the area of the former USSR. In other countries, potential material suppliers include all kinds of chemical plants, biological laboratories, RTG facilities and radioactive waste disposal sites, while the Internet offers unrestricted access to information about the production of such weapons.

Reasons for attempts to use weapons of this kind by terrorist groups include:

- The intention to kill as many people as possible in a terrorist attack;
- The intention to escalate violence through the use of fear, in order to create panic and enhance the psychological effects;
- The desire to gain a clear advantage before negotiations with state governments – political blackmail;
- Ensuring terrorists' anonymity: attack can remain undiscovered for a long time, allowing the terrorists to flee the area, and optionally spread the substances over a larger area;
- Causing economic and social damage.

Weapons of mass destruction (WMDs) were originally classified in the 1950s, during the Cold War, as ABC weapons (Atomic, Biological and Chemical). Today, the modern American acronym NBCR is used: Nuclear, Biological, Chemical and Radiological. These all refer to modern munitions that are lethal or non-lethal to

living organisms and, to some extent, inorganic matter, and act on a large scale, meaning large areas, large numbers, huge striking power with terrifying effects, leading to irreversible changes to the environment. Their agents can be far more effective than conventional weapons, and it is impossible to determine their harmful effects over time.

Mass killing agents released as a result of terrorist attacks might be dangerous to the health and life of people in contaminated or infected areas. It is very difficult to counteract the effects of such incidents and secure all of their potential sources – the greatest allies of terrorists are incompetent authorities, poorly organised rescue services, panic, and low levels of education about such incidents.

1.1. Nuclear Weapon (Laws Covering Nuclear Weapons)

After the Second World War, various other countries (following the United States) sought to obtain nuclear weapons. The atomic countries were joined by the Soviet Union, the United Kingdom, France, and China. As a result of this arms race, the world potentially faced global nuclear war. However, strong awareness of the threat of extermination of human civilisation among the decision-makers of the two largest powers, led to the taking of preventive actions. A significant role in the limiting of the nuclear proliferation and in the reduction of nuclear arsenals was played by the United Nations. Under the auspices of the UN, different working groups, committees and organisations prepared and ratified different treaties on nuclear weapons. The most important of these include:

- The Antarctic Treaty of August 4 1963, which forbade the military use of Antarctica, including the storing or testing of nuclear weapons;
- Partial Nuclear Test Ban Treaty of 5 August 1963, which forbade nuclear tests over ground, underwater and in outer space, but did not forbid underground nuclear tests;
- The Treaty on Principles Governing the Activities of States in the Exploration and Use of Outer Space, including the Moon and Other Celestial Bodies of 7 January 1967, which forbade placement of nuclear weapons in outer space;
- The Treaty on the Prohibition of Nuclear Weapons in Latin America and the Caribbean of 14 February 1967, which forbade the countries of Latin America and the Caribbean any work on the development of nuclear weapons;
- Nuclear Non-Proliferation Treaty (NPT) of 4 August 1963, which forbade the proliferation of nuclear weapons and technologies and conducting any further work in this respect;
- The Treaty on the Prohibition of the Emplacement of Nuclear Weapons and Other Weapons of Mass Destruction on the Sea-Bed and the Ocean Floor and in the Subsoil thereof of 11 February 1971;

- Strategic Arms Limitation Talks I (SALT I) of 26 May 1972, which was the first treaty on the limitation of strategic armaments. It determined the limits for armaments of both superpowers, however, it did not mention limitation of existing arsenals. Signatories: the United States and the Soviet Union;
- The Treaty on the Limitation of Underground Nuclear Weapon Tests of 18 May 1974, which limited underground nuclear tests to a yield of 150 kilotons. Signatories: the United States and the Soviet Union;
- Strategic Arms Limitation Talks II (SALT II) of 18 June 1979, which was the second treaty on the limitation of strategic armaments. Implementation of the treaty's provisions made it necessary to withdraw certain types of warheads. Signatories were the United States, the Soviet Union and the United Kingdom;
- The South Pacific Nuclear Free Zone Treaty of 6 August 1985, which forbade testing, developing or obtaining nuclear weapons by countries from the South Pacific region;
- The Intermediate-Range Nuclear Forces Treaty of 8 December 1987, which banned the possession of short- and intermediate-range missiles (500–5,500 km). All such missiles were eliminated (including the American Pershing missile and the Soviet SS-20 and SS-23);
- Strategic Arms Reduction Talks I (START I) of 5 December 1994, which was the first treaty on the reduction of strategic arsenals. It introduced a limitation on the number of strategic warheads of about 30% of then-current stocks. The signatories were the United States and the Soviet Union. However, on account of its dissolution in 1991, the protocol was signed by Russia, Belarus, Kazakhstan and Ukraine;
- Strategic Arms Reduction Talks II (START II), which was the second treaty on the reduction of strategic arsenals and constituted an entire package of documents. Ratified by the United States Senate in 1996, but not ratified by the Russian State Duma. It introduced further limitations on the number of nuclear warheads (to 3,000–3,500) of both signatories. Pursuant to the Treaty, the use of multi-head missiles (MIR V) in intercontinental ballistic missiles (ICBMs) was also banned;
- Comprehensive Test Ban Treaty (CTBT) of 10 September 1996, which banned the conduct of nuclear tests by all countries owning nuclear weapons;
- Strategic Offensive Reductions Treaty (SORT) of 24 May 2002 (it came into force on 1 June 2003), which concerned the reduction of strategic offensive possibilities. The Treaty introduced the limitation of the strategic arsenals of its signatories to 1,700–2,200 operationally deployed warheads each;
- Strategic Arms Reduction Treaty (START) of 8 April 2010 (it came into force on 5 February 2011), on the means of further reduction and limitation of strategic offensive forces (also referred to as START-3 or New START). This Treaty replaced the provisions of SORT and reduced the number of strategic nuclear missile launchers by half (including intercontinental ballistic missile

launchers, submarines and strategic air forces). The treaty did not limit the number of liquidated missiles.

The fundamental international treaty forbidding the proliferation of nuclear weapons is the Nuclear Non-Proliferation Treaty. It was signed on 1 July 1968. Until 2008, its signatories included 189 countries. Only three countries refused to ratify it: Israel, India and Pakistan, while North Korea signed the Treaty in 1985 but withdrew from it in 2003. The Treaty was concluded for 25 years, and with a decision of its signatories in May 1995, was extended indefinitely. The Treaty was signed by Poland on 1 July 1968 and ratified on 3 May 1969. According to Polish law, it came into force on 5 May 1970.

The Treaty obliges nuclear countries not to provide anyone, directly or indirectly, with nuclear weapons or other nuclear explosive devices or control over such weapons or such explosive devices, and not to assist, encourage, or induce any of the non-nuclear countries to produce or otherwise acquire such weapons or control over such weapons or explosive devices. Furthermore, it emphasises the principle of making available the benefits and scientific information connected with the use of atomic energy for peaceful purposes. Nuclear countries were also obliged to make efforts to prevent the outbreak of nuclear war. Non-nuclear countries were obliged not to accept from anyone, directly or indirectly, nuclear weapons or other nuclear explosive devices or control over such weapons or such explosive devices, and not to produce or otherwise acquire such weapons, and not to seek or accept any help in their production. But the countries that did not sign the treaty have not been excluded from international work in the nuclear field. In certain cases, their situation is more favourable than with the parties to the treaty, as they are not obliged to subject themselves to detailed international inspections.

Disarmament efforts by international organisations and individual states have not stopped nuclear testing. During the Vietnam War and the Cold War, work on a treaty that would regulate the ultimate cessation of nuclear testing was abandoned.

1.2. Chemical Weapons

The first milestone in the codification of the law of war is considered to be the negotiation of the 11 Hague Conventions, which combined the respect for law with the customs of war on land. The 2nd Convention, dated 29 July 1899, and the 4th Convention, dated 18 October 1907, banned in particular the use of poisons or poisoned arms, bullets and materials that might cause superfluous injuries.

Other diplomatic attempts to limit the use of chemical weapons during war led to the signing of the Geneva Protocol in 1925 for the Prohibition of the Use in War of Asphyxiating, Poisonous or other Gases, and of Bacteriological Methods of

Warfare, and the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction in 1972. However, these documents did not specify any ways in which it could be verified as to whether they are followed. In consequence, many countries, despite having ratified the conventions, more or less openly worked on chemical weapons. Thus, creation of a new treaty was proposed that would provide for methods of controlling the spread, stockpiling and destruction of chemical weapons.

On 13 January 1993, one of the most significant documents of international law, the Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction – a.k.a. The Chemical Weapons Convention (CWC), was signed. The Convention has been in force since 29 April 1993 and has been signed by 127 countries.

The CWC is the first treaty in history that assumes complete elimination of the proliferation and use of chemical weapons, which is supposed to be achieved by destroying all stockpiled chemical weapons and by completely stopping production. The Convention obliges its signatories to provide information about chemical weapons they possess and to destroy them, and to provide information about their chemical industry.

The general obligations of the CWC's parties can be found in Article 1 of the Convention, which reads as follows:

1. Each State Party to this Convention undertakes to never, under any circumstances:

- Develop, produce, otherwise acquire, stockpile or retain chemical weapons, or transfer, directly or indirectly, chemical weapons to anyone;
- Use chemical weapons;
- Engage in any military preparations to use chemical weapons;
- Assist, encourage or induce, in any way, anyone to engage in any activity prohibited to a State Party under this Convention.

2. Each State Party undertakes to destroy chemical weapons it owns or possesses, or that are located in any place under its jurisdiction or control, in accordance with the provisions of this Convention.

3. Each State Party undertakes to destroy all chemical weapons it has abandoned on the territory of another State Party, in accordance with the provisions of this Convention.

4. Each State Party undertakes to destroy any chemical weapons production facilities it owns or possesses, that are located in any place under its jurisdiction or control, in accordance with the provisions of this Convention.

5. Each State Party undertakes not to use riot control agents as a method of warfare.

Since 1997, the executive body of the Convention has been the Organisation for the Prohibition of Chemical Weapons – the OPCW, which is based in The

Hague. The Organisation supervises international adherence to the treaty, and is responsible for the implementation of international regulatory provisions and the elimination of global stocks of chemical weapons. A hundred and ninety countries are OPCW members.

Despite international conventions, there is a real danger of countries that have not signed the Convention using chemical weapons, and of their use by terrorist groups.

1.3. Biological Weapons

The role of the law is to support positive and to limit negative aspects of the development of new technologies, minimising threats and maximising their potential benefits. Every effort should be made to ensure that progress is not connected with increasing danger, but is used to improve the people's standard of living. Additionally, modern biotechnology shows enormous potential for improving people's well-being as long as it is developed using proper security measures with regard for the environment and people's health. Considering this, one has to highlight the necessity to make constant efforts to create such provisions that make it possible to fully exploit the potential of biotechnology, while at the same time ensuring protection against potential threats that arise out of its development.

Scientific research into the improvement of medical treatment methods and the prevention of infectious diseases is fully justified. However, the results might also be used for the wrong purposes. Some states or terrorist groups, under the pretext of undertaking scientific activity (to create vaccines against infectious diseases), use the developments of biological sciences, including microbiology and genetic engineering, in an attempt to create weapons of mass destruction. The possibility of obtaining and using highly infectious micro-organisms or their toxins for bioterrorist purposes makes it necessary to exercise strict control over such research – not only over materials that could be used as weapons, but also over the possibility of such agents being obtained by non-governmental groups. This security should ensure the prevention of the production and stockpiling of biological weapons, and its effectiveness should be guaranteed by signed international agreements forbidding the use of biological weapons.

The first attempt to develop regulations on biological weapons was the Geneva Protocol, signed on 17 June 1925, which banned the use of biological and chemical methods of warfare. The guarantee for the provisions of the treaty was the obligation of such countries as the United Kingdom, France, China and the United States to retaliate against countries that break the established rules by deciding to use biological or chemical weapons. The treaty was signed by 108 countries, and Poland ratified it on 4 February 1929.

The Geneva Protocol bans any use of bacteriological methods of warfare during armed conflict. Today, this ban is a commonly-binding standard of international law, which has been confirmed by numerous resolutions of the UN General Assembly. However, while it covers the use of bacteriological weapons during war, it does not cover activities preceding preparations for war, meaning research on biological weapons, their production and stockpiling. As such, it was necessary to sign an agreement that would also cover peacetime. Consequently, on 10 April 1972, the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction (a.k.a. the Biological Weapons Convention – BWC) was signed, and it was ratified on 26 March 1975. The most important obligations of the state parties to the BWC are as follows:

- Article I – Each State Party to this Convention undertakes never under any circumstances to develop, produce, stockpile or otherwise acquire or retain: microbial or other biological agents, or toxins whatever their origin or method of production, of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes; weapons, equipment or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict.

- Article II – Each State Party to this Convention undertakes to destroy, or to divert to peaceful purposes, all agents, toxins, weapons, equipment and means of delivery.

- Article III – Each State Party to this Convention undertakes not to transfer to any recipient whatsoever, directly or indirectly, and not in any way to assist, encourage, or induce any State, group of States or international organisations to manufacture or otherwise acquire any agents, toxins, weapons, equipment or means of delivery.

- Article IV – Each State Party to this Convention shall take any necessary measures to enforce the BWC's provisions.

- Article V – The State Parties to this Convention undertake to consult one another and to work together in solving any problems that may arise in relation to the objective of, or in the application of the provisions of, the Convention.

- Article VI – Any State Party to this Convention that finds that any other State Party is acting in breach of its obligations deriving from the provisions of the Convention may lodge a complaint with the Security Council of the United Nations.

- Article VII – Each State Party to this Convention undertakes to support or provide assistance, in accordance with the United Nations Charter, to any Party to the Convention that so requests it, if the Security Council decides that such Party has been exposed to danger as a result of violation of the Convention.

- Article X – The State Parties to this Convention undertake to implement the above provisions in a manner that encourages peaceful use of biological sciences and technology.

The provisions of the Convention also cover weapons that might be produced using genetic engineering and other developments of modern biotechnology. Consequently, the notion of 'black biotechnology' was introduced. This refers to the use of biotechnology in the context of such threats as biological war and bioterrorism to harm people, animals and plants. Activities were also undertaken based on other international agreements, to control black biotechnology and compliance with the ban on the use of biological weapons. One example of this is the so-called Australian Group (AG), which involves the cooperation of 41 countries (including Poland), and the European Commission. The aim of the Group is to minimise the threat of the spread of biological and chemical weapons through coordination of its members' export policies. This is achieved thanks to Common Control Lists – lists of goods and technologies that, because they can be used in the production of biological or chemical weapons, are subjected to special export control. The biological agents on the Australian Group's lists also include genetically modified organisms (GMOs). Moreover, the state parties to the Biological Weapons Convention undertake to submit to the UN annual reports on the outbreak of epidemics, on facilities conducting research on the methods of protection against biological weapons, scientific conferences organised in specific facilities, and the exchange of information and scientists.

The Convention was the first multilateral treaty that effectively banned developing, producing, acquiring, transferring, stockpiling, and using biological and toxin weapons. However, the BWC does not forbid conduct of research into, and improvement of, agents that ensure protection against such weapons, as such research is necessary to produce new drugs and protective agents. Thus, it makes it possible to conduct secret activities and makes it difficult to verify the compliance with its provisions.

Another international agreement – and the first to be directly connected and devoted in whole to the protection of the environment and human health against the potential dangers of modern biotechnology – is the Cartagena Protocol on Biosafety (CPB), which was drawn up on 29 January 2000 in Montreal. The Cartagena Protocol covers modified organisms being the products of modern biotechnology. These include organisms the characteristics of which pose a threat to the health of people, animals or plants, and which could be used as biological weapons.

There are also other initiatives aimed at preventing the use of, and controlling the production of, biological agents that could be used as biological weapons. However, these are mostly informal.

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2. CRISIS MANAGEMENT

Citizen safety is one of the main responsibilities of the State. State authorities should be prepared to protect people and critical infrastructure at all times, especially during disasters, accidents, or terrorist situations.

2.1. Basics of crisis management

2.1.1. The essence of crisis management

A crisis situation is a situation adversely affecting the level of safety and security of people, property and the environment, and causing significant restrictions on the operation of public authorities, if they are inadequately prepared and lacking capabilities and resources.

In crisis management, some of the work of the authorities can touch on elements of national security, but mainly consists in preventing crisis situations, preparing to assume control of crises through planned actions, reacting to crisis situations, restoring order and repairing the original character of the public infrastructure, as necessary.

Crisis management is understood as a complex of the activities taken by the competent authorities to control the crisis, as well in developing preventative measures. This involves several key principles:

- The principle of primacy of the territorial authorities – decisions should be made at the lowest level of the administrative division in the affected area;
- Single-person decision-making and responsibility – decisions (commands) should be made by a person at a high level of local government in the affected area;
- The principle of adequacy – the authorities at the lowest level of administration in the affected area should be competent first-responders;
- The principle of universality – all of the citizens in the affected area should be prepared to help the authorities deal with the crisis, with their local authorities able to ask everybody to help (through administrative decisions, decrees, etc).

2.1.2. Crisis management cycle

Activities in crisis management should be made as effective as possible. Proactively, public authorities should not wait until a crisis situation occurs – it is very important to reduce the probability of occurrence of threats and to reduce the potential effects. One of the most important attributes of crisis management is its division into four phases – Prevention, Preparedness, Response and Recovery. In the holistic view, these phases involve whole actions leading to elimination of threats, recovery from the threat, and development of the awareness and preparedness of citizens for further threats.

The objective of the **first phase** (Fig. 1) is to focus on preventing incidents before they happen. It is very important to be able to determine the symptoms of trouble, and thus initiate an appropriate response. This phase involves people who can identify danger, assess its risk and advance a response plan. This is the time for improving environmental protection, especially through local government policy. In this phase, the authorities can build people's knowledge of threats and raise awareness in local communities through public education.

The prevention phase includes:

1. Identification of threats;
2. Searching for the source of threats and recognition of risks;
3. A process of describing risks;
4. Analysis of the previous response phase and answering questions about it;
5. Analysis of the previous recovery phase;
6. Analysis of planning documents – making necessary changes as a result of the response and recovery phases;
7. Updating lists of threats – making necessary changes as a result of the response and recovery phases;
8. Updating of databases (addresses, materials, medical information, fuel etc);
9. Development of new educational programs.

Threat identification requires data to be collected and analysed. This can be done through:

- Analysis of statistical data;
- Analysis of historical data;
- Expert assessments;
- Field studies;
- Assessment of the international situation;
- Mathematical modelling;
- Analysis of data from threat monitoring systems;
- Analysis of trends;
- Examination of historical cases (case studies, 'lessons learned' etc.);
- Environmental diagnosis, etc.

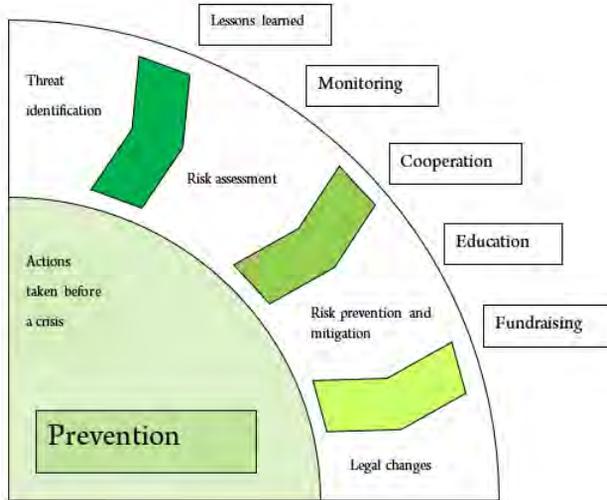


Figure 1. The first phase – Prevention

The process of determining sources of threats and recognizing risks is the first step in risk assessment. Threats can develop in several different ways, depending on, for example: the speed of the emergency services' response; the type of weapon used and the method of its release; the time, location and weather, and the preparedness of the authorities (their coordination, management and level of support, etc). Consequently, it is necessary to specify almost all of these factors in the prevention phase.

In terms of deciding whether a risk is acceptable or not, there are four categories:

A – Acceptable: no additional security measures are required; the current solutions and their assigned capabilities and resources, as well as their monitoring processes, are all strong.

T – Tolerable risk (permitted): alternatives must be considered, whether they be the introduction of small changes, legal or functional, and whether or not they will improve safety or the public's feeling of safety.

ToC – Tolerable on Condition: additional security measures should be introduced within the next six months, and existing solutions should be improved.

U – Unacceptable: immediate action should be taken to enhance security, and new solutions should be introduced.

Bear in mind that there are two possible areas of risk: risks resulting from actions taken directly by a person, and risks resulting from processes occurring in the human environment. This is particularly important when analysing risks from the point of view of CBRN incidents.

The concept of ‘Lesson learned’, which we refer to in this text, should be understood as an activity or methodology based on experience gained from evaluating projects, programmes and policies that are abstracted from specific circumstances to broader, more generalised situations. Frequently, the process of determining what lessons have been learned from experience can highlight strengths and weaknesses in preparation, design, and implementation that then affect performance, and the outcome and impact of future incidents.

Finally, it is important to note that there is no gap between the first phase (Prevention), and the second phase (Preparation).

Exercise 1

Working in groups of four, think about CBRN threats. Choose one and discuss:

1. Have any lessons be learned about this type of threat in your country?
2. What can we do to prevent or mitigate the risks posed by this threat?
3. Present the results and conclusions of your group discussion.
4. Are these conclusions positive, or not?

The **second phase** is the preparation phase (Fig. 2), and is dedicated to planning for incidents. The most important goal here is to prepare people and resources properly for direct use in the next (third) phase. All activities are focused on arranging and developing crisis management plans and improving the different documents needed to properly respond to diagnosed threats. In this phase, the authorities are focused on minimising potential damage or increasing public endurance to terrorist threats. It is the likelihood of the occurrence of threats and incidents is the centre of attention here.

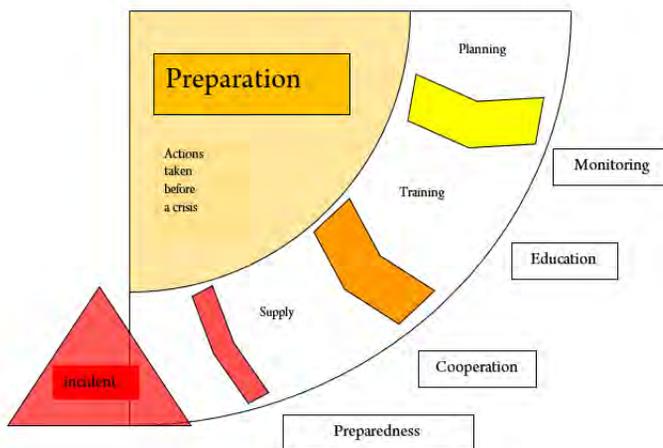


Figure 2. The second phase – Preparation

The principal parts of the preparation phase are:

1. Planning;
2. Preparation and implementation of response procedures;
3. Training of personnel in best procedures for dealing with threats;
4. Preparation of communication systems;
5. Preparation of logistics support, its assessment and supplementation (if required).

Some of the tasks involved are common to both the prevention and preparation phases. For example:

1. Continuous monitoring of sources of threats, and recognition of risks;
2. Educational activities by local authorities, NGOs, rescue services etc.;
3. Development of the cooperation between local authorities, state agencies, emergency services, NGOs etc.

Part of the preparation phase involves assigning responsibilities to various institutions and authorities in the event of an incident. There are three categories of participant, in terms of assigned responsibility:

- Leading institution (L) – The institution or authority tasked with preventing and combating threats. This is usually an executive authority in terms of actually reducing the level of risk and responding to the threat. There is only one Lead.
- Coordinating institution (C) – The institution or authority whose task is to coordinate (when needed) the activities of support and rescue entities involved in responding to crisis situations.
- Auxiliary institutions (A) – The institutions or authorities involved in the efforts to reduce the level of risk and respond to the threat.

The result of all of this analysis and preparation will be a set of tasks for, and responsibilities assigned to, the various participants involved in crisis management. This will take the form of a 'security matrix' (Table 1).

Table 1. Part of a security matrix

No.	CM Participant	Radiation	Infectious diseases	Hurricanes	...
1	Voivode	L	L	L	
2	Fire Service	A	A	A	
3	Police	A	A	A	
4	Ambulance Service	A	A	A	

A correctly prepared security matrix allows for determination of synergies between crisis management participants – whether a given institution or authority will, in a given scenario, fight a threat on its own or together with another authority or institution.

The **third phase** of crisis management is the response phase (Fig. 3). This begins when an incident occurs, and continues throughout it until the crisis/threat has been nullified. The goal of all actions taken in the response phase is saving the lives and health of people, property and the environment (including rendering aid to the injured and trapped). It is also very important to minimise economic damage and stop subsequent crime (theft, looting, public disorder, rioting etc.).

The practical work of the response phase lies in defining the priorities for responding to the threat, and to finding ways of minimising their impact. Existing CM plans and responses are put into action. The response phase includes:

1. Coordination of rescue and security measures;
2. Activation of necessary procedures and security entities;
3. Monitoring of current and subsequent threats and their consequences;
4. Implementation of decisions on the use of forces and means, with potential revision of plans for their use;
5. Coordination of the activities of the involved institutions and authorities;
6. Coordination of security and rescue services;
7. Coordination of the logistical protection of people affected;
8. Opening of information points for the population;
9. Introduction of necessary legal solutions.

Ultimately, the nature of the tasks carried out in the response phase is directly determined by the nature of the crisis, the extent of its effects and damage.

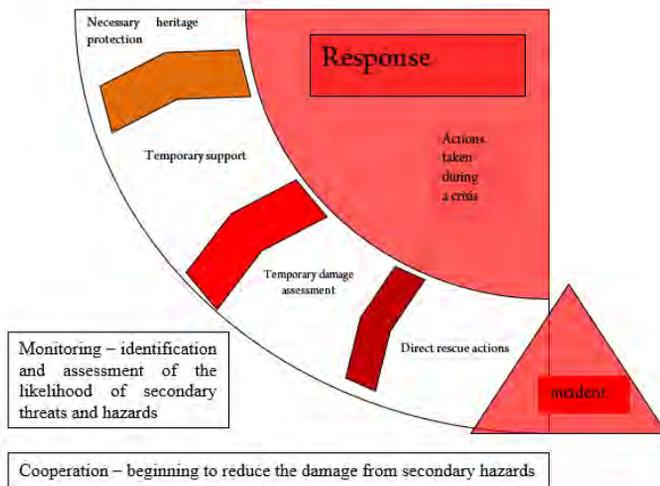


Figure 3. The third phase – Response

The **fourth phase** is the recovery phase (Fig. 4). The recovery phase begins when the activities of the response phase – to rescue and protect people, property

and the environment during a crisis – have run their course. Due to the nature of a crisis situation and its effects, this phase can have the longest duration. It can even overlap with the phases of prevention and preparation in the new crisis management cycle.

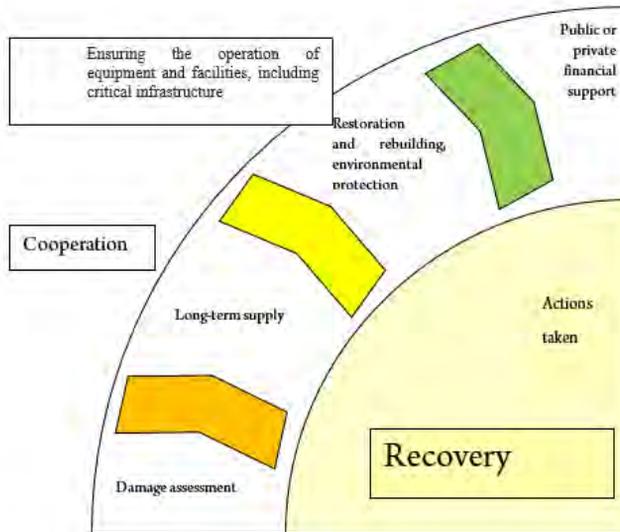


Figure 4. The fourth phase – Recovery

The most important tasks in the recovery phase are:

1. Damage assessment;
2. Running individual and collective aid programs for the affected populace;
3. Mobilizing financial assistance;
4. Ensuring the proper functioning of infrastructure, especially critical infrastructure.

Reconstruction after a crisis event is also an opportunity to correct previously made mistakes ('lessons learned'). It also allows for legislative work to improve security in the future.

2.1.3. Planning

In crisis management planning, the goal is always to secure the safety of the citizenry. The popular notion is that a CM plan is a list of definitive actions that should be taken in various crisis situations. But crisis management is a never-ending process, and so planning for crisis situations also never ends. A good CM plan should not be an all-encompassing document, as this creates false senses of security and preparedness. Additionally, it is not possible to anticipate every

threat. In fact, as soon as a CM plan is written, it begins losing its effectiveness. Furthermore, when the authorities need to implement their CM plan, they will find their options restricted from the outset – a CM plan can also be a barrier to flexibility.

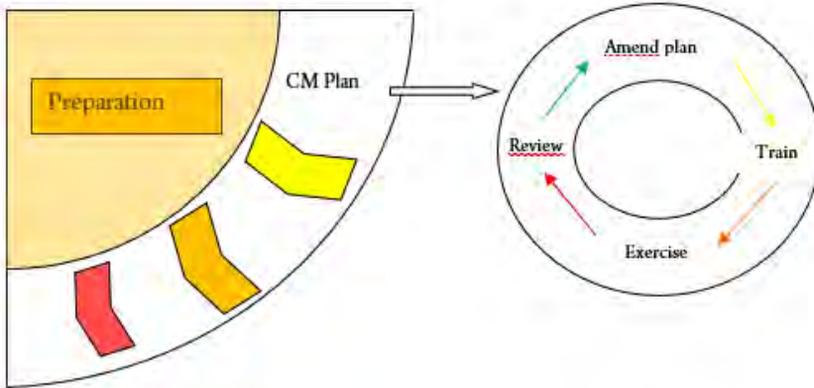


Figure 5. The preparation phase with the CM planning cycle

To begin thinking about a good plan, several important questions must be asked:

1. Why are making a CM plan?
2. Who or what necessitates having a CM plan?
3. Who is going to create the CM plan?
4. What has to go into it?
5. Do we need to test the plan?

And so on.

Effective crisis management planning is based on six activities:

1. Anticipation – What are the risks and threats out there? What new threats and hazards could arise? Having identified them, their potential impact must be assessed in order to effectively inform the level of planning needed to mitigate or eliminate them;
2. Assessment – Quantifying and analysing these threats by risk. A well-conducted risk analysis avoids the phenomenon of ‘over-planning’ which can paralyzed or choke the action when it needs to be taken;
3. Prevention – how to reduce the risk or prevent the incident from happening;
4. Preparation – training and exercises;
5. Response;
6. Recovery – return to normality.

CM plans need to satisfy a number of criteria:

1. There must be clear ownership of the plan;
2. The plan's actual, physical layout must be clear and easy to read;
3. The plan must be concise and to the point;
4. Roles and responsibilities must be clearly defined;
5. The plan must be revised and tested regularly;
6. All participants in the plan must be trained and regularly briefed.

A CM plan should have three parts:

1. The main plan, which lists:
 - a) A description of likely threats and a risk assessment of their occurrence in your country (including threats to critical infrastructure), with risk maps and a map of potential threat locations,
 - b) The responsibilities of the participants in crisis management procedures, in the form of a security matrix,
 - c) The capabilities and resources marked for use in crises.
2. A description of how to proceed in crisis situations:
 - a) Tasks related to threat monitoring,
 - b) Procedures for activating the necessary capabilities and resources marked for use in crisis situations,
 - c) Procedures for emergency responses, descriptions of what to do in crisis situations.
3. Functional annexes:
 - a) Procedures for initiating necessary in a crisis management situation,
 - b) Organisation of communications,
 - c) Organisation of threat monitoring, warning and alarm systems,
 - d) Rules for informing the population about threats and procedures in case of a crisis,
 - e) Procedures for evacuating endangered areas (this can also be a separate plan),
 - f) Rescue, medical care, and post-crisis social and psychological support procedures,
 - g) Procedures for protection against area-specific threats,
 - h) A list of all necessary signed contracts and agreements.

Exercise 2

Draw a 'bow-tie' diagram (as shown below) to visualise risk management and communicate the context of the controls in place, in your country. Identify the causes and potential consequences of a crisis situation (for example, CBRN). Try to identify what could help prevent dangerous situations from getting out of hand, and identify the indicators that will help to track potential changes.

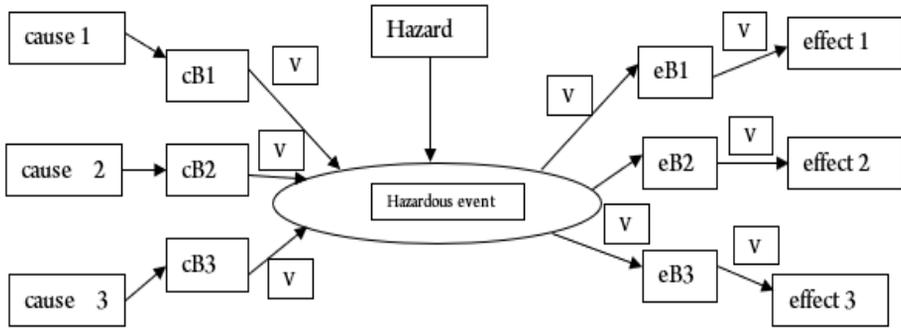


Figure 6. Bow-tie diagram: B – barrier; c – cause; e – effect; V – variable (control indicator)

Exercise 3

Based on exercise 2, discuss the causes of threats/hazards. Include people (crisis participants), places, times, authorities, emergency services, etc. Think about the variables behind the causes, such as the emergency services, their equipment, training, responsibilities etc.... (up to 5 items per variable). Then, using the identified items and variables, create a table like this:

Causes /variables	Small problem ☺	Middling problem ☹	Big problem ☠
Services: – Equipment – Training – Command			

As you can see, crisis management plans form the basis for the response of the various authorities and institutions involved in securing the safety of citizens in a crisis. And as discussed earlier, in the section on the fourth phase of crisis management, CM plans identify the key institutions and authorities involved in crisis response. These are categorised in one of three ways:

– **Leading institution (L)** – The institution or authority tasked with preventing and combating threats. This is usually an executive authority in terms of actually reducing the level of risk and responding to the threat. There is only one Lead.

– **Coordinating institution (C)** – The institution or authority whose task is to coordinate (when needed) the activities of support and rescue entities involved in responding to crisis situations.

– **Auxiliary institutions (A)** – The institutions or authorities involved in the efforts to reduce the level of risk and respond to the threat.

Exercise 4

Working in groups of three, think about the institutions and authorities in your own countries. Which of them would be Leading, Coordinating or Auxiliary in a given crisis situation? Make a list of your answers and compare it with your colleagues'. Are there any differences? If so, why?

2.2. Crisis management system

The safety of a nation's citizens is the main purpose and responsibility of the State. Protection of the citizenry is achieved through defence of the population and critical infrastructure during times of crisis, disaster, large-scale destruction or other extreme incidents.

National and civil protection systems are part of a state's national security infrastructure. Part of this is a crisis management system (CMS), which is a system for handling emergency responses, controlling them by means of pre-planned actions, responding to the occurrence of threats and, later, restoring comprised infrastructure and resources. A CMS is designed to cover both emergencies and long-term operations. It integrates information with decision-making processes and a set of necessary security matrices. The purpose of a CMS is to neutralise or eliminate crises and threats.

When comparing the crisis management systems of different countries, it is almost impossible to find two very similar. Every country has its own CMS, tailored to its own unique needs and abilities. However, beyond the specific there are some components of a CMS that are common to all nations:

At the top level of planning for the protection of people and infrastructure, common elements include:

1. Population protection systems as part of the national security infrastructure;
2. Unified systems of observation, early warning, announcement and management in crisis situations, with integration with other security forces;
3. Principles of resource management to mitigate weaknesses and improve reactions and post-crisis restoration processes;
4. Public Private Partnerships;
5. Use of civil organisations;
6. Effective international partnerships;
7. Use of the most effective combination of regular and voluntary groups.

At the national government level, common elements include:

1. Formulation and conduct of a national policy on common leadership in crisis management;
2. Development of risk evaluation procedures;
3. Definition of the legal persons and firms assigned roles in planning, preparing and carrying out activities for/in a crisis;
4. Signing international and bilateral agreements on information sharing, assistance and mutual cooperation;
5. Coordination of educational activities and training exercises, scientific research and technical developments related to the crisis management system;
6. Implementation of a national crisis information system.

At the Crisis Management centres' level, common elements include:

1. Twenty-four hour oversight of the flow of information for crisis management needs;
2. Preventive control – a complex of measures ranging from analysis and classification of risk, to development and implementation of programmes and measures preventing and mitigating the consequences of crises on health and life, nature and the national economy;
3. Response coordination – the fundamental purpose of a CM centre;
4. Flexible planning that is often actualised immediately;
5. Monitoring of potential threats and analysis and evaluation of threats and their development;
6. Warning;
7. Oversight;
8. Cooperation with various NATO and EU units, and other international organisations and authorities;
9. Conduct of training and exercises in crisis management, with participation in national and international field exercises.

At the regional government level (the middle tier of crisis management), common elements include:

1. Coordination and leadership of crisis management in the region;
2. Organisation and oversight of crisis management plans in the region;
3. Coordination of crisis management tasks between regional organisations/ authorities;
4. Responsibility for maintaining crisis management systems;
5. Preparation and implementation of information and warning systems.

At the street/community level (the lowest level of crisis management), common elements include:

1. Organisation and implementation of crisis management in the community;
2. Preparation of evacuation/safety plans;
3. Organisation and implementation of rescue teams, and fund-raising for the prevention and mitigation of crises;
4. Organisation and implementation of the evacuation of local residents etc.

As an example of a European system, Figure 8 below shows the Polish Crisis Management System.

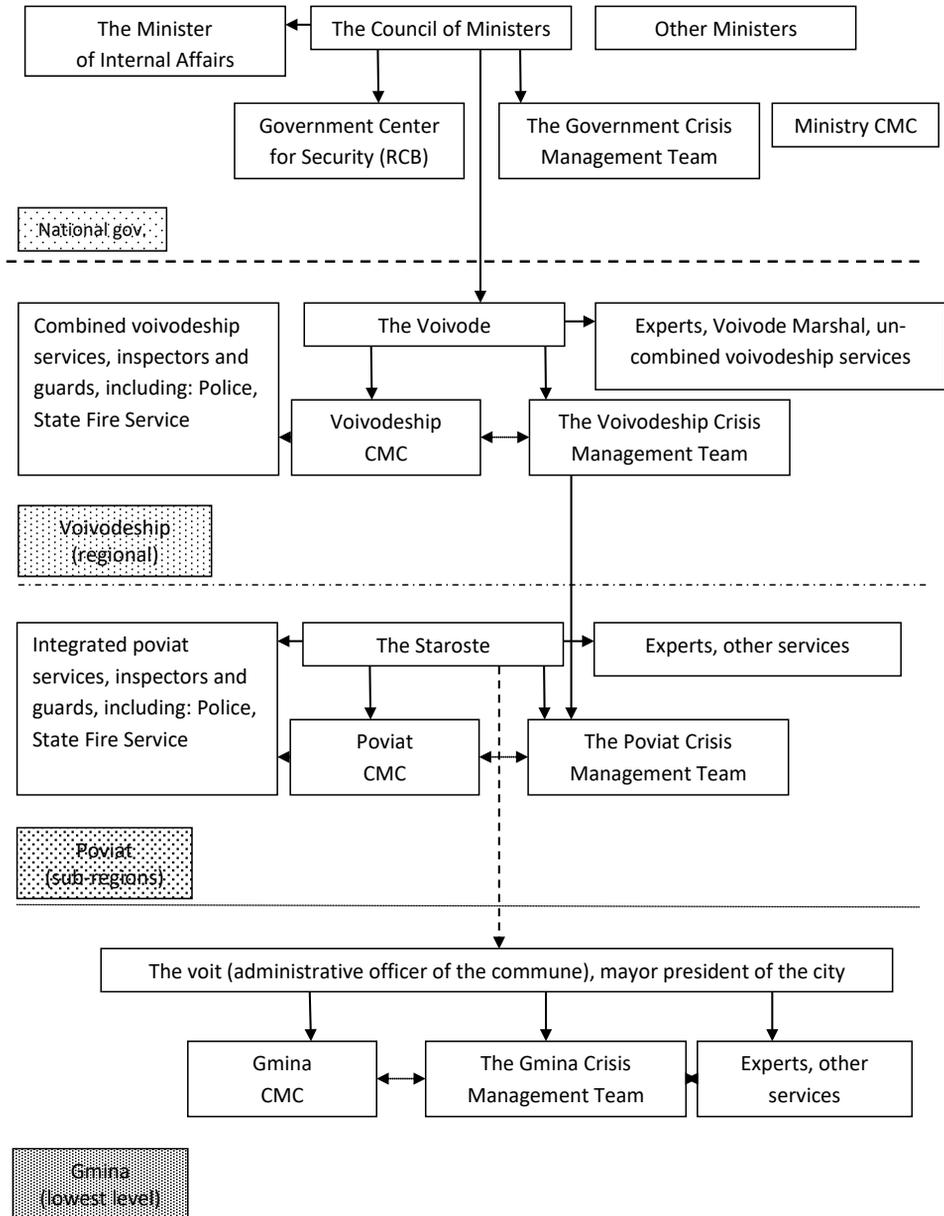


Figure 7. Structure of Poland’s Crisis Management System (based on The Act on Crisis Management of 26 April 2017)

The Government Centre for Security ('RCB' in Polish) is the top level of planning in Poland, established at the national level. The RCB provides the service for the Council of Ministers, The Prime Minister and the Government Crisis Management Team. Ministers and regional authorities then implement the internal security measures set up their own Crisis Management Centres (CMC).

The obligation to initiate crisis management procedures is born by the authority that first receives the information that a crisis/threat has, or will, occur. This information should be immediately shared with the higher level authorities.

Exercise 5

Think about your country.

1. How is its crisis management system structured?
2. Draw a diagram of the system.
3. Discuss the differences.
4. Based on a SWOT analysis, determine the Strengths, Weaknesses, Opportunities for and Threats to the CMS in your country.

2.3. Response in crisis management

Fortunately, crisis situations can be recovered from, giving affected communities the opportunity to prepare for the possibility of further incidents. The best preparation is training and practical exercises.

The competence of the authorities and emergency services is very important in crisis management. The knowledge, experience and level of training of the decision-makers are also important. Training of crisis management participants should include the decision-making process, knowledge of legal provisions and the use of available tools and technical solutions. Another important part of preparedness is creating new trainers; teaching how to train others to handle crisis situations.

Training should not gloss over or avoid the mistakes and shortcomings of crisis management bodies, nor should it be perceived simply as a 'show' without clear goals, skipping over the realities and experiences of crisis situations. Instead, CM training should be designed to ensure that participants are fully prepared to make difficult decisions in stressful, complex situations. Apart from the primary goal of instilling individual and organisational competences, CM training should also enable the validation of existing procedures and action plans in crisis situations. In crisis management training, the following types of participants are distinguished:

1. Executives of government and self-government;
2. Managers and staff of administrative units, including shops and businesses assigned roles in crisis situations;

3. Employees of academic crisis management bodies.

The people and experts who actually arrange for and run training sessions are usually the same people responsible for crisis management at a given level (national, regional, community, etc). They are usually heads of their level of administration (managers, executives, etc), and it is part of their job to arrange for appropriate assistance and resources to assist the decision-makers in a crisis situation.

Training should be planned on the basis of an analysis of needs. This analysis should include: possible threats; associated risks; the characteristics and circumstances of the entity for which the analysis is being made; the executives' knowledge and available resources, and any identified deficiencies in the entity's crisis management plans. A good training plan should be based on a well-formed analysis of these needs, and notification of the training should clearly state the subject of the training, the date of its conduct, the persons/teams required to participate in the training, and the trainers who will be conducting it.

Along with regular training sessions, another highly effective form of preparation for all crisis management entities are field exercises. 'Real-world' exercises allow for comprehensive acquisition and maintenance of required levels of knowledge and skills by the staff of public authorities, institutions and aid organisations assigned roles in crisis response situations. Exercises create the conditions necessary to properly familiarise participants with possible crisis situations, the different variations of their roles in those situations and how to select the most appropriate response to each situation.

Exercises should be conducted periodically at all levels of crisis management and include all involved management bodies (decision-making, consultative, administrative, etc).

Exercises should be used to close out preparatory cycles, meaning that they should be run after the necessary training sessions have been conducted, so that they can also be used to check the effectiveness of the training. In this way, the exercises can be used to check the actual preparedness of CM participants for implementation of their tasks in a crisis.

Exercises should not be designed to be unnecessarily complicated. If participants spend most of their time trying to understand what's going on around them, they will not be concentrating on their objectives. As such, some elements of a real-world exercise should be invisible to those being tested. The principles of well-planned and executed field exercises include the following:

- The principle of **realism** – creating conditions as close to how they would really be as possible;
- The principle of **continuity** – ensuring the appropriateness of the objectives during the exercise;
- The principle of **confidentiality** – as far as possible, the content of the exercise must remain unknown to the participants, so as to properly emulate the unpredictability of a real situation;

– The principle of **safety** – despite the nature of a real crisis situation, exercises should not actually endanger the health and safety of the participants and property involved.

In the organisation and conduct of field exercises, a number of different people are involved:

- Exercise leader/commander;
- Exercise team/staff and managers;
- Back-up/support team;
- Observers/‘referees’ to guard against cheating or short-cuts;
- Security/assistants;
- Actors and other ‘inside’ personnel (playing victims etc.).

The first step in creating any good, effective CM training sessions and field exercises is to set out the concept. This should consist of:

1. Legal basis.
2. General description of the exercise:
 - Subject/theme;
 - Type of training/exercise: staff training/seminars; command and staff exercises; desktop training/exercise; live exercise; simulations, demonstrations;
 - A list of the objectives for the training/exercise.
3. A detailed breakdown of the objectives:
 - For the exercise/training leader (a single, broadly-defined objective);
 - For the training staff.
4. Stages, place and time of the exercise/training.
5. Scenario of the exercise/training.
6. Assets and resources to be used in the exercise/training.
7. Final provisions.

A basic exercise scenario should be:

– Realistic (based on events that have recently occurred and could happen again);

- Flexible;
- Simple;
- Designed to not create alarm or panic in the local community.

After acceptance of the concept, the exercise leader should ask his team to prepare a more detailed document – a plan of the actual exercise on the day. This should contain:

1. Legal basis.
2. General characteristics of the exercise.
3. Detailed objectives of the exercise.
4. Entities involved and their cooperation with each other.
5. Stages, place and time of the exercise.
6. The assets and resources needed for the exercise.

7. Script (breakdown of the scenario, detailing actions and dialogue of actors etc.).
8. Participants' objectives.
9. Uniforms and equipment needed.
10. Safety conditions during the exercise.
11. Logistics of exercise first-aid and security resources.
12. Organisation of communications.

Exercise 6

Answer the following question: Do we really need exercises?

Exercise 7

Based on the preceding information, create an exercise concept. Use the following points as a guide:

- Step 1 – What is the reason for this exercise?
- Step 2 – What is the aim of the exercise?
- Step 3 – Decide the participants' objectives.
- Step 4 – Prepare the scenario.
- Step 5 – Prepare some questions to tease out the objectives.
- Step 6 – Driver inputs.

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3. SELECTED ISSUES IN FORENSIC TECHNIQUES

Forensic science falls within juridical science. It is an applied, practical science, which adapts developments of different branches of science, technology, art and even crafts to the requirements of the actual investigative practice, which is why it has the status of an interdisciplinary science. It concerns the tactics and technology of committing crimes, the tactics and technology of investigating crimes, and the tactics and technology of preventing crimes. Forensic tactics and technology are the two basic branches of this science. Forensic technology involves the examination of the technical means used for committing crimes and the development of means used by law enforcement bodies and the system of justice when investigating crimes as well as useful means for preventing crimes. The necessity to constantly improve the equipment and means of forensic technology and examination methods results not only from the development of crime but also from the technological developments taking place in our time. This branch of forensic science is aimed at obtaining, securing and using material sources of information, i.e. material evidence. It is useful whenever one deals with identification of a person or an object based on their traces.

From the point of view of the forensic technology, the key term is forensic evidence. It is the point of departure for any procedural and forensic activity. There is no doubt that each crime leaves forensic evidence. In a forensic sense, evidence includes all and any changes in the objective reality which – as discernible signs of events under investigation – form the basis for the recreation of the course of these events as they really happened. This definition of forensic evidence provided by Jan Sehn is most frequently used in forensic science.

One of the criteria for classifying forensic evidence is the evidence type. Classification based on type includes such evidence as: biological, mechanoscopic, trace, dactyloscopic, vermilion border, auricle, weapon use, and evidence subject to physiochemical examination.

3.1. Biological Evidences

Forensic genetics is a subspecialty of forensic biology, just like forensic medicine, forensic entomology, and bloodstain pattern analysis. Biological evidence is any trace coming from/related to a living organism that might be connected with the investigated incident.

Biological evidence may be:

A. Animal evidence: animal DNA (bloodstains, hair, tissues) making it possible to identify the genus, the species or the specimen (depending on the species);

B. Botanical evidence: fragments of conifer needles, plant tissues, remains of trunks etc.; allowing to identify the genus, the species or the specimen (depending on the species);

C. Human evidence:

- Bloodstains at the scene (bloodstain pattern analysis);
- Insect larvae (forensic entomology);
- Body, body fragments, injuries/analysis results being effects of a medical examination (forensic medicine);
- Genetic evidence (DNA), which might be:
 - Bodily fluids: blood, saliva, semen (there are initial tests allowing to confirm the presence of these substances in the evidence investigated);
 - Discharges and excretions of the human body, i.e. sweat, urine, faeces, tears;
 - Hair – discovered at the site as “fibres that look like human hairs” – only human hair with hair root sheath (bulb) preserved is the source of DNA with high discrimination power for the examination of the so-called nuclear DNA;
 - The so-called contact evidence or the so-called potential biological material – DNA traces, where the sources of DNA are cells of peeled epithelium on surfaces that could have had contact with the skin of the perpetrator/victim.

3.1.1. Methods of Securing DNA Evidence

- Evidence for DNA testing:

DNA traces might have the form of substances of a specific colour and state of aggregation (e.g. a brown substance that might be human blood) or of the so-called potential biological material (surfaces where one expects to find cells of peeled human epithelium, however, without complete tests one is unable to say whether they are present). When securing evidence, the operator should be wearing the basic personal protective equipment, i.e. a mask, gloves and coveralls, so that they do not transfer their own DNA.

DNA evidence may include:

- **Objects** that can be carriers of biological evidence **as a whole** (within the boundaries of the law and common sense), such as: furniture handles and knobs, bottles, cans, butt ends, cable ends, clothing of the perpetrator or the victim (properly dried), crime weapons etc. Such objects should be secured in air-permeable containers (paper envelopes, paper-foil packaging, wrapping paper, cardboard packaging) and properly immobilised so as not to spoil the evidence that may be subject to comprehensive analyses (e.g. dactyloscopic analysis);

- **Evidence secured together with the base**, e.g.: furniture upholstery, carpet fragments, fragments of furniture veneer etc.; the securing method as above;

- **Swab tests** – performed on handles, surfaces of objects that cannot be secured as a whole, bodies, samples collected during medical examinations (swabs from the reproductive tract, from under the foreskin, from under nails etc.). Swab tests are performed by wiping the examined surface with a sterile swab moistened with sterile distilled water. Thus prepared swabs should be secured in transport containers (protected against foreign biological material, air-permeable), e.g. in paper-foil packaging or paper envelopes.

- Any evidence in the form of fluid or semi-fluid material should be dried completely or stored in cold storage.

B. Reference material for DNA analysis:

- Reference material is biological material as to which one is certain it comes from a specific person/body. Reference material for DNA analysis includes:

- **Cells scraped off the mucous membrane of the inside of the cheek** – the best material (collected in accordance with the law of a given country),

- **Blood:**

- Peripheral blood that might come from the sample tested for alcohol/ other illegal drugs,

- A blood sample taken for the aforementioned purposes or blood secured on a swab/FTA card, taken by a forensic doctor from an open cavity,

- **Hair** – hair pulled out with root sheath (bulb) from the body in case it is impossible to collect reference material in the manner described above,

- **Bones, teeth** – in the case of bodies that are badly decomposed or charred, fragments of prepared bones/teeth might be sources of DNA for a comparative analysis.

3.2. Physicochemical Examination of Forensic Evidence

Forensic physicochemical examination is one of the forensic disciplines that uses the experience of chemistry and partially also physics, and is closely connected with law. The aim of forensic physicochemical examination is to

identify unknown substances, to determine the properties of substances, to compare substances, and to identify and determine the properties of traces found in relation to such incidents as explosions, fires, the use of firearms, traffic accidents or illegal production of controlled substances. In the case of the analysis in question, identification and comparative analysis can be distinguished; the difference between them is the type of the issue examined. Forensic identification analysis allows to determine the identity of materials based on their characteristics, i.e. their composition and properties. In the opinion based on such an analysis, the expert witness answers the question “What is it?”. On the other hand, comparative analysis is aimed at determining the identity of an object that left the trace by way of comparison. Conclusions from comparative analysis that assesses the degree of similarity between the evidence and the reference material are formulated in terms of probability. Forensic physicochemical examinations only allow for group identification.

The scope of physicochemical identification analysis is broad and it includes:

- Examination of narcotics, psychoactive substances and precursors;

the scope of an expert witness’s activity in this case involves: qualitative and quantitative analysis, profiling synthetic drugs and their precursors, identifying the methods of production of synthetic drugs and their precursors, and examining contact evidence of narcotics and psychoactive substances;

- Examination of micro-traces, fibres, paints, varnishes, plastic, adhesives, glass, metals and their alloys, and unusual traces;

- Examination of post-fire and flammable substances, irritants, alcohols, and unknown substances;

- Examination of explosive materials and devices and chemically unstable substances;

- Examination of post-explosion remains;

- Examination of contact evidence of explosives;

- Comparative and identification analysis of car paints, and car selection;

- Examination of traces left by a firearm shot;

- Analysis of ethyl alcohol in bodily fluids;

- Qualitative and quantitative analysis of narcotics and psychoactive substances and their metabolites in bodily fluids.

The above examination scope was determined in the Decision No. 16/2014 of the Head of the Central Forensic Laboratory of the Police dated 4 February 2014 on the typical scope of the activity of a forensic expert and specialist.

For the purposes of physicochemical identification, experts use the following methods: chromatography, spectroscopy, electron microscopy, optical microscopy, and combined methods, such as Gas chromatography–mass spectrometry (GC-MS). These methods ensure high sensitivity, which allows to test very small material samples. In the case of some of these methods,

the process of preparing the sample for the test is laborious, however, the test itself is fast, results are documented in the form of a graph or digital records, while evaluation and analysis use mathematical and statistical methods. The possibility of physicochemical identification is to a large extent determined by the way the material is secured for examination, which is an absolute principle. Packaging serves a significant function in the technical securing of evidence for physicochemical examination. It should be clean, dry and tight, chemically neutral towards the secured substance, it should be properly mechanically resistant, and its size should be adjusted to the dimensions or volume of the secured evidence. Incorrectly performed collection and securing of the evidence sample, e.g. during inspection, have consequences during laboratory identification analysis.

3.3. Dactyloscopic Evidences (Fingerprints)

Dactyloscopy is considered a classical branch of the forensic technology. Fingerprints, discovered and secured during procedural and forensic activities (such as an inspection of the site) are treated as evidence of unquestionable identification value due to the possibility of directly identifying an individual. The identification value of fingerprints is based on the use of their properties, such as their immutability, indestructibility (permanence), and uniqueness (individual character). It is commonly believed they they form valuable evidence, which is of significance when establishing a link between a given person and a given crime. Thanks to these possibilities of dactyloscopy, law enforcement bodies and the system of justice obtain irrefutable evidence of the individual's presence in a given place, while the discovered and secured fingerprints might be used to identify persons and bodies, register the images of fingerprint reproductions and images of fingerprint copies in AFIS (*Automated Fingerprint Identification System*) as well as draw conclusions and come up with investigative theories.

A significant benefit of dactyloscopic examination is the relatively simple methodology based on comparative analysis which makes use of evidence (usually in the form of the fingerprints discovered at the site or during visualisation examinations in the laboratory) and the reference material (in the form of fingerprints and/or palm prints taken from a specific person).

Fingerprints at the site come in one of the following three forms: **layered fingerprints** (transferred with sweat and sebum, "bloody" fingerprints, and "greasy" fingerprints), **delayed fingerprints** (left on a hard, non-absorbent surface in dust or a different loose substance, such as spilled flour, icing sugar, coal dust etc.), and **hollow fingerprints, the so-called prints** (left in plastic mass, such as Plasticine®, modelling clay, putty, wax etc.).

3.3.1. Methods of Securing Fingerprints

Evidence

The development method selected must be adjusted to the given type of fingerprints. In forensic science, the most commonly used methods are physical (optical and adhesive), chemical, physicochemical, and biological. The application of a proper method and its effectiveness to a large extent depend on the type of the substance creating fingerprints, the type and properties of the base, the development conditions, and the time lapse. The method for developing fingerprints during an inspection of the site commonly used by forensic scientists is the proven method that has been known for years, based on the adhesiveness of the particles of the dactyloscopic powder to sweat and sebum creating the fingerprint. It is worth highlighting that the development and securing of fingerprints must be performed in accordance with certain principles namely the contrast principle and the principle of the order of developing evidence (i.e. biological evidence first, and then dactyloscopic evidence). What matters a lot when selecting proper dactyloscopic powder is the effectiveness of the development of fingerprints on different surfaces, good quality of the fingerprints developed, and the so-called non-smearing of the base. In the forensic practice, depending on the properties of the prints and the base type, the following dactyloscopic powders are used: **light powders** (e.g. argenterat), **heavy powders** (e.g. ferromagnetic powders) and **fluorescent powders**. Fingerprints developed using light and heavy powders are usually secured on dactyloscopic foil, in accordance with the principle of contrast. According to this principle, in the case of a light base dark powders are used (such as iron oxide black powder, black ferromagnetic powder) and thus developed fingerprints are secured on transparent or white dactyloscopic foil. In the case of a dark base, argenterat is usually used (classified as a light powder), while the developed fingerprints are secured on black dactyloscopic foil. When fluorescent powders are used to develop fingerprints, then they are secured using the photographic method. Its point is to photograph fingerprints in conditions corresponding to the process of their development (which is usually UV light). The best effects when it comes to contrast and legibility of fingerprints are achieved when colour photography is used for their documentation.

However, one has to remember that the above method is effective in cases when fingerprints are developed on relatively smooth, level and clean surfaces and when it is necessary to develop “fresh” fingerprints. The specific character of an inspection of the site proves that the actual conditions of developing fingerprints are far from perfect. Surfaces at the site tend to be uneven, porous, colourful and dirty, while the fingerprints are “old” or poorly visible. In such cases, the physical method usually proves to be insufficient.

An inspection of the site allows to uncover objects that might be connected with the incident in question. On the surfaces of these objects, there might be invisible “fresh” and “old” fingerprints, which is why the use of the powder method in order to develop them might destroy or damage the “old” fingerprints. In such a situation, it is justified to secure the whole object, following the applicable procedures of commissioning forensic examination, and to send the evidence to the visualisation laboratory being part of the dactyloscopy laboratory in a forensic laboratory. Under laboratory conditions, an expert in dactyloscopy makes an attempt to develop fingerprints on the surfaces of these objects, using a proper chemical examination method. Selection of this method depends on a number of factors including the surface type (absorbent and non-absorbent bases, thermosensitive paper, adhesive side of adhesive tape) and the type of the fingerprints developed (whether they are bloody fingerprints, layered fingerprints left with sweat and sebum on wet or damp surfaces, or layered fingerprints left with a substance including fats, such as technical maintenance agents, edible fats).

When selecting the method, a significant role is played by the principles the dactyloscopic expert needs to follow when developing fingerprints. Among other things, the point is to apply several complementary development methods and to keep their proper order; to register evidence before the next method is used and, in the case of any doubts about the effectiveness of a given method, to use test evidence; to ensure that the method applied will not hinder or prevent other forensic examinations (this usually concerns DNA testing); and to strictly follow any applicable OHS regulations. When these principles are followed, a properly applied method might not only reveal both “fresh” and “old” fingerprints but also improve the legibility of barely visible or even fragmentary evidence discovered visually during the initial inspection of the object in visible light. Such clear evidence with visible individual characteristics can be used in procedural activities (e.g. comparative and identification analysis as part of the dactyloscopic analysis commissioned by a given body) or extra-procedural activities (e.g. searching the AFIS).

Reference Material

Reference material consists of prints of fingerprints and palm prints that are usually taken using the classical, traditional dactyloscopic method, the so-called ink method. It is based on the use of ceramic ink pads soaked with black dactyloscopic ink. The fact of taking fingerprints is documented using dactyloscopic cards.

The dactyloscopic practice more and more frequently uses the new method of taking fingerprints and palm prints, which is based on an electronic fingerprinting device *LiveScanner*. The device uses an inkless fingerprinting system that allows to quickly create high-quality images of fingerprints. The registration element of the scanner is equipped with a flexible membrane, which makes it easier to copy

“difficult” fingerprints, when, for example, one has to fingerprint someone with dry or damaged skin or someone with wet or sweaty hands. In such cases, the traditional “ink” method unfortunately does not produce the expected results.

3.4. Mechanoscopic Evidence

The term “mechanoscopy” is a combination of Greek words *mechane* – tools and *skopeo* – I examine, and it was introduced into the literature by a Czech forensic scientist L. Havlicek in 1939. Traditionally, it is assumed that mechanoscopy is a branch of the forensic science, dealing with examinations aimed at the **identification of tools based on the marks they leave**. According to T. Hanausek, mechanoscopy is a branch of the forensic science covering all methods and means used to identify, secure and examine for identification purposes evidence created as a result of interaction of two or more objects, or the effect one of them had on the other. According to J. Bieniek: “mechanoscopy is a branch of the forensic science, which deals with the examination of evidence created as a result of mechanical interaction between different objects (marks created as a result of the effect of force, after resistance has been overcome) and with the determination of the identity of crime tools based on the marks they left.”

Mechanoscopic evidence is trace evidence and it can be found on different bases, such as metal, glass, rubber, wood, plastic, fabric, human and animal bones, food etc. This evidences are divided into evidences in the form of **base deformation**, which are divided into:

- **Static**, created as a consequence of imprinting one object/tool on the material of another (base), without longitudinal translation. These are imprints;
- **Dynamic**, created as a result of depressing one tool into a material and its simultaneous longitudinal translation against the base surface. This group includes: scratches, slips, cuts, processing marks.

Mechanoscopic evidence is usually found on:

1. Objects being security measures the perpetrator had to overcome in order to get inside a room, such as doors, anti-burglary roller shutters, windows, security bars in windows and balconies etc.;
2. Other objects, in places directly acted upon by a force (on vehicles, seals, safes, cabinet safes, trees etc.);
3. The person or the body (e.g. injuries inflicted by a tool);
4. Clothing in the form of wear, tears, cuts, chafes etc.

Procedural Securing of the Above Evidence Includes:

1. Description in the inspection report considering:
 - The evidence type and characteristics (marks given by the manufacturer, shape, dimensions, individual characteristics resulting from, for example, wear, damage etc.);

- Location;
- The revealing and securing method;
- Description of other forensic activities undertaken in connection with the evidence (e.g. dismantling method, marking of the sides);

2. Photographic documentation of the evidence including photographs of the evidence taken using the scale technique, and of its location – situational photographs. The evidence is photographed with a centimetre or millimetre scale and a number. Lens axis should be perpendicular to the evidence surface;

3. Description and the securing of the packaging into which the evidence has been put;

4. Evidence information sheet attached to the packaging;

5. Marking of the evidence location on the site layout drawing.

Methods of the Technical Securing of Evidence:

1. Securing together with the base (or its part) on which the evidence is located;

2. Modelling, creating a replica (cast) of the evidence using special mass (usually silicone of different colours and densities);

3. Collecting from the base and placing in a proper container;

4. Photographic documentation.

Drawing Conclusions Based on Mechanoscopic Evidence:

1. The evidence allows to recreate the appearance of the tool and to classify it into a specific group;

2. Location and the order of creation of mechanoscopic evidence allow to conclude about the place where the perpetrator entered the site, to retrace their steps, and to determine the place they left the site;

3. The type, methodology and time of use of certain tools allow to select potential perpetrators.

The reference material is usually obtained during searches of rooms, stops and searches of persons, vehicle controls, and inspections of different sites. Working parts of tools need to be particularly protected as they might bear substances from the base upon which the given tool was used.

Sources of reference material can also be forensic collections including broken lock cylinders installed in doors in apartments, counterfeit circulation and collector coins, counterfeit hallmarks etc.

Reference materials can be reference marks made with the tool selected. Reference marks should be made on a base the properties of which are the same as or similar to the properties of the material bearing the evidence marks.

The technique of making the reference marks should be similar to the technique of creating the evidence.

3.4.1. Types of Mechanoscopic Examinations

1. Identification of evidence, which allows to:
 - Determine the properties of the evidence examined;
 - Conclude about the way it was created;
 - Select the tools used;
 - Highlight the usefulness of the evidence for further examinations.
2. Such examinations use forensic templates in the form of, for example, catalogues with data and original products provided to expert witnesses by their manufacturers.
3. Comparison making it possible to determine whether the characteristics of the evidence and reference material are the same or different, which is done by comparing the evidence with the reference material. Such examinations usually make use of a stroboscopic microscope.
4. “Analysis of the whole”, making it possible to determine whether the given parts, elements, pieces or fragments used to form one object (a whole) before they were separated.
5. What is examined is the concordance of the parts compared in terms of their shape, thickness, fracture structure, processing method, micro- and macro-roughness of the external surfaces of splits etc.

3.4.2. Scope of Mechanoscopic Examinations

Such examinations include:

1. Object identification based on the analysis of:
 - Marks of mechanical impact of an object on the base;
 - Secured fragments of the objects;
 - Reconstruction.
2. Determination of the cause-and-effect relationship between the object and the incident including the determination whether the damage is the cause or the effect of the incident.

Mechanoscopic Examinations Include:

1. Identification of tools allowing to determine the type of the tool used, its shape, dimensions, and production technology etc. (group identification).
2. Group characteristics include tool characteristics that are created in the course of most manufacturing processes, the so-called manufacturing characteristics. They include casting, embossing, threading, and all kinds of processing, except for grinding and polishing.
3. Trace the mark back to a specific tool (individual identification of the tool).
4. Individual characteristics include characteristics created in the course of some manufacturing processes and all characteristics created during use, such as

damage, and as a result of repair, e.g. sharpening. Manufacturing processes that give tools individual characteristics are grinding and polishing.

5. Determination the mechanism of the creation of the mark (whether it was created as a result of one-time or multiple uses of the tool; determination of the direction, the force and the place where the tool touched the base).

3.5. Tools as Subjects of Forensic Examinations

In the forensic practice, tools are divided into:

1. Cutting tools:
 - One-blade tools, the blade of which can act upon the base with one side;
 - Two-blade tools, the blade of which can act upon the base with both sides;
 - Glass cutters.
2. Cutters used for processing objects by removing a certain layer of material from their surface.
3. Two- and multi-jaw tools, with two fixed or movable jaws for gripping, clenching, crushing and turning objects of different section shapes (e.g. vices, crowbars). Working surfaces of jaws can be smooth, grooved or notched.
4. Seals and sealing pliers which are mechanical security devices the aim of which is not to directly protect the building but to register marks of forced entry by unauthorised persons.
5. Blunt and blunt-edged tools, objects and fixed surfaces.
 - Blunt tools are the ones that leave marks of their surface unlimited by any edge, of cylindrical section, such as reinforcing bars.
 - Blunt-edged tools leave marks of their surface limited by at least one edge, such as crowbars, hammers, planks etc.
6. Mechanical security devices and skeleton keys, divided into:
 - Locks;
 - Blocking mechanisms (cylinders);
 - Padlocks.
7. Examinations of mechanical locking devices (padlocks, locks, blocking mechanisms) and keys, allowing to:
 - Determine the device effectiveness;
 - Determine the method of opening based on the traces left on locking device elements;
 - Identify tools used by perpetrators for forced entry;
 - Determine the originality of keys sent for examination.
8. Examination of broken panes and glass. The extensiveness of breakage is usually influenced by the following correlated factors: type and dimensions

of the tool used, the size, thickness and internal structure of the glass sheet (tempered or non-tempered glass, bulletproof including wired glass etc.), its fixtures, setting in a frame, and the method and force of the strike. When determining the side from which the glass was broken, the following issues are considered:

- Radial fractures extend along relatively straight lines outward from the point at which the glass was struck (the hole). They suggest that the glass was struck with a small pressure force;
- Concentric fractures form circles around the point at which the glass was struck, and cross radial fractures;
- In the case of small holes in glass, one can determine the direction from which the force was applied;
- If the hole has the shape of a truncated cone – the force was applied from the side of its shorter base;
- On the dividing surface of the broken glass (shard fractures) there are multiple short curves, ribbed or arched, in the shape of fan-like “waves”;
- These waves always widen from the side of the application of the force.

9. Examination of separated objects in order to see whether before separation they formed a whole, e.g. pieces of glass, wood, parts of tools or different objects, fragments of paint, examination in terms of the whole. Identification in terms of the whole most frequently concerns: chips of toothed blades of hacksaws; fragments of fabrics; glass shards; fragments of paint; splinters of wood. Examinations of wood also cover characteristics of its morphological structure and defects.

10. Examinations of counterfeit coins in order to establish the production technology and to identify the tools used. The main elements protecting coins against counterfeiting:

- Rare material the coins are made of;
- Precise image;
- Proper dimensions with very low tolerance (additional elements of the image on coins frequently include colours);
- Additional security features, e.g. grooves on the edge, inscriptions including convex inscriptions etc.;
- Narrowly defined magnetic properties of coins;
- Company’s logo.

11. Identification of punches, seals and sealing pliers, number punches and markers used for the permanent marking of vehicles, machines, technical devices, cut down trees. Seals consist of an element on the surface of which identification marks are pressed using sealing pliers, and wire (sealing wire). On the surfaces of sealing pliers matrices there are identification marks including letters, numbers and graphic designs. Marks indicating seal breakage can be found inside the seal and on its outside surfaces.

12. Examination of identification marks of different objects in order to determine the authenticity, alteration method, and the content of the original marking;

13. Technical examinations of the way different objects and bases are damaged;

14. Metal science - elements of steering systems, suspension, tow hooks of vehicles, pressure cylinders and containers, and gas fittings;

15. Identification of devices used for the production and copying of CDs and DVDs and writers installed on computers (burners). These examinations include identification of parts of injection moulding machines used in production processes – injection moulding machine mirror, and to determine whether the records sent for examination, secured as part of operational or procedural activities, were produced using the same devices. This allows to link those selling illegal records to their producer.

Experts in mechanoscopy also answer questions about whether car bulbs were on during the crash. This is possible thanks to the analysis of a tungsten filament which heats to over 2000 Celsius degrees when the bulb is glowing. When a glowing bulb is broken, oxygen from the air reacts with tungsten producing tungsten oxides in characteristic yellow colour.

After touching the heated filament, glass shards from the broken bulb melt and stick to it, while the ends of the divided filament become cylindrical. A cold filament is elastic, which is why during the crash it is temporarily deformed but then returns to its original shape.

3.6. Methodology of Revealing and Securing Traceological Evidence

Traceology – is a branch of forensic science dealing with the mechanism of creating reproductions in the form of prints and imprints as a result of walking or running of people or animals, the movement of vehicles, for example, on wheels, tracks, skids, and of moving and dragging objects.

Traceological evidence – this is evidence created as a result of the movement of people, animals and objects being means of transport.

Types of Traceological Evidence:

1. Footprints:
 - Bare;
 - With shoes on;
 - Clothed on hard and soft surfaces.
2. Vehicle tracks of:
 - Wheels (rubber and iron);
 - Skids;
 - Caterpillars on hard and soft surfaces.

3. Tracks left by animal legs:

- Hoofs;
- Paws on hard and soft surfaces.

4. Tracks of tools used by people to move e.g. stilts, crutches, skateboards, wheelchairs.

Traceological evidence also includes tracks of dragging, for example, a body or a killed animal, marks of shoes on human skin in the form of bruises (called internal evidence), and a shoe or a pair of shoes (lost, abandoned).

The Division of Traceological Evidence by Location of the Evidence on the Surface

1. Surface evidence (prints):

- Layered, visible – colourful, not requiring development;
- Layered, invisible – requiring development using different means;
- Delayed – visible, colourless, not requiring development using any means.

2. Hollow traces (imprints):

- Left in;
- Loose base;
- Cohesive base;
- Snow;
- A different plastic base.

Mechanism of Creation of Traceological Evidence (Location)

Human and animal footprints are created when a foot (e.g. a hoof) touches the ground, which might be soil (clayey, sandy or other), snow (loose/dry, compact/wet) or a hard surface (asphalt, concrete, floor or other). If the surface accepting the trace is soft, usually hollow tracks are created - imprints, and if it is hard, surface marks are created - prints. Prints can be layered or unlayered.

Tracks of vehicles are created in static and dynamic conditions. In static conditions, they are created in the form of reproductions: prints and imprints. In dynamic conditions, tracks of blocked wheels or skids are created: the object creating the tracks moves on the surface. In this case, longitudinal elements of treads are reproduced on the base.

Vehicle tracks should be found on roads or nearby surfaces, and on victims.

Tracks found on roads and shoulders are most frequently:

- Driving marks;
- Braking marks;
- Blocking marks;
- Skid marks;
- Marks of wheel rims;
- Fishtailing marks;
- Drag marks;
- Stopping marks.

Methods of Technical Securing of Traceological Evidence

- Plaster or silicone cast;
- Securing together with the base (e.g. a sheet of paper with the shoe's sole copied);
- Transfer onto black gel foil measuring 13x36 mm;
- Photographic documentation of traceological evidence in accordance with the scale technique;
- The use of MES device, applying the electrostatic method, and transfer onto black gel foil.

3.7. Revealing and Securing Methods

Evidence is revealed visually. When revealing invisible prints, layered with such substances as fats or oils, light falling at an angle on the observed surface and a magnifying instrument, such as a magnifying glass, are used. Sometimes, fluorescent properties of the substance creating the print (fat) can be used in UV light. Invisible marks are developed using dactyloscopic powder. In order to develop footprints on carpets or flooring, the phenomenon of changing the electrostatic charge (pouring fine plastic particles on the surface from a certain height) can be used.

Each developed mark should be **documented photographically in accordance with the scale technique.**



Figure 1. Hollow print of a shoe sole in a soft base

1. Forensic securing:

Hollow marks are secured through modelling. For this purpose, plaster, silicone mass, metal alloys, sulphur, or paraffin are used. The technical securing method depends on the surface on which the mark has been developed (heavy soil, loose soil, loose snow, compact snow) and the agent applied.

2. Surface marks are secured:

- Together with the surface on which they are found, e.g. marks on paper, polystyrene foam, cardboard etc.,
- By transferring them onto traceological foil,
- Using the electrostatic method, with a MES kit producing static electricity, transferring the mark onto black gel foil.

Evidence should be secured in procedural terms by describing it in detail in the inspection report, giving their dimensions, location and marking. For each developed and secured evidence, information sheet should be drawn up, filled in and attached to the packaging, thus becoming a procedural document.

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MODULE II

THE BIOLOGICAL BASIS OF CBRN

1. MICROBIOLOGY

1.1. Structure of the bacterial cell

Bacteria are single-celled prokaryotic microorganisms, and their DNA is not contained within a separate nucleus as in eukaryotic cells. They are approximately 0.1–10.0 μm in size (Fig. 1) and exist in various shapes, including spheres (cocci), curves, spirals and rods (bacilli) (Fig. 2). These characteristic shapes are used to classify and identify bacteria. The appearance of bacteria following a Gram stain is also used for identification. Bacteria which stain purple/blue are termed Gram-positive, whereas those that stain pink/red are termed Gram-negative. This difference in response to the Gram stain results from the composition of the cell envelope (wall) (Fig. 3).

Cytoplasmic membrane

A cytoplasmic membrane surrounds the cytoplasm of all bacterial cells and is composed of protein and phospholipid; it resembles the membrane surrounding mammalian (eukaryotic) cells, but it lacks sterols. The phospholipids form a bilayer into which proteins are embedded, some spanning the membrane. The membrane carries out many functions, including the synthesis and export of cell-wall components, respiration, secretion of extracellular enzymes and toxins, and the uptake of nutrients by active transport mechanisms. Mesosomes are intracellular membrane structures, formed by folding of the cytoplasmic membrane. They occur more frequently in Gram-positive than in Gram-negative bacteria. Mesosomes present at the point of cell division of Gram-positive bacteria are involved in chromosomal separation; at other sites they may be associated with cellular respiration and metabolism.

Cell wall

Bacteria maintain their shape by a strong rigid outer cover, the cell wall (Fig. 3). Gram-positive bacteria have a relatively thick, uniform cell wall, largely composed of peptidoglycan, a complex molecule consisting of linear repeating sugar sub-units cross-linked by peptide side chains (Fig. 4a). Other cell-wall polymers, including teichoic acids, teichuronic acids and proteins, are also present.

Gram-negative bacteria have a thinner peptidoglycan layer and an additional outer membrane that differs in structure from the cytoplasmic membrane (Fig. 4b). The outer membrane contains lipopolysaccharides on its outer face, phospholipids on its inner face, and proteins and lipoproteins that anchor it to the peptidoglycan. Porins are a group of proteins that form channels through which small hydrophilic molecules, including nutrients, can cross the outer membrane. Lipopolysaccharides are a characteristic feature of Gram-negative bacteria and are also termed ‘endotoxins’ or ‘pyrogen’. Endotoxins are released on cell lysis and have important biological activities involved in the pathogenesis of Gram-negative infections; they activate macrophages, clotting factors and complement, leading to disseminated intravascular coagulation and septic shock.

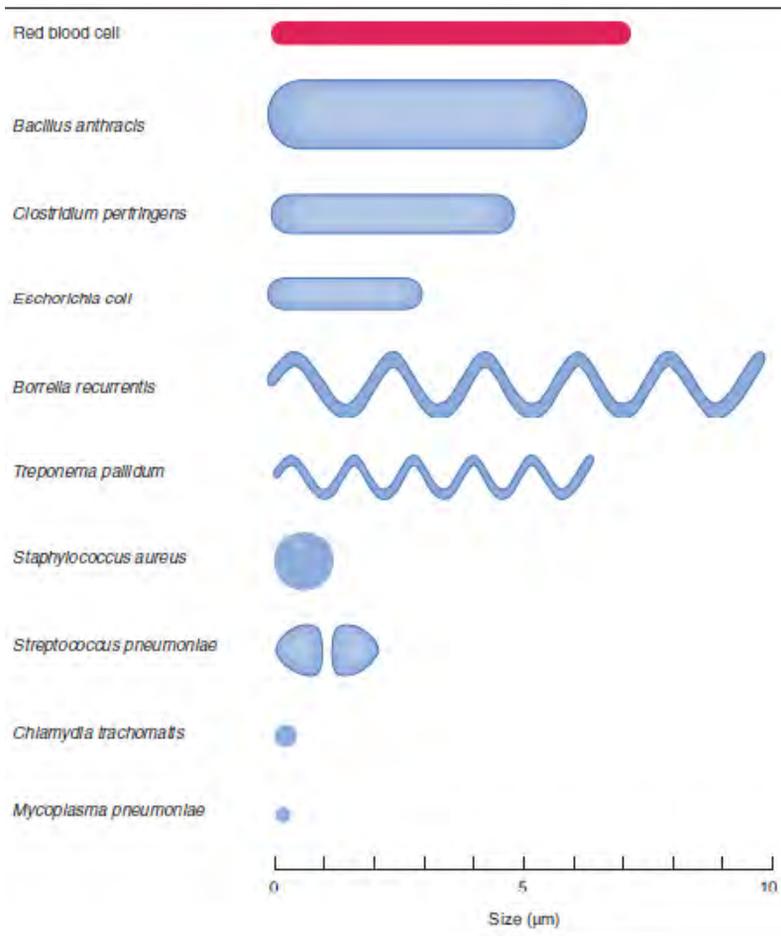


Figure 1. The shape and size of certain clinically important bacteria (figure used with permission under Creative Commons license)

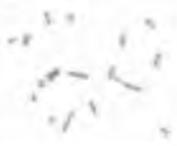
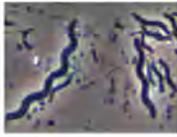
Common Prokaryotic Cell Shapes			
Name	Description	Illustration	Image
Coccus (pl. cocci)	Round		
Bacillus (pl. bacilli)	Rod		
Vibrio (pl. vibrios)	Curved rod		
Coccobacillus (pl. coccobacilli)	Short rod		
Spirillum (pl. spirilla)	Spiral		
Spirochete (pl. spirochetes)	Long, loose, helical spiral		

Figure 2. Various bacterial shapes (*figure used with permission under Creative Commons license*)

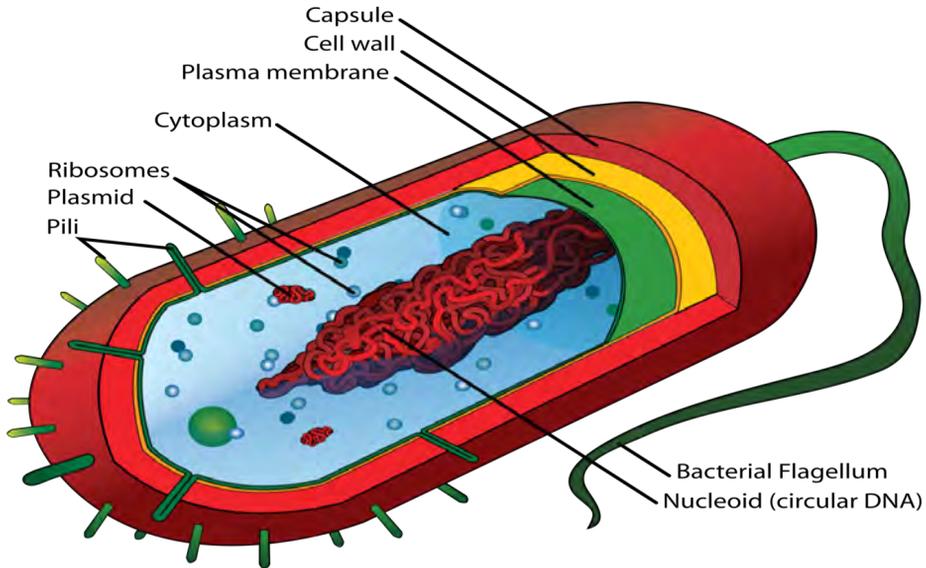


Figure 3. Section of a typical bacterial cell (*figure used with permission under Creative Commons license*)

Mycobacteria have a distinctive cell wall structure and composition that differs from that of Gram-positive and Gram-negative bacteria. It contains peptidoglycan but has large amounts of high molecular weight lipids in the form of long chain length fatty acids (mycolic acids) attached to polysaccharides and proteins. This high lipid content gives the mycobacteria their acid-fast properties (retaining a stain on heating in acid), which allows them to be distinguished from other bacteria (e.g. positive Ziehl-Neelsen stain). The cell wall is important in protecting bacteria against external osmotic pressure. Bacteria with damaged cell walls, such as after exposure to β -lactam antibiotics such as penicillin, often rupture. However, in an osmotically balanced medium, bacteria deficient in cell walls may survive in a spherical form called protoplasts.

Under certain conditions some protoplasts can multiply and are referred to as L-forms. Some bacteria, such as mycoplasmas, have no cell wall at any stage in their life cycle. The cell wall is involved in bacterial division. After the nuclear material has replicated and separated, a cell wall (septum) forms at the equator of the parent cell. The septum grows in, produces a cross-wall and eventually the daughter cells may separate. In many species the cells can remain attached, forming groups, such as staphylococci form clusters and streptococci form long chains (Fig. 5).

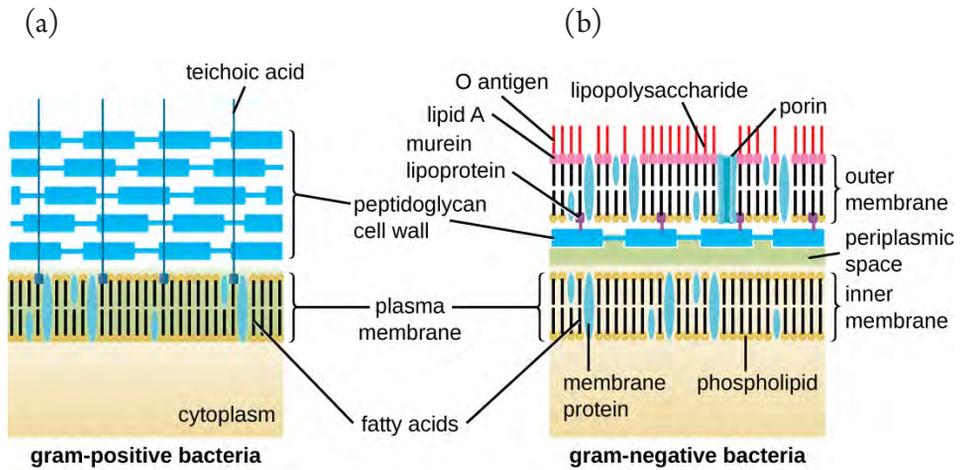


Figure 4. Cell wall and cytoplasmic membrane of (a) Gram-positive bacteria, (b) Gram-negative bacteria. The Gram-positive bacterial cell wall has a thick peptidoglycan layer with associated molecules (teichoic acids, teichuronic acids and proteins). The Gram-negative bacterial cell wall contains lipopolysaccharides, phospholipids and proteins in an outer membrane linked to a thin inner peptidoglycan layer. The mycobacterial cell wall contains long chain length fatty acids (mycolic acids) (figure used with permission under Creative Commons license)

Capsules

Some bacteria have capsules external to their cell walls. These structures are bound to the bacterial cell and have a clearly defined boundary. They are usually polysaccharides with characteristic compositions that can be used to distinguish between microorganisms of the same species (e.g. in serotyping). Capsular antigens can be used to differentiate between strains of the same bacterial species, such as in the typing of *Streptococcus pneumoniae* for epidemiological purposes. The capsules are important virulence determinants in both Gram-positive and Gram-negative bacteria, because they may protect the bacteria from host defences and, in some bacteria, aid attachment to host cells.

Bacterial slime and biofilm

Extracellular slime layers are produced by some bacteria. They are more loosely bound to the cell surface than capsules and do not form a clearly defined surface boundary. The slime layer is composed predominantly of complex polysaccharides (glycocalyx), which acts as a virulence factor through the formation of biofilm, for example, by facilitating the attachment of *Staphylococcus*

epidermidis onto artificial surfaces, such as intravascular cannulae, replacement joints and heart valves. Once formed, biofilms present a major problem for treatment and may require removal of the biomedical device.

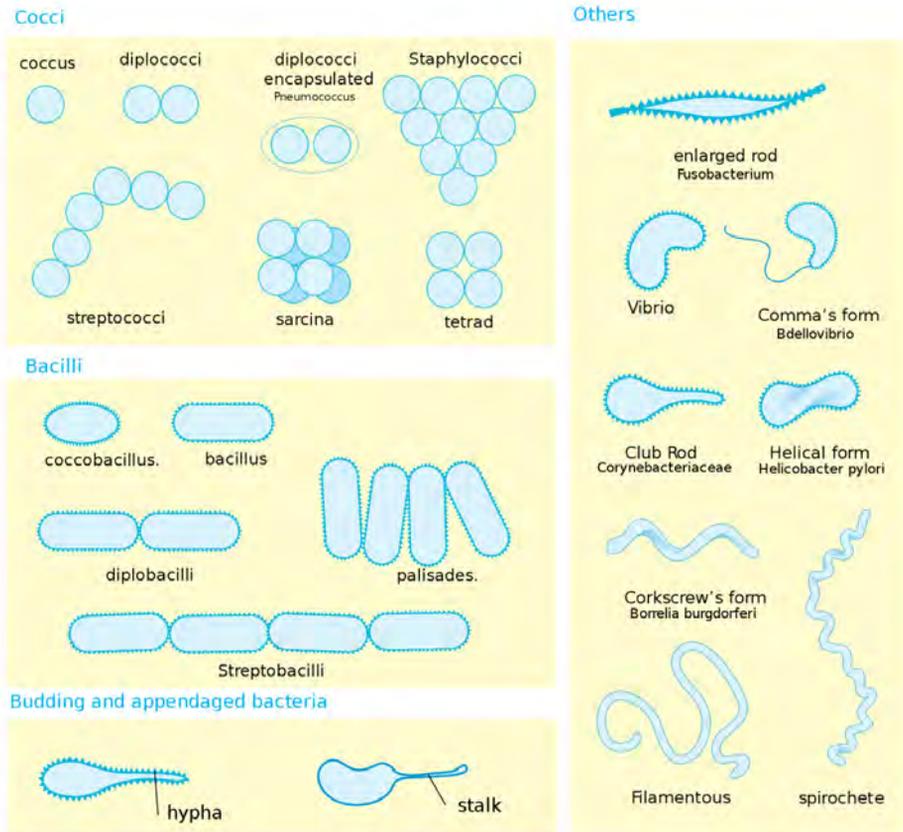


Figure 5. Various groups of bacteria (figure used with permission under Creative Commons license)

Flagella

Bacterial flagella are spiral-shaped surface filaments consisting mainly of the protein, flagellin. They are attached to the cell envelope as single (monotrichous) or multiple (peritrichous) forms (Fig. 6). Flagella facilitate movement (motility) in bacteria by rapid rotation. They can be observed under the light microscope with special stains. Flagella are usually detected for diagnostic purposes by observing motility in a bacterial suspension or by spreading growth on solid media. The antigenic nature of the flagella may be used to differentiate between and identify strains of *Salmonella sp.*

Fimbriae

Fimbriae (also termed pili) are thin, hair-like appendages on the surface of many Gram-negative, and some Gram-positive, bacteria. They are approximately half the width of flagella, and are composed of proteins called pilins. In some bacteria they are distributed over the entire cell surface. Fimbriae are virulence factors enabling bacteria to adhere to particular mammalian cell surfaces, an important initial step in colonisation of mucosal surfaces. For example, *Neisseria gonorrhoeae* produce fimbriae that bind to specific receptors of cervical epithelial cells, whereas *Streptococcus pyogenes* have fimbriae containing 'M' protein, which facilitates adhesion to human cells in the pharynx. Specialised fimbriae are involved in genetic material transfer between bacteria, a process called conjugation.

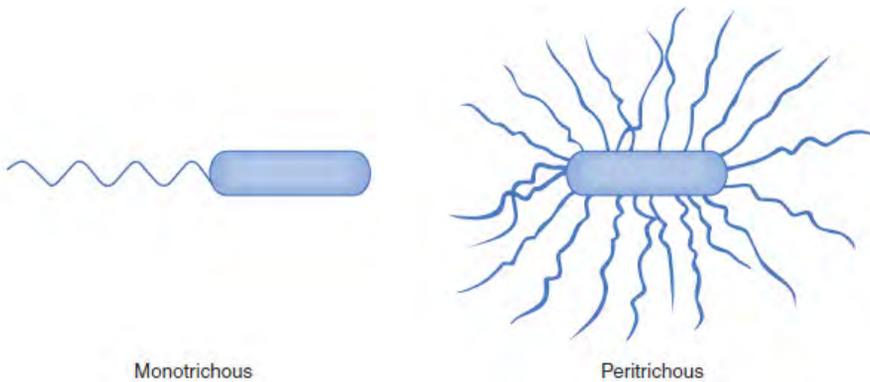


Figure 6. Arrangements of bacterial flagella (figure used with permission under Creative Commons license)

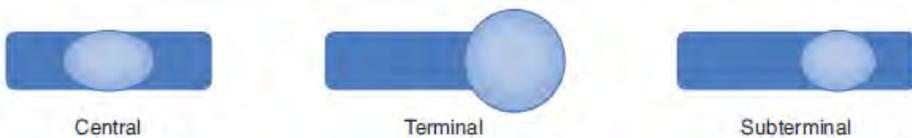


Figure 7. Size, shape and position of bacterial spores (from left to right): non-projecting, oval, central, e.g. *Bacillus anthracis*; projecting, spherical, terminal, e.g. *Clostridium tetani*; non-projecting, oval, sub-terminal, e.g. *C. perfringens* (figure used with permission under Creative Commons license)

Nuclear material

The bacterial chromosome consists of a single circular molecule of double-stranded DNA, which is maintained in a compact form within the cell by supercoiling. When released from the cell and uncoiled the DNA would be about

1 mm long (10 to 100-times the length of the cell). Additional, smaller extra-chromosomal DNA molecules, called plasmids, may also be present in bacteria. The chromosome usually codes for all the essential functions required by the cell; some plasmids control important phenotypic properties of pathogenic bacteria, including antibiotic resistance and toxin production. Extracellular nuclear material for encoding virulence and antibiotic resistance may also be transferred between bacteria and incorporated into the recipient's chromosome or plasmid. Transfer of genes encoding for virulence or antibiotic resistance may account for bacteria becoming resistant to antibiotics and for low-virulent bacteria becoming pathogenic.

Endospores

Endospores (spores) are small, metabolically dormant cells with a thick, multi-layered coat, formed intracellularly by members of the genera *Bacillus* and *Clostridium* (Fig. 8). They are highly resistant to adverse environmental conditions and may survive desiccation, disinfectants or boiling water for several hours. Spores are formed in response to limitations of nutrients by a complex process (sporulation) involving at least seven stages. When fully formed, they appear as oval or round cells within the vegetative cell. The location is variable, but is constant in any one bacterial species. Spores can remain dormant for long periods of time. However, they are able to revert to actively-growing cells (i.e. germinate) relatively rapidly in response to certain conditions, such as the presence of specific sugars, amino acids or bile salts. Spores also have an important role in the epidemiology of certain human diseases, such as anthrax, tetanus, gas gangrene and infection caused by *Clostridium difficile*. The eradication of spores is of particular importance in certain processes, for example the production of sterile products including pharmaceuticals and surgical instruments, in routine hospital ward and care centre cleaning, and in food preservation.

1.2. Bacterial growth

Most bacteria will grow on artificial culture media prepared from extracts from animal or plant tissues, which supply pre-formed nutrients and vitamins. However, some bacteria, e.g. *Mycobacterium leprae* (leprosy) and *Treponema pallidum* (syphilis), cannot yet be grown in vitro; other bacteria, e.g. *Chlamydia sp.* and *Rickettsia sp.*, only replicate intracellularly within host cells and are therefore grown in tissue culture.

Under suitable conditions (nutrients, temperature and atmosphere), a bacterial cell will increase in size and then divide by binary fission into two identical cells. These two cells are able to grow and divide at the same rate as

the parent cell, provided that conditions including nutrient supply remain stable. This results in an exponential or logarithmic growth rate. The time required for the number of bacteria in a culture to double is called the generation time, e.g. *Escherichia coli* has a generation time of about 20 minutes under optimal conditions. By contrast, *Mycobacterium tuberculosis* has a generation time of 24 hours.

Most bacteria of medical importance require carbon, nitrogen, water, inorganic salts and a source of energy for growth. They have various gaseous, temperature and pH requirements, and can utilize a range of carbon, nitrogen and energy sources. Some bacteria also require special growth factors, including amino acids and vitamins. Growth requirements are important in selecting the various culture media required in diagnostic microbiology and in understanding the tests for identifying bacteria.

Carbon, oxygen and nitrogen sources

Bacteria are classified into two main groups according to the type of compounds that they can utilise as a carbon source:

1. Autotrophs utilise inorganic carbon from carbon dioxide and nitrogen from ammonia, nitrites and nitrates; they are of minor medical importance.
2. Heterotrophs require organic compounds as their major source of carbon and energy; they include most bacteria of medical importance.

Bacteria require CO₂ for growth; adequate amounts are present in the air or are produced during metabolism by the microorganisms themselves. A few bacteria, however, require additional CO₂ for growth, such as *Neisseria meningitidis*, *Campylobacter jejuni*.

Bacteria can also be classified into four groups according to their O₂ requirements:

1. Obligate (strict) aerobes: grow only in the presence of oxygen, e.g. *Pseudomonas aeruginosa*.
2. Microaerophilic bacteria: grow best in low oxygen concentrations, e.g. *Campylobacter jejuni*.
3. Obligate (strict) anaerobes: grow only in the absence of free oxygen, e.g. *Clostridium tetani*.
4. Facultative anaerobes: grow in the presence or absence of oxygen, e.g. *Escherichia coli*.

Temperature

Most pathogenic bacteria grow best at 37°C. However, the optimum temperature for growth is occasionally higher, e.g. for *C. jejuni*, it is 42°C. The ability of some bacteria to grow at low temperatures (0–4°C) is important in food

microbiology; *Listeria monocytogenes*, a cause of food poisoning, will grow slowly at 4°C and has resulted in outbreaks of food poisoning associated with cooked, chilled products. Bacteria can be classified into four major types on the basis of their temperature response:

1. Psychrophilic bacteria: grow just above the freezing temperature, and can cause contamination of food stored in refrigerators. Example – *Pseudomonas*.
2. Mesophilic bacteria: grow at normal temperature in water and food products, liberate gas and cause changes in texture. Example – *Lactobacillus*.
3. Thermophilic bacteria: can survive at higher temperatures and can withstand pasteurization. Example – *Clostridium*, *Bacillus*.
4. Thermophilic bacteria: can survive pasteurization but cannot grow at pasteurization temperature. Example – *Micrococcus*, *Streptococcus*.

pH

Most pathogenic bacteria grow best at a slightly alkaline pH (pH 7.2–7.6). There are a few exceptions: *Lactobacillus acidophilus*, present in the Gram-stain of *Clostridium sporogenes* (showing oval sub-terminal spores), and a *Clostridium tetani* with a terminal spore (arrowed). The vagina of post-pubescent females prefers an acid medium (pH 4.0). It produces lactic acid, which keeps the vaginal secretions acidic, thus preventing many pathogenic bacteria from establishing infections. *Vibrio cholerae*, the cause of cholera, prefers an alkaline environment (pH 8.5).

Growth in liquid media

When bacteria are added (inoculated) into a liquid growth medium, subsequent multiplication can be followed by determining the total number of live microorganisms (viable counts) at various time intervals. The growth curve produced normally has:

1. Lag phase (A): the interval between inoculation of a fresh growth medium with bacteria and the commencement of growth;
2. Log phase (B): the phase of exponential growth; the growth medium becomes visibly turbid at approximately 1,000,000 cells/ml;
3. Stationary phase (C): the growth rate slows as nutrients become exhausted, waste products accumulate, and the rate of cell division equals the rate of death; the total viable count remains relatively constant;
4. Decline phase (D): the rate of bacterial division is slower than the rate of death, resulting in a decline in the total viable count (Fig. 8).

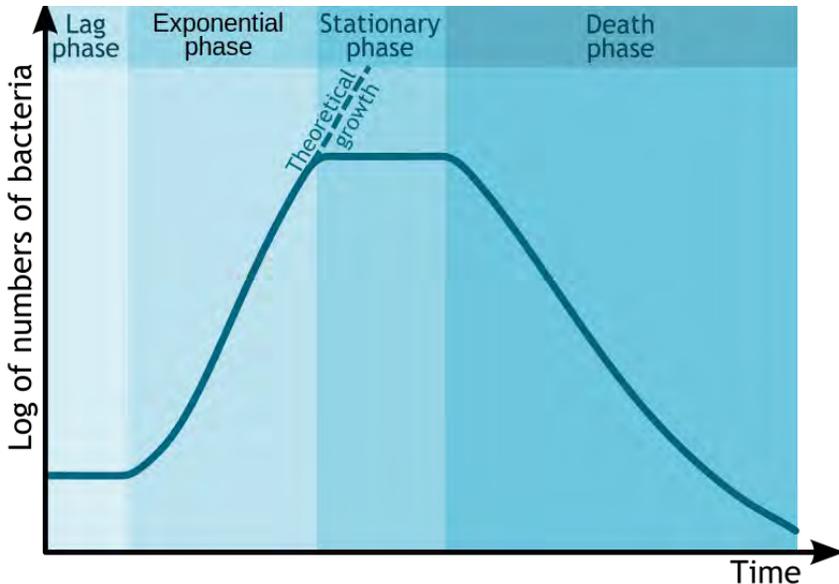


Figure 8. Bacterial growth curve showing the four phases: (A) lag; (B) log or exponential; (C) stationary, and (D) decline (death) – (figure used with permission under Creative Commons license)

Growth on solid media

Liquid growth media containing the nutrients needed for bacterial growth can be solidified with agar, a polysaccharide extracted from seaweed. Heating during sterilisation of the medium melts the agar, which then remains liquid until the temperature falls to approximately 40°C, when it produces a transparent solid gel. Solid media are normally set in Petri dishes ('agar plates'). When spread across the surface of an agar plate, most bacteria grow as visible colonies. Each colony comprises millions of bacterial cells that emanated from either a single cell or a cluster of cells. The appearance of the bacterial colony (colonial morphology) assists in identification.

Growth on laboratory media

To grow bacteria *in vitro*, the microbiologist has to take into account the physiological requirements. Various types of liquid and solid media have been developed for the diagnostic microbiology laboratory.

Simple media

Many bacteria will grow in or on simple media, for example nutrient broth/nutrient agar that contains 'peptone' (polypeptides and amino acids from the enzymatic digestion of meat), and 'meat extract' (water-soluble components of meat containing mineral salts and vitamins).

Enriched media

These contain additional nutrients for the isolation of more fastidious bacteria that require special conditions for growth, e.g. agar containing whole blood (blood agar) or agar containing lysed blood (chocolate agar).

Selective media

These are designed to facilitate growth of some bacteria, while suppressing the growth of others, and include: mannitol salt agar which contains increased NaCl (salt) concentration for the recovery of staphylococci; MacConkey agar, which contains bile salts and allows the growth of bile-tolerant bacteria only, and antibiotics, which are frequently added to media to allow only certain bacteria to grow while suppressing or killing others.

Indicator media

These are designed to aid the detection and recognition of particular pathogens. They are often based on sugar fermentation reactions that result in production of acid and the subsequent colour change of a pH indicator. For example, MacConkey agar contains lactose and a pH indicator (neutral red); lactose-fermenting bacteria (e.g. *Escherichia coli*) produce acid and form pink colonies, and non-lactose fermenting bacteria (e.g. *Salmonella spp.*) do not produce acid and form pale yellow colonies. This property facilitates the recognition of possible *Salmonella* colonies among normal bowel flora. Note that indicator media may also contain selective agents including antibiotics or substances such as bile salts and crystal violet to suppress growth of most Gram-positive microorganisms. MacConkey agar is therefore both a selective medium and an indicator medium.

1.3. Classification of bacteria

The classification of bacteria serves a variety of different functions. Because of this variety, bacteria may be grouped using many different typing schemes. The critical feature for all these classification systems is an organism identified

by one individual (scientist, clinician, epidemiologist), is recognized as the same organism by another individual. At present the typing schemes used by clinicians and clinical microbiologists rely on phenotypic typing schemes. These schemes utilize the bacterial morphology and staining properties of the organism, as well as O₂ growth requirements of the species combined with a variety of biochemical tests. For clinicians, the environmental reservoir of the organism, the vectors and means of transmission of the pathogen are also of great importance.

The Gram stain is a test used to identify bacteria by the composition of their cell walls. It is named for Hans Christian Gram, who developed the technique in 1884. Bacteria are first stained with a purple dye called crystal violet, which specifically binds to peptidoglycan, a complex structure of amino acids and sugars found in the cell wall. This is followed by a series of steps that ultimately remove any unbound or loosely bound crystal violet. Then the cells are stained with a second red-coloured dye called safranin. Gram-positive bacteria stain purple because their cell walls are rich in peptidoglycan. On the other hand, Gram-negative bacteria whose cells walls have two layers take on a red colouring. The outer layer of lipids does not bind strongly to crystal violet and the dye is easily washed away during the staining process. For example, *Streptococcus pneumoniae*, which causes pneumonia, is a Gram-positive bacterium, while *Escherichia coli* and *Vibrio cholerae*, which causes cholera, are Gram-negative bacteria.

Microorganisms can be grouped on the basis of their need for oxygen to grow. Facultative anaerobic bacteria can grow in high oxygen or low oxygen content and are among the more versatile bacteria. In contrast, strictly anaerobic bacteria grow only in conditions where there is minimal or no oxygen present in the environment. Bacteria such as bacteroides found in the large bowel are examples of anaerobes. Strict aerobes only grow in the presence of significant quantities of oxygen. *Pseudomonas aeruginosa*, an opportunistic pathogen, is an example of a strict aerobe. Microaerophilic bacteria grow under conditions of reduced oxygen and sometimes also require increased levels of carbon dioxide. *Neisseria* species (for example, those that cause gonorrhoea) are examples of microaerophilic bacteria.

There are three basic bacterial shapes, according to 'Mims Medical Microbiology'. Round bacteria are referred to as cocci (singular: coccus); cylindrical, capsule-shaped bacteria as bacilli (singular: bacillus); and spiral bacteria are aptly called spirilla (singular: spirillum). Cocci can also associate with one another in different configurations: combinations of two or diplococcus; a linear chain or streptococcus; and a cluster or staphylococcus. The shapes and configurations of bacteria are often reflected in their names.

In 1872, German scientist Ferdinand Cohn classified bacteria to 4 major types, depending on their shapes. Examples of differently-shaped bacteria include:

1. Coccus: These types of bacteria are unicellular, spherical or elliptical. They either remain as a single cell, or can aggregate together in various configurations.
2. Monococcus: Also called micrococcus and represented by a single, discrete round cell. Example: *Micrococcus flavus*.
3. Diplococcus: The cell of the Diplococcus divides once in a particular plane and after division, the cells remain attached to each other. Example: *Diplococcus pneumoniae*.
4. Streptococcus: Here the cells divide repeatedly in one plane to form chain of cells. Example: *Streptococcus pyogenes*.
5. Tetracoccus: Consists of four round cells, which divided in two planes at a right angles to one another. Example: *Gaffkya Tetragena*.
6. Staphylococcus: Here the cells divided into three planes forming a structured like bunches of grapes giving an irregular configuration. Example: *Staphylococcus aureus*.
7. Sarcina: These cells divide in three planes but they form a cube like configuration consisting of eight or sixteen cells but they have a regular shape. Example: *Sarcina Lutea*.
8. Bacilli: Rod-shaped or cylindrical bacteria which either remain singly or in pairs. Example: *Bacillus cereus*.
9. Vibrio: curved, comma-shaped bacteria represented by a single genus. Example: *Vibrio cholerae*.
10. Spirilla: spiral or spring-like, with multiple curvature and terminal flagella. Example: *Spirillum volutans*.

On the basis of nutrition, bacteria are classified as follows:

1. Autotrophic bacteria: these bacteria are non-pathogenic, free living, self-sustaining in nature, which prepare their own food by utilisation of solar energy and inorganic components like carbon dioxide, nitrogen etc.
2. Photoautotrophs: these bacteria contain bacterio-chlorophyll and bacterioviridin and can prepare their own food by fixing carbon dioxide in nature by the utilisation of solar energy.
3. Chemoautotrophs: these bacteria prepare food by deriving the energy from oxidation of inorganic substances like nitrogen dioxide, carbon dioxide etc. They can also fix carbon dioxide and water for their nutrition.
4. Heterotrophic bacteria: this type of bacteria cannot fix inorganic Carbon but rather depend on external organic Carbon for their nourishment. They also can be classified on the basis of presence and absence of flagella and on the basis of the media on which the bacteria are growing.

Classification of bacteria on the basis of number of flagella:

On the basis of their flagella, bacteria can be classified as:

1. Atrichos: These bacteria has no flagella. Example: *Corynebacterium diptheriae*.
2. Monotrichous: One flagellum is attached to one end of the bacteria cell. Example: *Vibrio cholera*.
3. Lophotrichous: Bunch of flagella is attached to one end of the bacteria cell. Example: *Pseudomonas*.
4. Amphitrichous: Bunch of flagella arising from both end of the bacteria cell. Example: *Rhodospirillum rubrum*.
5. Peritrichous: The flagella are evenly distributed surrounding the entire bacterial cell. Example: *Bacillus*.

The classification criteria mentioned thus far are based on physiological properties and morphology. However, classification of bacteria based on their evolutionary relationships to one another, that is to say, drawing a sort of family tree of all bacterial species, is a relatively new development. This type of phylogenetic classification became possible with the advent of nucleotide sequencing technology (the ability to read the order of nucleotides in DNA or RNA). Since ribosomes are present in all living organisms, one can look at similarities and differences in the RNA sequences that encode certain ribosomal proteins and determine the degree of relatedness of different organisms.

Classification of Bacteria Based on Metabolic Characteristics

Bacteria can be divided into 2 major groups, based on their metabolic properties. The two most important metabolic properties used to classify bacteria into groups include:

- How the organism deals with oxygen;
- What the organism used as a carbon and energy source.

Obligate aerobes have all the faculties to carry out oxidative phosphorylation to obtain energy with oxygen quite perfectly. They use glycolysis, the Krebs Cycle and the electron transport chain, just as we do, to obtain the energy they need for their metabolism. Noteworthy is the fact that they are unable to carry out anaerobic respiration, and thus they will definitely die in the absence of oxygen.

Bacteria that are obligate aerobes include:

1. *Nocardia*;
2. *Bacillus cereus*;
3. *Neisseria*;
4. *Pseudomonas*;
5. *Bordetella*;
6. *Legionella*;

7. *Brucella*;
8. *Mycobacterium*;
9. *Leptospira Interrogans*;
10. *Branhame llatatarrhalis*;
11. *Burkholderiacepacia*;
12. *Francisella tularensis*;
13. *Spirillum minus*;
14. *Coxiellaburnetti*.

Facultative anaerobes are the closest analogy to humans. They are able to carry out aerobic respiration quite perfectly, possessing both superoxide dismutase and catalase (not peroxidase). However, their most noteworthy feature is that they are also able to carry out anaerobic respiration. Thus, they are mainly aerobic, but they have the faculty to carry out anaerobic respiration. This is why they are called facultative anaerobes. When the need arises, they have the faculty to carry out fermentation to obtain energy in the absence of oxygen. This is very similar to the anaerobic respiration carried out by human muscle cells during strenuous activity, such as sprinting. Facultative Anaerobes include:

1. *Listeria*;
2. *Actinomyces*;
3. *Bacillus anthracis*;
4. *Coryne bacterium*;
5. *Staphylococcus*;
6. Most other gram negative rods.

Microaerophilic bacteria are aerobic bacteria that require only a very small amount of oxygen to survive, and are poisoned by excessively high oxygen tension. Microaerophilic bacteria include:

1. *Enterococcus*;
2. some *Streptococci* (although some species of *streptococci* are facultative anaerobes);
3. *Helicobacter pylori*;
4. *Spirochetes*;
5. *Treponema*;
6. *Borrelia*;
7. *Leptospira* (except *Leptospira interrogans*);
8. *Campylobacter*.

Obligate Anaerobes don't like oxygen. They have no electron transport chain, and have no enzymes to prevent against oxidative stress. Thus, if they are exposed to oxygen, they die. Obligate Anaerobes include:

1. *Clostridium*;
2. *Bacteroides*;
3. *Fusobacterium*;
4. *Streptobacillus moniliformis*;

5. *Porphyromonas*;
6. *Prevotella*;
7. *Veillonella*;
8. *Peptostreptococcus*.

There is also a division of obligate anaerobes, known as aerotolerant anaerobes. These bacteria require no oxygen as they respire anaerobically, but unlike obligate anaerobes, they can survive in the presence of oxygen.

1.4. Reproduction of bacteria

Most bacteria multiply by a process called binary fission. A single bacterial cell, the 'parent', makes a copy of its DNA and grows large in size by doubling its cellular content. The doubled contents are pushed out to either end of the cell. Then a small fissure emerges at the centre of the parent, eventually splitting it into two identical 'daughter' cells. Some bacterial species such as cyanobacteria and firmicutes reproduce *via* budding. During budding, the daughter cell grows as an offshoot of the parent. It starts off as a small nub, grows until it is the same size as its parent, and splits off.

The DNA found in parents and offspring after binary fission or budding is exactly the same. Therefore bacterial cells try to introduce some variation into their genetic material by integrating additional DNA into their genome. This is known as horizontal gene transfer, and the resulting genetic variation ensures that bacteria can adapt and survive as their environment changes. There are three ways by which this occurs: transformation, transduction and conjugation.

During transformation, bacterial cells integrate short fragments of DNA from their surrounding environment. These fragments may be released by nearby bacteria that have ruptured. On the other hand, transduction occurs when bacteria are infected by special viruses known as bacteriophages that can carry bacterial DNA.

Conjugation requires physical contact between two bacteria. Genetic material, usually a duplicated plasmid, will transfer from a donor to a recipient. This plasmid copy travels out through a physical extension called the pilus and enters the recipient bacterial cell. Donor bacteria contain a sequence of DNA called the F-factor that enables pilus formation. Conjugation can aid in the spread of antibiotic resistance genes.

1.5. Pathogenic bacteria

Pathogenic bacteria are bacteria that can cause infection. This article deals with human pathogenic bacteria. Although most bacteria are harmless or often beneficial, some are pathogenic, with the number of species estimated as fewer

than 100 that are seen to cause infectious diseases in humans. By contrast, several thousand species exist in the human digestive system. One of the bacterial diseases with the highest disease burden is tuberculosis, caused by the bacterium *Mycobacterium tuberculosis*, which kills about 2 million people a year, mostly in sub-Saharan Africa. Pathogenic bacteria contribute to other globally important diseases, such as pneumonia, which can be caused by bacteria such as *Streptococcus* and *Pseudomonas*, and foodborne illnesses, which can be caused by bacteria such as *Shigella*, *Campylobacter*, and *Salmonella*. Pathogenic bacteria also cause infections such as tetanus, typhoid fever, diphtheria, syphilis, and leprosy. Pathogenic bacteria are also the cause of high infant mortality rates in developing countries.

Some pathogenic bacteria cause disease under certain conditions, such as entry through the skin *via* a cut, through sexual activity or through a compromised immune function. *Streptococcus* and *Staphylococcus* are part of the normal skin microbiota and typically reside on healthy skin or in the nasopharyngeal region. Yet these species can potentially initiate skin infections. They are also able to cause sepsis, pneumonia or meningitis. These infections can become quite serious creating a systemic inflammatory response resulting in massive vasodilation, shock, and death. Other bacteria are opportunistic pathogens and cause disease mainly in people suffering from immunosuppression or cystic fibrosis. Examples of these opportunistic pathogens include *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, and *Mycobacterium avium*. Bacterial pathogens often cause infection in specific areas of the body. Others are generalists.

1.5.1. Bacterial toxins

There are two types of bacterial toxins:

- Lipopolysaccharides: associated with the cell walls of Gram- bacteria. The lipopolysaccharide (LPS) component of the Gram- bacteria outer membrane bears the name endotoxin because of its association with the cell wall of bacteria.
- Proteins: may be released into the extracellular environment of pathogenic bacteria. Most of the protein toxins are thought of as exotoxins, since they are 'released' from the bacteria and act on host cells at a distance.

The protein toxins are soluble proteins secreted by living bacteria during exponential growth. The production of protein toxins is specific to a particular bacterial species. For example, only *Clostridium tetani* produces tetanus toxin, and only *Corynebacterium diphtheriae* produces the diphtheria toxin. Usually, virulent strains of the bacterium produce the toxin (or range of toxins) while non-virulent strains do not. Toxin is the major determinant of virulence. Both Gram-positive and Gram-negative bacteria produce soluble protein toxins. Bacterial protein toxins are the most potent poisons known and may show activity at very high dilutions.

The protein toxins resemble enzymes. Like enzymes, bacterial exotoxins are: proteins denatured by heat, acid, proteolytic enzymes have a high biological activity (most act catalytically) exhibit specificity of action. Bacterial protein toxins are highly specific in the substrate utilized and in their mode of action. Usually the site of damage caused by the toxin indicates the location of the substrate for that toxin. Terms such as 'enterotoxin', 'neurotoxin', 'leukocidin' or 'hemolysin' are used to indicate the target site of some well-defined protein toxins. Certain protein toxins have very specific cytotoxic activity (i.e. they attack specific cells, for example, tetanus or botulinum toxins). Some (as produced by *staphylococci*, *streptococci*, *clostridia*, etc.) have fairly broad cytotoxic activity and cause the nonspecific death of tissues (necrosis). Toxins that are phospholipases may be relatively nonspecific in their cytotoxicity. This is also true of pore-forming 'hemolysins' and 'leukocidins'. A few protein toxins cause death of the host and are known as 'lethal toxins' (e.g. anthrax toxin).

Protein toxins are strongly antigenic. *In vivo*, specific antibody (antitoxin) neutralizes the toxicity of these bacterial proteins. *In vitro*, specific antitoxin may not fully inhibit their enzymatic activity. Protein toxins are inherently unstable: in time they lose their toxic properties but retain their antigenic ones. Toxoids are detoxified toxins which retain their antigenicity and their immunizing capacity (first discovered by Ehrlich). The formation of toxoids can be accelerated by: treating toxins with a variety of reagents including formalin, iodine, pepsin, ascorbic acid, ketones, etc. The mixture is maintained at 37°C at pH range 6 to 9 for several weeks. Toxoids can be used for artificial immunization against diseases caused by pathogens where the primary determinant of bacterial virulence is toxin production. E.g. immunizing against diphtheria and tetanus that are part of the DPT vaccine.

Many protein toxins consist of two components: Sub-unit A, which is responsible for the enzymatic activity of the toxin, and Sub-unit B, which is concerned with binding to a specific receptor on the host cell membrane and transferring the enzyme across the membrane. The enzymatic component is not active until it is released from the native toxin. Isolated A sub-units are enzymatically active but lack binding and cell entry capability. Pertussis toxin produced by *Campylobacter* is a member of the A-B bacterial toxin superfamily. These are cells that even block the binding of a hexameric protein comprising native A+B toxin. Endotoxins are part of the outer cell wall of bacteria. Invariably associated with Gram-negative bacteria as constituents of the outer membrane of the cell wall. Endotoxin: Occasionally used to refer to any 'cell-associated' bacterial toxin. But should be reserved for the lipopolysaccharide complex associated with the outer envelope of Gram-negative bacteria such as *E. coli*, *Salmonella*, *Shigella*, *Pseudomonas*, *Neisseria*, *Haemophilus*, and other leading pathogens. Lipopolysaccharide (LPS) participates: in a number of outer membrane functions essential for bacterial growth and survival, especially within the context of a host-parasite interaction.

The biological activity of endotoxin is associated with the lipopolysaccharide (LPS). Toxicity is associated with the lipid component (Lipid A) and Immunogenicity (antigenicity) is associated with the polysaccharide components. The cell wall antigens (O antigens) of Gram- bacteria are components of LPS. LPS activates complement by the alternative (properdin) pathway and may be a part of the pathology of most Gram-negative bacterial infections. Most part of endotoxins remain associated with the cell wall until disintegration of the bacteria. In vivo, this results from autolysis, external lysis, and phagocytic digestion of bacterial cells. Small amounts of endotoxin may be released in a soluble form, especially by young cultures. Compared to the classic exotoxins of bacteria, endotoxins are less potent and less specific in their action, since they do not act enzymatically. Endotoxins are heat stable (boiling for 30 minutes does not destabilize endotoxin), but certain powerful oxidizing agents such as, superoxide, peroxide and hypochlorite degrade them. Endotoxins, although strongly antigenic, cannot be converted to toxoids.

Endotoxin lipopolysaccharides: are complex amphiphilic molecules with a mw of about 10 kDa, that vary widely in chemical composition both between and among bacterial species. In a basic ground plan common to all endotoxins, LPS consists of three components:

- Lipid A;
- Core polysaccharide;
- Polysaccharide.

Lipid A is the Lipid component of LPS. Contains the hydrophobic, membrane-anchoring region of LPS. Lipid A consists of a phosphorylated N-acetylglucosamine (NAG) dimer with 6 or 7 fatty acids (FA) attached. 6 FA are found. All FA in Lipid A are saturated. Some FA are attached directly to the NAG dimer and others are esterified to the 3-hydroxy fatty acids that are characteristically present. The structure of Lipid A is highly conserved among Gram- bacteria. Among *Enterobacteriaceae* Lipid A is virtually constant.

Core (R) polysaccharide is attached to the 6 position of one NAG. The R antigen consists of a short chain of sugars. For example: KDO – Hep – Hep – Glu – Gal – Glu – GluNAc. Two unusual sugars, present Heptose and 2-keto-3-deoxyoctonic acid (KDO), in the core polysaccharide. KDO is unique and invariably present in LPS and so has been an indicator in assays for LPS (endotoxin). Core polysaccharide is common to all members of a bacterial genus with minor variations (for example, *Salmonella*). But it is structurally distinct in other genera of Gram-negative bacteria. *Salmonella*, *Shigella* and *Escherichia* have similar but not identical cores.

O polysaccharides (also referred to as the O antigen or O side chain) are attached to the core polysaccharide. Consists of repeating oligosaccharide sub-units made up of 3–5 sugars. The individual chains vary in length ranging up to 40 repeat units. O polysaccharide is much longer than the core polysaccharide

and it maintains the hydrophilic domain of the LPS molecule. A unique group of sugars, called dideoxyhexoses, occurs in the O polysaccharide. A major antigenic determinant (antibody-combining site) of the Gram-negative cell wall resides in the O polysaccharide. Great variation occurs in the composition of the sugars in the O side chain between species and even strains of Gram-negative bacteria. LPS and virulence of Gram-negative bacteria Endotoxins are toxic to most mammals. They are strong antigens but they seldom elicit immune responses which give full protection to the animal against secondary challenge with the endotoxin. Endotoxins released from multiplying or disintegrating bacteria significantly contribute to the symptoms of Gram- bacteraemia and septicaemia important pathogenic factors in Gram-negative bacteria infections.

Regardless of the bacterial source all endotoxins produce the same range of biological effects in the animal host. Injection of living or killed Gram-cells, or purified LPS, into experimental animals wide spectrum of nonspecific pathophysiological reactions related to inflammation such as: fever changes in white blood cell counts disseminated intravascular coagulation tumour necrosis hypotension shock.

Physiological activities of endotoxins are mediated mainly by the Lipid A component of LPS. Its biological activity depends on a peculiar conformation determined by the glucosamine disaccharide, PO₄ groups, Acyl chains, and KDO-containing inner core. Lipid A is known to react at the surfaces of macrophages release cytokines that mediate the pathophysiological response to endotoxin.

1.5.2. Pathogenicity Islands

Pathogenicity Islands (PAI) are a distinct class of genomic islands which are acquired by horizontal gene transfer. Incorporated in the genome of pathogenic bacteria usually absent from non-pathogenic organisms of the same or closely related species. They occupy relatively large genomic regions ranging from 10–200 kb encode genes which contribute to virulence of the pathogen. Typical examples: adhesins, toxins, iron uptake systems, invasins, etc.

One species of bacteria may have more than one pathogenicity island. For example, in *Salmonella*, five pathogenicity islands have been identified. Found mainly in Gram negative bacteria, but have been shown in a few Gram-positives. Found in pathogens that undergo gene transfer by plasmid, phage, or a conjugative transposon and are typically transferred through mechanisms of horizontal gene transfer (HGT). May be located on the bacterial chromosome or may be a part of a plasmid. They are rich in Guanine + Cytosine content. They are flanked by direct repeats i.e. the sequence of bases at two ends are the same. Are associated with tRNA genes, which target sites for the integration of DNA. Have characteristics of transposons in that they carry functional genes e.g. integrase, transposase, or part of insertion sequences may move from one tRNA locus to another on the chromosome or plasmid.

Pathogenicity Islands play a vital role in the virulence of bacterial pathogens of humans, animals and plants. The availability of a large number of genome sequences of pathogenic bacteria and their non-pathogenic relatives identification of novel pathogen-specific genomic islands. PAI apparently -acquired during the speciation of pathogens from their non-pathogenic or environmental ancestors. The acquisition of PAI is an ancient evolutionary event that led to the appearance of bacterial pathogens on a timescale of millions of years May also represent a mechanism that contributes to the appearance of new pathogens. Pathogenicity Islands Knowledge about PAI, their structure, their mobility, and the pathogenicity factors they encode is helpful in gaining a better understanding of bacterial evolution and interactions of pathogens with eukaryotic host cells.

PAI can also have important practical implications such as providing delivery systems for vaccination, and tools for the development of new strategies for bacterial infection therapy.

Pathogenicity Islands represent distinct genetic elements encoding virulence factors of pathogenic bacteria. Belong to a more general class of genomic islands common genetic elements sharing a set of unifying features. Genomic islands have been acquired by horizontal gene transfer. In recent years many different genomic islands have been discovered in a variety of pathogenic as well as non-pathogenic bacteria. Because they promote genetic variability, genomic islands play an important role in microbial evolution.

1.5.3. Mechanism of pathogenesis

An infectious disease is a clinically evident deviation from health. It occurs when there is a parasitic relationship between a host and a microorganism. Several different factors influence a microorganism's relationship to its host and level of severity. These include:

- Pathogenicity: The ability to produce disease in a host organism.
- Virulence: The degree of pathogenicity of a microorganism. Determinants of virulence for a pathogen include a pathogen's genetic, biochemical, or structural features. For example, one strain of influenza may only cause a fever and sore throat, while another may cause pneumonia or other serious respiratory condition.
- Infectivity: The level at which a microorganism is able to infect or invade a host.
- Transmissibility: The measure of a microorganism's ability to spread from one host to the next. This can include both distance and number of affected individuals.

Over a century ago, Robert Koch established that infectious diseases were caused by microbes. He was looking for the causative agent for anthrax. Koch's

postulates are experimental criteria that are used to determine if a microbe caused a specific disease. The criteria include:

1. The microbe must be present in every case of the disease.
2. The organism must be grown in a pure culture from diseased hosts.
3. The same disease must be produced when a pure culture of the organism is introduced into a susceptible host.

4. The organism must be recovered from the experimentally infected hosts.

However, there are some exceptions to these criteria. These include:

1. Some organisms cannot be cultured in a lab and grown on artificial media.
2. Some pathogens can cause several disease conditions such as *M. tuberculosis*, which can cause lung disease and other diseases of the skin, bone, and internal organs.

3. There may be ethical reasons that do not allow testing, (i.e. human diseases with no animal model – smallpox, rubella).

Pathogenesis is the method by which a disease can develop. This can occur through foodborne intoxication where the causative agent produces toxins in the body (e.g. botulism). Another route is the colonization of an invading pathogen on the host surface, which allows the pathogen to increase in numbers and produce toxins that are damaging to the host's cells (e.g. *Vibrio* and *Corynebacterium*). Pathogenesis can also occur by pathogens invading and breaching the body's barrier in order to multiply. These organisms have mechanisms that will not allow macrophages (the body's defence against pathogens) to destroy them. They can also evade antibody detection (e.g. tuberculosis and plague). Finally, organisms can invade tissues within the body and produce toxins (e.g. *Shigella*). The relationship between a host and pathogen is dynamic. Production of disease occurs through a process of steps. The first five mechanisms make up a pathogen's invasiveness (i.e. ability to invade tissues).

Transmission

In order to begin infection and eventually cause disease, pathogens must find a transmission route. Transmission of an infectious agent can occur in many ways, but it is typically through exposed skin (e.g. a cut, abrasion, puncture, or wound) or mucous membranes (e.g. gastrointestinal tract, respiratory tract, or urogenital tract).

Adherence

Once the pathogen has gained access to the body, it must have some means of attaching itself to the host's tissues. This attachment is called adherence and is a necessary step in pathogenicity. Microbes contain ligands, which are projections that attach host receptors or surface proteins. Pathogens may have

specific adherence mechanisms to attach to cells or tissue surfaces. Examples of this include:

1. Tissue tropism (i.e. pathogens that prefer specific tissues over others),
2. Species specificity (i.e. pathogens that only infect certain species), and
3. Genetic specificity (i.e. surface mutations that occur so previous antibodies do not recognize the invading pathogen). If a microorganism cannot adhere to a host cell membrane, disease will not occur.

Invasion

At this point, microbes begin to invade the host and produce a bacteraemia (i.e. presence of bacteria in the bloodstream) or viremia (presence of a virus in the bloodstream). Microorganisms are exposed to many barriers after introduction into the host. Some bacteria are able to cause disease while remaining on the epithelial barriers, while many need to penetrate that barrier. Once this barrier has been penetrated, these pathogens can multiply without competition.

Colonization

Colonization is the multiplication of pathogenic organisms where toxins are produced and the normal flora are overcome. During this stage, pathogens compete with normal flora for space and nutrients. Pathogens usually colonize host tissues that are in contact with the external environment. During colonization, the host begins to show signs of septicaemia (i.e. blood infection where bacteria are reproducing). For infection to proceed an infectious dose should be determined. This is the minimal number of microbes necessary to establish infection. Certain pathogens are less contagious and therefore require larger numbers of pathogens to cause disease (i.e. 10–100 for *Shigella* and 1,000,000 for *Salmonella*).

Evasion of Host Defences

After colonization, pathogens circumvent the host's innate and adapted defences by phagocytosis. Multiple mechanisms are used by pathogens to evade a host's immune system. For the innate system this includes:

- Intracellular pathogens that live inside a host cell;
- Avoid phagocyte recognition by producing capsules prevents phagocytosis;
- Producing membrane damaging toxins which can kill phagocytes (e.g. leukocidins);
- Interfere with complement activation;
- Survive in the phagocyte.

Pathogens must also avoid adapted defences. Pathogens can produce proteases (i.e. allow each pathogen to avoid antibodies), or catalases (i.e. prevent the digestion of an engulfed pathogen). They can also utilize antigenic variation to alter the antigen structure. In addition, pathogens can mimic host molecules, which can cause disease-related damage.

Exiting the Host

A pathogen must exit the body. This occurs through various routes. Examples include sneezing, coughing, diarrhoea, coitus, pus, blood, or insect bites.

Survival Outside the Host

Finally, a pathogen must be able to survive in the environment long enough to be transmitted to another host. Some are hardy and can survive for several weeks before a new host is found. There are others that survive in animal reservoirs or require direct contact because they are fragile.

1.5.4. Antibiotic resistance

Antibiotics are typically used to treat bacterial infections. However in recent years, the improper or unnecessary use of antibiotics has promoted the spread of several strains of antibiotic-resistant bacteria.

Antibiotic resistance is a phenomenon where infectious bacteria are no longer susceptible to previously effective antibiotics. According to the CDC, each year in the United States, at least 2 million people are infected with antibiotic resistant bacteria, leading to the death of at least 23,000 each year.

One of the more notorious antibiotic resistant bacterial strains is methicillin-resistant *Staphylococcus aureus* (MRSA), which resists methicillin and other antibiotics used to treat *Staphylococcus* infections. It spreads primarily through skin contact. MRSA infections occur in health care settings such as hospitals and nursing homes, where it can lead to pneumonia or bloodstream infections. MRSA also spreads in the community, especially in situations where there is a lot of skin contact or the use of shared equipment; for example, among athletes, in tattoo parlours, or in day care facilities and schools. Community-acquired MRSA most often causes skin infections.

1.6. Viruses

The concept behind modern virology can be traced back to Adolf Mayer, Dimitri Ivanofsky and Martinus Beijerinck who, independently in the late 1880's, discovered what was later to be called tobacco mosaic virus (TMV). Their

discoveries led to the descriptions of filterable agents, too small to be seen with the light microscope, that could be grown in living cells and cause disease. The first filterable agent from animals, foot and mouth disease virus, was described by Loeffler and Frosch in 1898, and the first human filterable agent discovered yellow fever virus, discovered by Walter Reed in 1901. The term 'virus' derives from the Latin for slimy liquid or poison and was gradually introduced during this period to replace the term 'filterable agents'. The first virus to be visualized by x-ray crystallography and electron microscopy was TMV, reported in 1941 and 1939, respectively. These advances introduced the notion that viruses were structurally composed of repeating sub-units. Frederick Twort and Felix d'Herelle, working independently, are credited with the discovery of viruses which could infect and lyse bacteria in 1915. D'Herelle introduced the term 'bacteriophages' for these agents and also described the concepts of virus adsorption to its target, cell lysis and release of infectious particles. Over the next 35–40 years, work with phages led to numerous discoveries including how the introduction of DNA into a target cell could reproduce itself and the regulation of cellular macromolecular synthesis directed by viruses. In essence, the field of molecular biology was opened up during this period. Advances in animal virology were noted throughout the 20th century but the major breakthrough came through the development of tissue culture systems that led, for example, to the isolation of poliovirus by Enders et al. in 1949. This markedly facilitated detailed study of this agent and, most importantly, the development of poliovirus vaccines. The ensuing 60 years have seen diagnostic virology mature as a field with the discovery of new agents and diseases and the parallel determination of the importance of viruses in our understanding of molecular biology and cancer.

1.6.1. Virus structure, classification

A. Virus particle or virion. An infectious agent composed of nucleic acid (RNA or DNA), a protein shell (capsid) and, in some cases, a lipid envelope. Virions have full capacity for replication when a susceptible target cell is encountered.

– Capsid and capsomeres. The protein coat that surrounds the viral nucleic acid. This is composed of repeating protein sub-units called capsomeres. Generally, capsids have either helical or icosahedral symmetry.

– Nucleocapsid. The complete protein-nucleic acid complex.

B. Satellite or Defective Viruses. Viruses which require a second virus (helper virus) for replication. Hepatitis delta virus is the major human pathogen example. It requires the presence of hepatitis B virus to complete its replication cycle.

C. Viroids. Viroids are the smallest known autonomously replicating molecules. They consist of single-stranded, circular RNA, 240–375 residues in length and are plant pathogens.

D. Prions. Prions are not viruses but are often discussed within this microbiologic category. Prions are infectious protein molecules that contain

no definable nucleic acid and are responsible for the transmissible and familial spongiform encephalopathies: Creutzfeldt-Jakob disease, kuru, fatal familial insomnia, Gerstmann-Straussler-Sheinker syndrome, and bovine spongiform encephalopathy ('mad cow disease'). The pathogenic prion protein, PrP^{Sc}, is formed from a normal human protein, PrP^C.

Viral classification has been confusing and oft-changing over the years. In the past, viruses were often classified by host, target organ or vector and these are still used vernacularly (e.g. the hepatitis viruses). Modern classification is based on the following three characteristics:

1. The type of viral nucleic acid (RNA or DNA, single-stranded or double-stranded).
2. Its replication strategy.
3. The capsid symmetry (icosahedral or helical).

DNA viruses

– Double-stranded DNA viruses include poxviruses, herpesviruses, adenoviruses, papova viruses and polyomaviruses.

– Single-stranded DNA viruses include parvoviruses. DNA viruses usually replicate in the nucleus of host cells by producing a polymerase that reproduces viral DNA. Viral DNA is not usually incorporated into host chromosomal DNA.

RNA viruses

RNA viruses possess a single strand of RNA and adopt different reproductive strategies:

– RNA sense (positive) may serve directly as mRNA and be translated into structural protein and an RNA-dependent RNA polymerase.

– RNA antisense (negative) contains an RNA-dependent RNA polymerase that transcribes the viral genome into mRNA. Alternatively, the transcribed RNA can act as a template for further viral (antisense) RNA.

– Retroviruses have single-stranded sense RNA that cannot act as mRNA. This is transcribed into DNA by reverse transcriptase and incorporated into host DNA. The subsequent transcription to make mRNA and viral genomic RNA is under the control of host transcriptase enzymes.

1.6.2. Pathogenesis of Viral Diseases

As with other infectious agents which cause human disease, the outcome of the interaction of a particular virus with the human host is dependent on both pathogen and host factors. Viral strains within a genus may have differential cell tropisms, replication capacities and cytopathogenic effects. As an example, strains of HIV may

preferentially target monocyte/macrophages or T-lymphocytes, may use different co-receptors (e.g. the chemokine receptors, CCR5 or CXCR4) on the cell surface, may replicate to different levels and may induce different degrees of cell killing. These traits have direct clinical correlates for HIV infected persons with respect to the rates of CD4 cell decline and progression to clinical AIDS. On the host side, the nature of the exposure and the host immune status are probably the two most important determinants of outcome. Thus, the key elements of the virus host interaction are:

1. Viral strain.
2. Inoculum size.
3. Route of exposure.
4. Susceptibility of the host (i.e. is there pre-existent immunity from past exposure or vaccination?).
5. Immune status and age of host.

A generalized schema of viral infection leading to disease in the human host is as follows:

1. Depending upon the agent, the virus enters through the skin, mucous membranes, respiratory tract, gastrointestinal tract, *via* a transfusion or transplanted organ or *via* maternal-foetal transmission.

2. There is local replication at the site of the inoculation. Certain agents exhibit pathology at the skin or mucous membrane surface – e.g. herpes simplex virus, human papillomavirus.

3. For some neurotropic viruses there may be spread along peripheral nerve routes to ganglia (e.g. herpes simplex virus) or the central nervous system (e.g. rabies virus). For other neurotropic agents, the central nervous system is seeded following viremia.

4. For many agents, there is replication in regional lymph nodes with subsequent viremia and spread to target organs. Some viruses travel in the bloodstream free in plasma (e.g. picornaviruses); others are cell associated (e.g. cytomegalovirus (CMV)).

5. Replication in target organs may lead to local damage and further rounds of viremia.

6. Non-specific and specific host immune responses come into play to try to control and downregulate the viral replicative process.

1.6.3. Immune Responses to Viral Infections

1. Innate (non-specific) immunity refers to those elements of the immune system that can clear virus or virus infected cells immediately upon or shortly after viral exposure and which are not dependent upon immunologic memory.

Non-specific immunity may include:

- a) Phagocytic cells (neutrophils and monocyte/macrophages).
- b) Cytokines (e.g. interferons) and chemokines.

- c) Natural killer cells.
 - d) Poorly defined antiviral factors that may exist in blood or body fluids.
2. Adaptive (specific) immunity refers to antigen specific B and T cell responses that lead to the development of antibodies, cytotoxic T cells and antibody dependent cellular cytotoxicity.
3. In some instances, an intense immunologic reaction to a viral agent can result in immunopathology and a serious clinical syndrome. A prime example is dengue haemorrhagic fever which is likely due to antibody dependent enhancement and T cell activation on re-exposure to dengue virus.

1.6.4. Mechanisms of Viral Persistence

Viruses may cause chronic, persistent infection with continuous viral replication in the face of an immune response. Examples include HIV, hepatitis B virus and hepatitis C virus. Some viruses may demonstrate persistent infection in immune compromised hosts. These include the herpesviruses, human papillomavirus and rubella virus, among others. Some viruses are able to cause latent infection. Latency is characterized by a quiescent or minimally transcriptionally active viral genome with periods of reactivation. Latent viruses include the herpesviruses (cytomegalovirus, Epstein-Barr virus, herpes simplex virus, varicella-zoster virus), human papillomavirus, human retroviruses. Recurrent herpes labialis (cold sores) or genital herpes due to HSV, or herpes zoster due to varicella zoster virus are classic examples of latency and reactivation. Viruses which exhibit latency may also exhibit chronic, persistent replication in the setting of immune compromise of the host.

Mechanisms of persistence of viruses which produce chronic infections include antigenic variation to escape antibody or cytotoxic T cell responses, downregulation of class I major histocompatibility antigens resulting in diminished recognition by cytotoxic T cells and modulation of apoptosis. Viruses which establish latent infection escape recognition by the immune system through decreased viral antigen expression and presentation. Sites of persistence include the nervous system (herpes simplex virus, varicella zoster virus, measles virus, poliovirus, JC virus), the liver (hepatitis B virus, hepatitis C virus), and leukocytes (HIV, cytomegalovirus, Epstein-Barr virus).

1.6.5. The Viral Life Cycle

All viruses depend on cells for reproduction and metabolic processes. By themselves, viruses do not encode for all of the enzymes necessary for viral replication. But within a host cell, a virus can commandeer cellular machinery to produce more viral particles. Bacteriophages replicate only in the cytoplasm, since prokaryotic cells do not have a nucleus or organelles. In eukaryotic

cells, most DNA viruses can replicate inside the nucleus, with an exception observed in the large DNA viruses, such as the poxviruses, that can replicate in the cytoplasm. RNA viruses that infect animal cells often replicate in the cytoplasm.

The Lytic Cycle

During the lytic cycle of virulent phage, the bacteriophage takes over the cell, reproduces new phages, and destroys the cell. T-even phage is a good example of a well-characterized class of virulent phages. There are five stages in the bacteriophage lytic cycle. Attachment is the first stage in the infection process in which the phage interacts with specific bacterial surface receptors (e.g. lipopolysaccharides and OmpC protein on host surfaces). Most phages have a narrow host range and may infect one species of bacteria or one strain within a species. This unique recognition can be exploited for targeted treatment of bacterial infection by phage therapy or for phage typing to identify unique bacterial subspecies or strains. The second stage of infection is entry or penetration. This occurs through contraction of the tail sheath, which acts like a hypodermic needle to inject the viral genome through the cell wall and membrane. The phage head and remaining components remain outside the bacteria.

The third stage of infection is biosynthesis of new viral components. After entering the host cell, the virus synthesizes virus-encoded endonucleases to degrade the bacterial chromosome. It then hijacks the host cell to replicate, transcribe, and translate the necessary viral components (capsomeres, sheath, base plates, tail fibres, and viral enzymes) for the assembly of new viruses. Polymerase genes are usually expressed early in the cycle, while capsid and tail proteins are expressed later. During the maturation phase, new virions are created. To liberate free phages, the bacterial cell wall is disrupted by phage proteins such as holin or lysozyme. The final stage is release. Mature viruses burst out of the host cell in a process called lysis and the progeny viruses are liberated into the environment to infect new cells.

The Lysogenic Cycle

In a lysogenic cycle, the phage genome also enters the cell through attachment and penetration. A prime example of a phage with this type of life cycle is the lambda phage. During the lysogenic cycle, instead of killing the host, the phage genome integrates into the bacterial chromosome and becomes part of the host. The integrated phage genome is called a prophage. A bacterial host with a prophage is called a lysogen. The process in which a bacterium is infected by a temperate phage is called lysogeny. It is typical of temperate phages to be latent or inactive

within the cell. As the bacterium replicates its chromosome, it also replicates the phage's DNA and passes it on to new daughter cells during reproduction. The presence of the phage may alter the phenotype of the bacterium, since it can bring in extra genes (e.g. toxin genes that can increase bacterial virulence). This change in the host phenotype is called lysogenic conversion or phage conversion. Some bacteria, such as *Vibrio cholerae* and *Clostridium botulinum*, are less virulent in the absence of the prophage. The phages infecting these bacteria carry the toxin genes in their genome and enhance the virulence of the host when the toxin genes are expressed. In the case of *V. cholera*, phage encoded toxin can cause severe diarrhoea; in *C. botulinum*, the toxin can cause paralysis. During lysogeny, the prophage will persist in the host chromosome until induction, which results in the excision of the viral genome from the host chromosome. After induction has occurred the temperate phage can proceed through a lytic cycle and then undergo lysogeny in a newly infected cell.

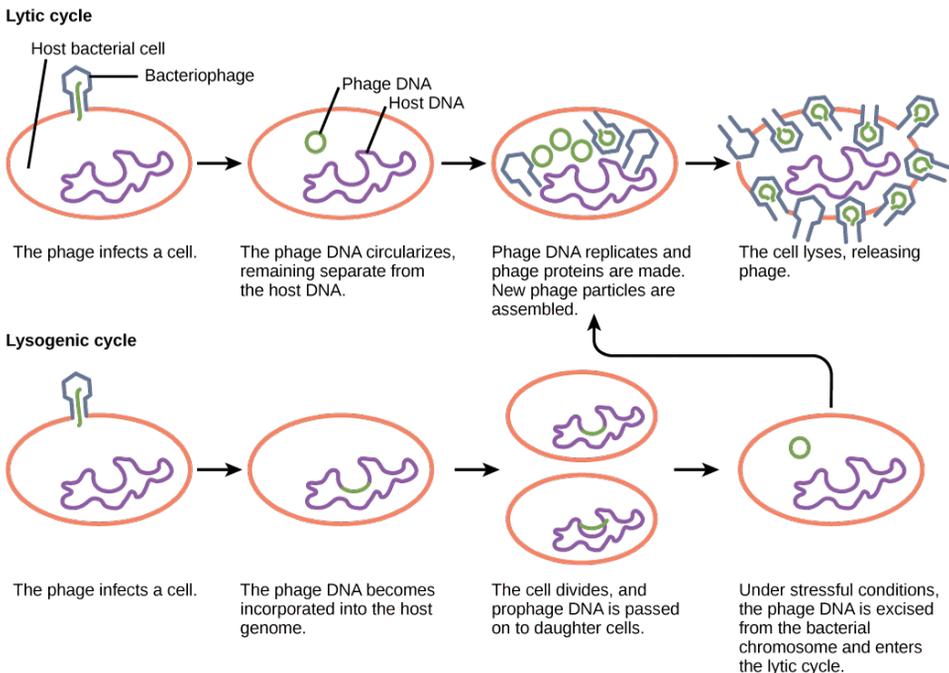


Figure 9. Scheme of both lytic and lysogenic cycles. In the lysogenic cycle, phage DNA is incorporated into the host genome, forming a prophage, which is passed on to subsequent generations of cells. Environmental stressors such as starvation or exposure to toxic chemicals may cause the prophage to be excised and enter the lytic cycle (figure used with permission under Creative Commons license)

Life Cycle of Viruses with Animal Hosts

Lytic animal viruses follow similar infection stages to bacteriophages: attachment, penetration, biosynthesis, maturation, and release. However, the mechanisms of penetration, nucleic-acid biosynthesis, and release differ between bacterial and animal viruses. After binding to host receptors, animal viruses enter through endocytosis (engulfment by the host cell) or through membrane fusion (viral envelope with the host cell membrane). Many viruses are host specific, meaning they only infect a certain type of host; and most viruses only infect certain types of cells within tissues. This specificity is called a tissue tropism. Examples of this are demonstrated by the poliovirus, which exhibits tropism for the tissues of the brain and spinal cord, or the influenza virus, which has a primary tropism for the respiratory tract.

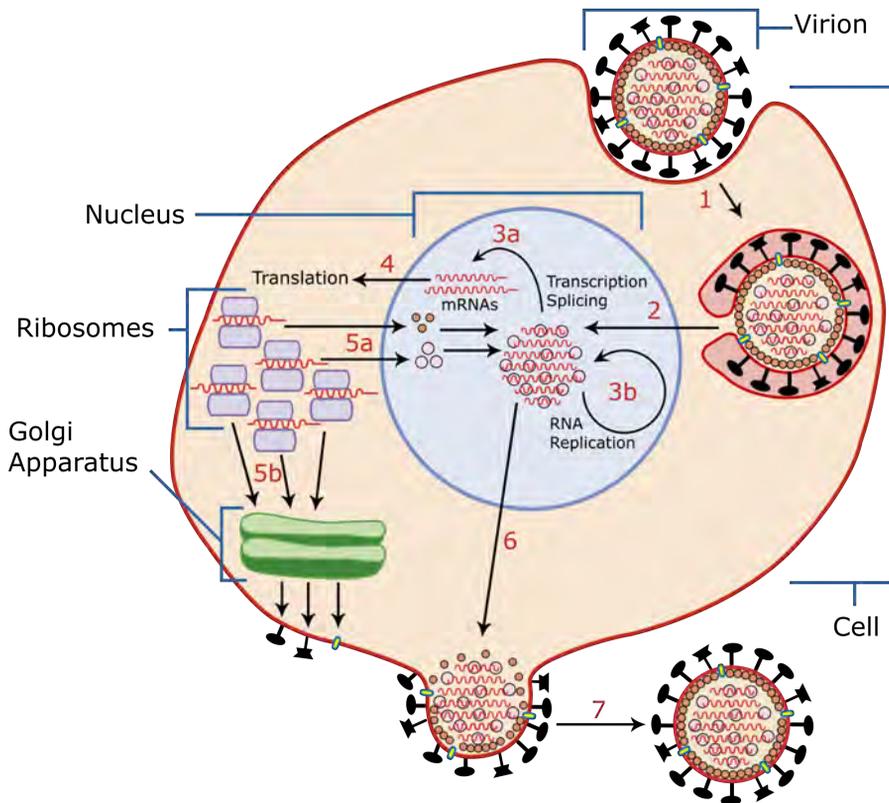


Figure 10. Virus cycle in animal cell. Viral glycoproteins attach the virus to a host epithelial cell. As a result, the virus is engulfed. Viral RNA and viral proteins are made and assembled into new virions that are released by budding (*figure used with permission under Creative Commons license*)

Animal viruses do not always express their genes using the normal flow of genetic information – from DNA to RNA to protein. Some viruses have a dsDNA genome like cellular organisms and can follow the normal flow. However, others may have ssDNA, dsRNA, or ssRNA genomes. The nature of the genome determines how the genome is replicated and expressed as viral proteins. If a genome is ssDNA, host enzymes will be used to synthesize a second strand that is complementary to the genome strand, thus producing dsDNA. The dsDNA can now be replicated, transcribed, and translated similar to host DNA.

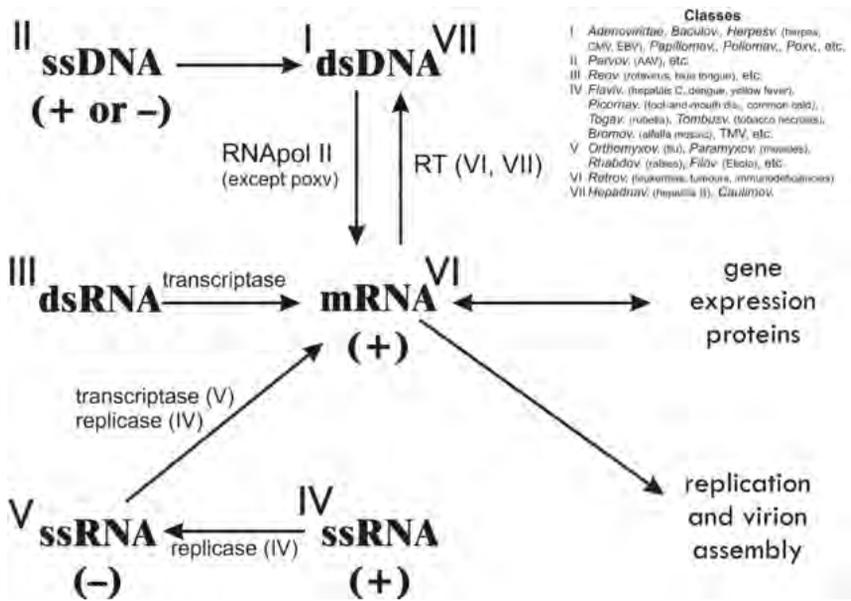


Figure 11. Animal virus classification (figure used with permission under Creative Commons license)

If the viral genome is RNA, a different mechanism must be used. There are three types of RNA genome: dsRNA, positive (+) single-strand (+ssRNA) or negative (-) single-strand RNA (-ssRNA). If a virus has a +ssRNA genome, it can be translated directly to make viral proteins. Viral genomic +ssRNA acts like cellular mRNA. However, if a virus contains a -ssRNA genome, the host ribosomes cannot translate it until the -ssRNA is replicated into +ssRNA by viral RNA-dependent RNA polymerase (RdRP). The RdRP is brought in by the virus and can be used to make +ssRNA from the original -ssRNA genome. The RdRP is also an important enzyme for the replication of dsRNA viruses, because it uses the negative strand of the double-stranded genome as a template to create +ssRNA. The newly synthesized +ssRNA copies can then be translated by cellular ribosomes.

An alternative mechanism for viral nucleic acid synthesis is observed in the retroviruses, which are +ssRNA viruses. Single-stranded RNA viruses such as HIV carry a special enzyme called reverse transcriptase within the capsid that synthesizes a complementary ssDNA (cDNA) copy using the +ssRNA genome as a template. The ssDNA is then made into dsDNA, which can integrate into the host chromosome and become a permanent part of the host. The integrated viral genome is called a provirus. The virus can now remain in the host for a long time to establish a chronic infection. The provirus stage is similar to the prophage stage in a bacterial infection during the lysogenic cycle. However, unlike prophage, the provirus does not undergo excision after splicing into the genome.

1.7. The action of physical and chemical agents on microorganisms

Sterilization is the killing or removal of all microorganisms, including bacterial spores which are highly resistant. Sterilization is an absolute term, i.e. the article must be sterile meaning the absence of all microorganisms.

Disinfection is the killing of many, but not all microorganisms. It is a process of reduction of number of contaminating organisms to a level that cannot cause infection, i.e. pathogens must be killed. Some organisms and bacterial spores may survive.

Disinfectants are chemicals that are used for disinfection. Disinfectants should be used only on inanimate objects.

Antiseptics are mild forms of disinfectants that are used externally on living tissues to kill microorganisms, e.g. on the surface of skin and mucous membranes.

Sterilization and disinfection are done by:

- A. Physical Agents
 1. Heat
 2. Radiation
 3. Filtration
- B. Chemical Agents

In practice, certain methods are placed under sterilization which in fact do not fulfil the definition of sterilization such as boiling for 1/2 h and pasteurization which will not kill spores.

1.7.1. Sterilization by heat

Heat is most effective and a rapid method of sterilization and disinfection. Excessive heat acts by coagulation of cell proteins. Less heat interferes metabolic reactions. Sterilization occurs by heating above 100°C which ensure lolling of bacterial spores. Sterilization by hot air in hot air oven and sterilization by autoclaving are the two most common method used in the laboratory.

Types of Heat treatment:

- Sterilization by moist heat;
- Sterilization by dry heat.

Sterilization by Moist Heat

Moist heat acts by denaturation and coagulation of protein, breakage of DNA strands, and loss of functional integrity of cell membrane. Boiling at 100°C for 30 minutes is done in a water bath. Syringes, rubber goods and surgical instruments may be sterilized by this method. All bacteria and certain spores are killed. It leads to disinfection.

I. Steaming

Steam (100°C) is more effective than dry heat at the same temperature as: (a) Bacteria are more susceptible to moist heat, (b) Steam has more penetrating power, and (c) Steam has more sterilizing power as more heat is given up during condensation. A steam Sterilizer works at 100°C under normal atmospheric pressure i.e. without extra pressure. It is ideally suitable for sterilizing media which may be damaged at a temperature higher than 100°C. It is a metallic vessel having 2 perforated diaphragms (Shelves), one above boiling water, and the other about 4 feet above the floor. Water is boiled by electricity, gas or stove. Steam passes up. There is a small opening on the roof of the instrument for the escape of steam. Sterilization is done by two methods:

Single Exposure for 1 1/2 hours. It leads to disinfection.

Tyndallization (Fractional Sterilization). Heat labile media like those containing sugar, milk, gelatine can be sterilized by this method. Steaming at 100°C is done in steam sterilizer for 20 minutes followed by incubation at 37°C overnight. This procedure is repeated for another 2 successive days. That is 'steaming' is done for 3 successive days. Spores, if any, germinate to vegetative bacteria during incubation and are destroyed during steaming on second and third day. It leads to sterilization.

II. Sterilization above 100°C

Autoclaving is one of the most common methods of sterilization. Principle: In this method sterilization is done by steam under pressure. Steaming at temperature higher than 100°C is used in autoclaving. The temperature of boiling depends on the surrounding atmospheric pressure. A higher temperature of steaming is obtained by employing a higher pressure. When the autoclave is closed and made air-tight, and water starts boiling, the inside pressures increases and now the water boils above 100°C. At 15 lb per sq. inch pressure, 121°C temperatures is obtained. This is kept for 15 minutes for sterilization to kill spores. It works like a pressure cooker.

III. Sterilization below 100°C

1. Pasteurization.

Pasteurization is heating of milk to such temperature and for such a period of time so as to kill pathogenic bacteria that may be present in milk without changing colour, flavour and nutritive value of the milk. *Mycobacterium bovis*, *Salmonella species*, *Escherichia coli* and *Brucella species* may be present in milk. It does not sterilize the milk as many living organisms including spores are not destroyed.

Methods of Pasteurization:

- Flash Method. It is 'high temperature – short time method'. Heating is done at 72°C for 15 seconds.
- Holding Method. Heating is done between 63°C and 66°C for 30 minutes.

2. Inspissation. Inspissation is done between 75°C to 80°C. Inspissation means stiffening of protein without coagulation as the temperature is below coagulation temperature. Media containing serum or egg is sterilized by heating for 3 successive days. It is done in a Serum Inspissator machine.

Sterilization by Dry Heat

Dry heat at 160°C (holding temperature for one hour is required to kill the most resistant spores). The articles remain dry. It is unsuitable for clothing which may be spoiled.

1. Red Heat. Wire loops used in microbiology laboratory are sterilized by heating to 'red' in a Bunsen burner or spirit lamp flame. Temperature is above 100°C. It leads to sterilization.

2. Flaming. The article is passed through flame without allowing it to become red hot, e.g. scalpel. Temperature is not high to cause sterilization.

3. Sterilization by Hot Air. It is one of the most common method used for sterilization. Glass wares, swab sticks, all-glass syringes, powder and oily substances are sterilized in hot air oven. For sterilization, a temperature of 160°C is maintained (holding) for one hour. Spores are killed at this temperature. It leads to sterilization. Hot Air Oven is an apparatus with double metallic walls and a door. There is an air space between these walls. The apparatus is heated by electricity or gas at the bottom. On heating, the air at the bottom becomes hot and passes between the two walls from below upwards, and then passes in the inner chamber through the holes on the top of the apparatus. A thermostat is fitted to maintain a constant temperature of 160°C.

1.7.2. Filtration

Many of the biological fluids (liquids) or gases that need to be sterilized cannot be done so by the application of heat. Their sterilization is achieved by filtration.

Filtration of Biological Fluids (Biological Filters)

When ingredients of a culture medium are thermolabile, i.e. easily destroyed by heat, the use of heat sterilization is not practicable. For instance, biological fluids such as solutions of antibiotics, vitamins, tissue extracts, animal serum, etc. come under this category. In such cases, however, the process of filtration is used. The filters suitable for the purpose are Seize filter (Asbestos filter), Chamberland-Pasteur filter (Porcelain filter), Berkefeld filter (Diatomaceous earth filter) and Membrane or Molecular filter. The first three filters are bacteriological filters, i.e. they allow liquid to pass but retain bacteria. Contrary to this, the membrane filters retain all forms of organisms whatever small they may be (even viruses). The mean pore diameter in these filters ranges from one to several micrometres. These filters do not merely serve the mechanical prevention but other factors such as electric charges of the filter, electric charge of the microorganisms, and the nature of the fluid being filtered.

Important biological filters

1. Seitz Filter (Asbestos filter)

This filter consists of 2–6 mm compressed asbestos fibre filter sheet. A variety of filter sheets containing different pore sizes are available in discs or squares ready for use and work satisfactorily only for a few hours. The medium to be filtered (sterilized) is poured into the funnel-like structure and drawn through filter sheet by vacuum. When the filtration is complete the filter sheet is discarded and the filtrate is obtained. A modified Seitz filter in which vacuum-drawn filtrate technique has been replaced by centrifugal technique is also used now-a-days where the filter is mounted on a centrifuge which forces the filtrate into the tube.

2. Chamberland-Pasteur Filter. (Porcelain Filter)

These filters consists of hollow unglazed cylinders having a short open end. The cylinders are composed of oxides of silicon, aluminium, potassium and sodium with traces of oxides of iron, calcium and magnesium (the mixture commonly called 'porcelain'). The cylinders are baked at a temperature as high as possible without sintering the porcelain. These filters are prepared to various degrees of porosity from 0.65 to 15 μm and are used to remove bacteria and other coarse materials.

Membrane or Molecular Filter

A new type of filter called 'membrane' or 'molecular' filter has been developed in recent years. Unlike bacteriological filters which retain only bacteria, membrane filter retains all forms of microorganisms whatever their size be. These filters, are made up of biologically inert cellulose esters, and are prepared as

circular membranes of about 150 μm diameter consisting of millions of pores of an uniform and specifically predetermined size. Membrane filters were originally manufactured by the Millipore Filter Corporation (USA) and therefore they are also known as 'Millipore' or 'Ultra filters'. At the time of filtration, membrane filters of various porosity are used on the principle of 'graded filtration' and finally fluid free of all organisms larger than 10 μm is obtained.

1.7.3. Radiation

Radiation refers to the transmission of energy in a variety of forms through space or through a medium. The most effective type of radiation to sterilize or reduce the microbial burden in almost any substance is through the use of electromagnetic radiation. Various types of electromagnetic radiations are separated within the electromagnetic on the basis of their wavelengths. The radiations of shorter wavelengths are more damaging to microorganisms. Thus, two types of radiations of primary interest in sterilization are—electromagnetic waves and streams of minute particles. The electromagnetic waves in decreasing orders of wavelengths are infrared, ultra-violet light, X-rays and gamma rays, whereas the streams of minute particles of matter are alpha and beta radiation. However, the sterilization by electromagnetic radiation is commonly called 'cold sterilization' and is ideal for disposable materials made up of plastics, wool, cotton, etc., which can be sterilized using a high dose of irradiation without altering the material. For others, complete sterilization is difficult without causing changes in colour and flavour of the materials which occur at higher doses of radiations. Microbial cells possess various vital molecules, which are made up of atoms. An atom consists of a small nucleus surrounded by planetary electrons. When the electromagnetic rays and radiation particles pass through the matter, they give energy called radiant energy to the electrons of constituent atoms.

1.8. Bacteria as a potential tool in bioterrorism

Bacterial pathogens have been identified as agents that have been, or could be, used as weapons of biological warfare and/or biological terrorism. These agents are relatively easily obtained, prepared and dispersed, either as weapons of mass destruction or for more limited terrorist attacks.

According to the U.S. Centers for Disease Control and Prevention (CDC), bioterrorism is the deliberate release of viruses, bacteria, toxins or other harmful agents to cause illness or death in people, animals, or plants. These agents are typically found in nature, but could be mutated or altered to increase their ability to cause disease, make them resistant to current medicines, or to increase their ability to be spread into the environment. Biological agents can be spread

through the air, water, or in food. Terrorists tend to use biological agents because they are extremely difficult to detect and do not cause illness for several hours to several days. Some bioterrorism agents, like the smallpox virus, can be spread from person to person and some, like anthrax, cannot.

Bioterrorism is an attractive weapon because biological agents are relatively easy and inexpensive to obtain, can be easily disseminated, and can cause widespread fear and panic beyond the actual physical damage. Military leaders, however, have learned that as a military asset, bioterrorism has some important limitations; it is difficult to deploy a bioweapon in a way that only affects the enemy and not friendly forces. A biological weapon is useful to terrorists mainly as a method of creating mass panic and disruption to a state or country. However, technologists such as the American Bill Joy, co-founder of Sun Microsystems, have warned of the potential power that genetic engineering might place in the hands of future bio-terrorists. Setting up a laboratory in which bio-agents can be prepared does not require great financial or practical effort. The equipment and reagents for producing bio-agents are fully available on the civilian market (even from Internet shops), and are not always sold under the supervision of relevant security services. A properly prepared homemade laboratory can have the dimensions of a cargo container, or caravan. Additionally, the production of bio-agents can also be incorporated into the daily activity of analytical medical or scientific laboratories, as well as within the infrastructure of the cosmetic and pharmaceutical industries. Production costs for bio-agents are exceptionally low, and for this reason they are known as the 'Arms of the poor'. Calculations performed by The United Nations show that the financial cost of causing 1 km² of human casualties using conventional arms is about USD 2,000. For nuclear weapons it's USD 800; chemical weapons USD 600, and for biological weapons just USD 2. Bioweapons are easy to hide, and in comparison to chemical weapons, are colourless and odourless. In addition, actual attacks can initially appear to be untypical of mass terror actions, with the first symptoms of an attack even occurring sometime after their deployment. For these reasons it can be difficult to find the source of the attack. Finally, biological agents remain relatively stable after dissemination, at least for periods long enough to infect humans.

The use of agents that do not cause harm to humans but disrupt the economy has been discussed. A highly relevant pathogen in this context is foot-and-mouth disease (FMD), which affects cloven-hoofed animals is capable of causing widespread economic damage and public concern (as witnessed in the 2001 and 2007 FMD outbreaks in the UK), whilst having almost no capacity to infect humans.

The CDC defines bioterrorism as "deliberate release of viruses, bacteria or other germs (agents) used to cause illness or death in people, animals, or plants." The Biological Weapons Convention (BWC), opened for signature in 1972, was the first disarmament treaty, which banned development, production, and storage of this entire category of weapons of mass destruction.

1.8.1. The CDC categories

The CDC maintains a list of potential critical biological agents, including those usable in bioweapons. These are classified into three categories – A, B and C – based on their ease of transmission, morbidity and mortality rates, and likelihood of actually being used.

Category A

Anthrax

Anthrax is a non-contagious zoonotic disease caused by the spore-forming bacterium *Bacillus anthracis*. *Bacillus anthracis* is a gram-positive, rod-shaped bacterium, and one of the most likely agents to be used in a biological attack. *Bacillus anthracis*, the only obligate pathogen within the genus *Bacillus*, has a large size at 1.0–1.5 μm width and 3–10 μm length. *B. anthracis* is an aerobic bacterium, meaning that in anaerobic conditions it is able to survive only a few days. However, its spores are highly resistant to adverse environmental conditions including heat, both ultraviolet and ionizing radiation, high pressure and chemical agents. It has been established that spores are able to survive in soil for up to 40 years.



Figure 12. *Bacillus anthracis* (figure used with permission under Creative Commons license)

Anthrax takes three different forms: cutaneous anthrax, which is acquired through cuts and lesions in the skin; gastrointestinal anthrax, and pulmonary (inhalation) anthrax. The most dangerous and potentially useful bioterror form is pulmonary anthrax. It is caused by alveolar deposits of Anthrax spores, which are less than 5 µm in size. This mainly affects the lungs but can also be accompanied by meningitis. Inhaled spores are phagocytosed by macrophages and carried to local mediastinal lymph nodes, wherein it is sufficient to absorb 8,000 to 50,000 spores of *B. anthracis* to induce the disease. The spores then germinate into vegetative forms, replicate, and produce haemorrhagic mediastinitis. General symptoms include fever, coughing and chest pains about 12–24 hours after infection. There then follows a sudden increase in shortness of breath, swelling of the neck area and the appearance of the first signs of sepsis. Next comes necrosis of the lymph nodes, pulmonary oedema and a characteristic mediastinal widening, with fluid in the pleural cavity. In the absence of treatment, mortality is 97% and death usually occurs 3 days after the onset of symptoms. The only way to cure anthrax is to administer the antibiotics (such as ciprofloxacin) within 12 hours of infection. However, in this short period the symptoms of the disease may not yet even present. The pulmonary form of anthrax, even if treated with antibiotic therapy, has a 75% mortality rate. An anthrax vaccine does exist but requires many injections for stable use. In the US there is an anthrax vaccine, Anthrax Vaccine Adsorbed (AVA), which requires five injections for a stable result.

The first modern incidence of anthrax use in biological warfare occurred in 1916 when Scandinavian ‘freedom fighters’, supplied by the German General Staff, used anthrax (with unknown results), against the Imperial Russian Army in Finland. In 1993, the Japanese Aum Shinrikyo cult deployed anthrax in an unsuccessful attack in Tokyo, which had zero fatalities. Anthrax was again used in a series of attacks on the offices of several United States Senators in late 2001. This anthrax was sent in a powder form and delivered by the US mail, infecting 22 people, including 12 postmen, and killing five. Additionally, there was one documented accidental use in Sverdlovsk, Russia, in 1979, when approximately 1 or 2 grams of spores in aerosol form leaked into the atmosphere. The wind then carried them into the (fortunately) sparsely-populated suburbs of the town, killing 68 of 79 infected people.

There is no doubt that *Bacillus anthracis* is one of the most dangerous pathogens with potential use in bioterrorism. Its greatest advantages are that it is easy to acquire natural strains, as well as its ease of handling and low associated costs. Production of 1kg of spores can cost just USD 50. Based on scientific calculations by the World Health Organization, release of 50 kg of dried anthrax powder by aerosolization for two hours in a city of 500,000 inhabitants would cause 95,000 deaths and incapacitation of 125,000 individuals. The strain on medical resources would be tremendous, leading to a need for bed space

for 12,500 individuals (10% of those incapacitated), 60-day antibiotic courses for 125,000, and the disposal of 95,000 corpses. This would almost certainly lead to a rapid total breakdown in medical resources and civilian infrastructure.

Tularemia or ‘rabbit fever’

Tularemia has a very low fatality rate if treated, but it can severely incapacitate those infected. The disease is caused by the *Francisella tularensis* bacterium, contracted through contact with fur, inhalation, ingestion of contaminated water or insect bites. This pathogen is a gram-negative, rod-shaped coccobacillus. *Francisella tularensis* is highly infectious and requires only a small number of organisms (about 10–50) to cause disease. However, in comparison to other potential bio-agents it is very sensitive to chemicals and high temperatures. Even the sun’s rays can kill the bacteria within 30 minutes. But at low temperatures it exhibits considerable resistance, and can last up to 3 years.

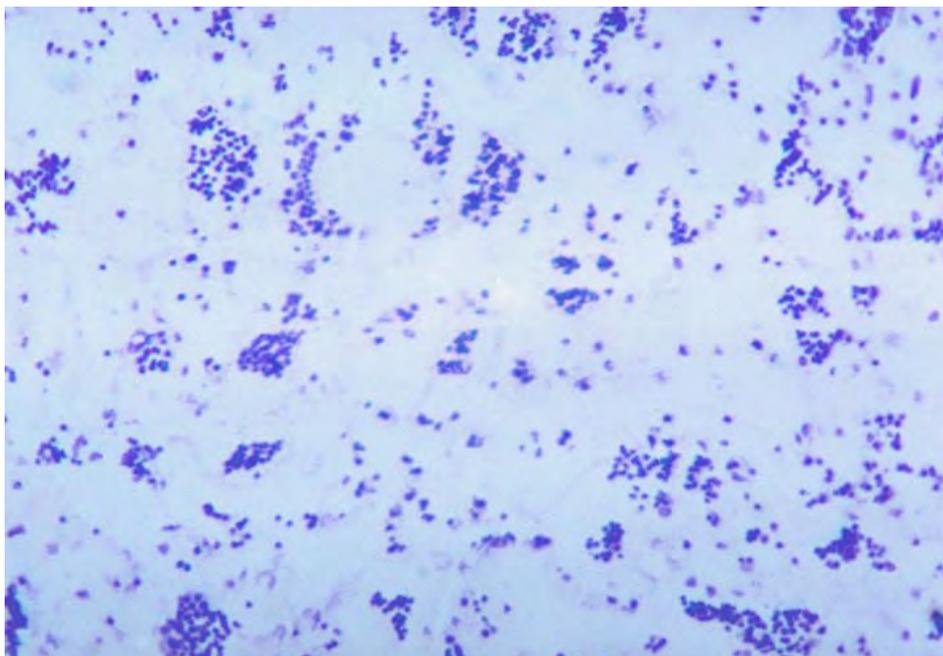


Figure 13. *Francisella tularensis* (figure used with permission under Creative Commons license)

F. tularensis has been classified as a Tier 1 Select Agent by the U.S. government, along with other potential agents of bioterrorism such as *Yersinia pestis*, *Bacillus anthracis* and the Ebola virus. The bacteria that cause tularemia occur widely in nature and can be isolated and grown in quantity in a laboratory,

although manufacturing an effective aerosol weapon to deploy them would require considerable sophistication.

There are several types of tularemia. The most common is transmitted *via* skin contact or bites by infected ticks, and this is ulceroglandular tularemia. However, if *F. tularensis* is used as a weapon, the bacteria would likely be made airborne for exposure by inhalation. People who inhale it as an infectious aerosol would generally experience severe respiratory illness, including life-threatening pneumonia and systemic infection. The pneumonic form is the most potentially lethal form of tularemia, and is caused by inhalation of the bacteria. Besides the general symptoms, patients with the pneumonic form also experience chest pain, bloody sputum and can have trouble breathing – and even sometimes stop breathing. The mortality rate of this form is about 30%.

WHO simulations have shown that bioterrorism using *F. tularensis* would be very effective. Dispersion of 50 kg of bacteria in a city of 5,000,000 inhabitants would cause the deaths of 19,000 people and infection of 250,000 people. In simulations run by Kaufmann et al., the financial costs of infecting 100,000 people ran to USD 5.4 billion.

Smallpox

Smallpox was a severe human disease caused by the variola virus (VARV), which was both highly lethal and highly contagious. VARV is a member of the genus Orthopoxvirus. A characteristic feature of this virus is its strict specificity to humans. It is transmitted easily through the atmosphere and has a high mortality rate (20–40%). It was once one of the most devastating diseases known to mankind. The first symptoms of smallpox usually appear 10 to 14 days after infection. During the incubation period of 7 to 17 days, the infected looks and feels healthy and is themselves non-infectious. Smallpox generally begins with fever, headaches, body aches, and weakness on day 1. Then in days 2–3 small, round pox (blisters) appear and spread on the face, arms, legs, and inside the mouth. By day 7 the pox turns into bigger blisters that fill with pus. On day 12 the blisters crust over; stomach pain and confusion can also occur. By week 3–4 the blisters have turned into scabs and fall off, leaving pitted scars on the skin. There is no proven treatment for smallpox once the rash appears, but research is underway to find an effective anti-viral medication. Those who are ill with smallpox can benefit from supportive care in a hospital setting. People who recover from smallpox usually have severe scars, especially on the face, arms and legs. In some cases, smallpox can also cause blindness.

The virus can be transmitted in several ways, including directly, from person to person. Direct transmission of the virus requires fairly prolonged face-to-face contact. The virus can also be transmitted through the air by droplets that escape when an infected person coughs, sneezes or speaks. Indirectly from an infected

person. In rare instances, the airborne virus can spread farther, possibly through the ventilation system in a building, infecting people in other rooms or on other floors. Smallpox can also infect through contaminated items, including contaminated clothing and bedding, although the risk of infection from these sources is less common. Historically, people have received smallpox by touching or inhaling the smallpox virus. Smallpox is not spread by insects or animals, and there is no naturally-occurring smallpox as it was eradicated in the world in the 1970s, thanks to a global vaccination program. However, some virus samples are still available in Russian and American laboratories. Some believe that after the collapse of the Soviet Union, cultures of smallpox have become available in other countries. Although people born pre-1970 will have been vaccinated for smallpox under the WHO program, the effectiveness of vaccination is limited as the vaccine provides a high level of immunity for only 3 to 5 years. Revaccination protection lasts longer. So as a biological weapon, smallpox is dangerous because of the highly contagious nature of both the infected and their pox. Also, the infrequency with which vaccines are administered among the general population since the eradication of the disease generally would leave most people unprotected in the event of an outbreak.

In the case of terrorist use in a public setting, the virus would be spread by:

- Breathing in the virus from a cough, sneeze, or saliva (spit) of a smallpox carrier;
- Touching skin that has smallpox blisters;
- Touching contaminated body fluids or objects, such as bedding or clothing;
- Breathing in the virus in a room, bus, or train shared with someone who has smallpox;
- Sharing a razor, tableware or toothbrush with someone who has smallpox.

Even if all the stocks of naturally-occurring, lab-stored smallpox virus are destroyed, it is now possible to genetically engineer a similar viral agent in a laboratory setting. This capability requires that the medical and public health communities maintain smallpox preparedness into the foreseeable future.

Plague

Plague is a disease caused by the *Yersinia pestis* bacterium. This bacteria is a Gram-negative, rod-shaped coccobacillus, a facultative anaerobic organism which does not produce spore forms, but has very high vitality. It can survive up to 6 months within the bodies of dead animals, in water for less than a month, and for a few days in dry conditions. *Y. pestis* is maintained in nature as a zoonotic infection in rodent hosts and their fleas in large areas of Asia, Africa and the Americas. Rodents are the normal host of plague, and the disease is transmitted to humans by flea bites and occasionally by aerosol in the form of pneumonic plague. This was the disease that caused the 'Black Death' in Medieval Europe.

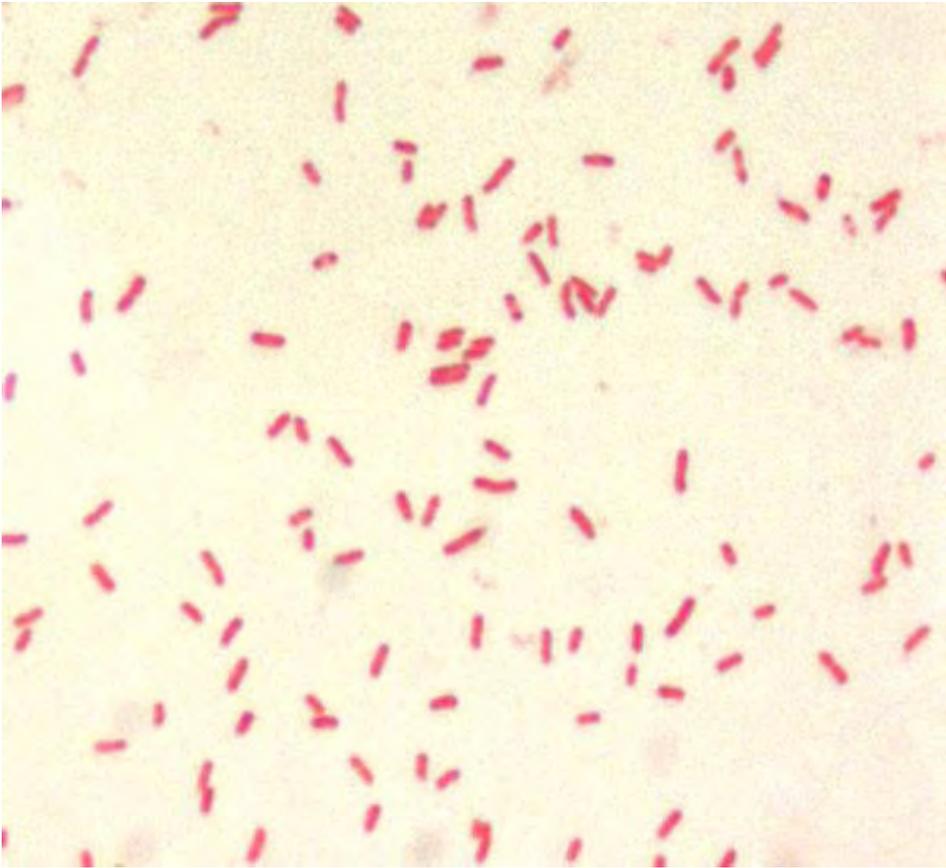


Figure 14. *Yersinia pestis* (figure used with permission under Creative Commons license)

There are three clinical forms of this disease: classical bubonic plague; primary septicaemic plague, and pneumonic plague, determined largely by the way the pathogen enters the body. The weaponized threat comes mainly in the form of pneumonic plague (infection by inhalation). The pneumonic plague occurs when *Y. pestis* infects the lungs. Pneumonic plague can spread from person to person through the air. Transmission occurs when someone breathes in aerosolized bacteria, which could happen during a bioterrorism incident. The most obvious symptom of pneumonic plague is coughing, often accompanied by haemoptysis (coughing up blood). With pneumonic plague, the first signs of illness are fever, headache, weakness and rapidly-developing pneumonia, characterised by shortness of breath, chest pains, coughing and sometimes bloody or watery sputum. The pneumonia plague progresses for two to four days and can cause respiratory failure and shock. This is a very aggressive infection

requiring early antibiotic treatment (within 24 hours of presentation of the first symptoms, in order to reduce the risk of death). Without therapy, the mortality rate approaches 100%.

The disease has a history of use in biological warfare dating back many centuries. This pathogen has great potential as a very useful bio-agent. It is naturally occurring and can be isolated and grown in bulk in a laboratory. Because of the delay between being exposed to the bacteria and becoming sick, carriers can travel over large areas before becoming contagious and possibly infecting others. World Health Organisation simulations has shown that bioterrorism using *Y. pestis* would be very effective. Dispersion of 50 kg of bacteria in a city of 5,000,000 inhabitants would causes 36,000 deaths, and plague infection of 150,000. *Y. pestis* can also be used to contaminate soil, food and water. Antibiotic-resistant strains of the bacteria, isolated in the 2014 outbreak in Madagascar, are very dangerous.

Viral haemorrhagic fevers

This includes haemorrhagic fevers caused by members of the Filoviridae family (Marburg virus and Ebola virus), and by the Arenaviridae family (for example, Lassa virus and Machupo virus). There are all families of negative-stranded and lipid-enveloped ribonucleic acid (RNA) viruses. The Ebola virus in particular has caused high fatality rates, ranging from 25–90%, with a 50% average. Ebola is one of the classic zoonotic diseases, with index transmission occurring from animals to human hosts. The Ebola virus is also transmitted through contact with the full range of bodily fluids of infected individuals (blood, urine, saliva, sweat, faeces, vomit, breast milk and semen). Ebola viruses enter the human body *via* mucosal surfaces, abrasions and lesions in the skin. The incubation period for Ebola ranges from 2 to 21 days, normally occurring after 4–10 days. The first signs are flu-like symptoms (fever, myalgia, chills), vomiting and diarrhoea. The disease can rapidly evolve into a severe state with a rapid clinical decline. Lethal Ebola cases generally succumb between days 6 and 16 from the onset of symptoms. Death from Ebola is commonly due to multiple organ failure and hypovolemic shock. No cure currently exists, although vaccines are being developed. The Ebola virus is useful as a bio-agents thanks to its high virulence. Studies performed on rhesus monkeys have shown that a low dose of virus introduced into the body in aerosol form rapidly leads to an almost 100% death rate. Additionally, the first symptoms are not typical of serious diseases. This significantly delays the detection of infection, and consequently initiation of treatment and the possibility of the outbreak spreading further. The Soviet Union investigated the use of filoviruses in biological warfare, and the Japanese Aum Shinrikyo cult unsuccessfully attempted to obtain cultures of Ebola virus.

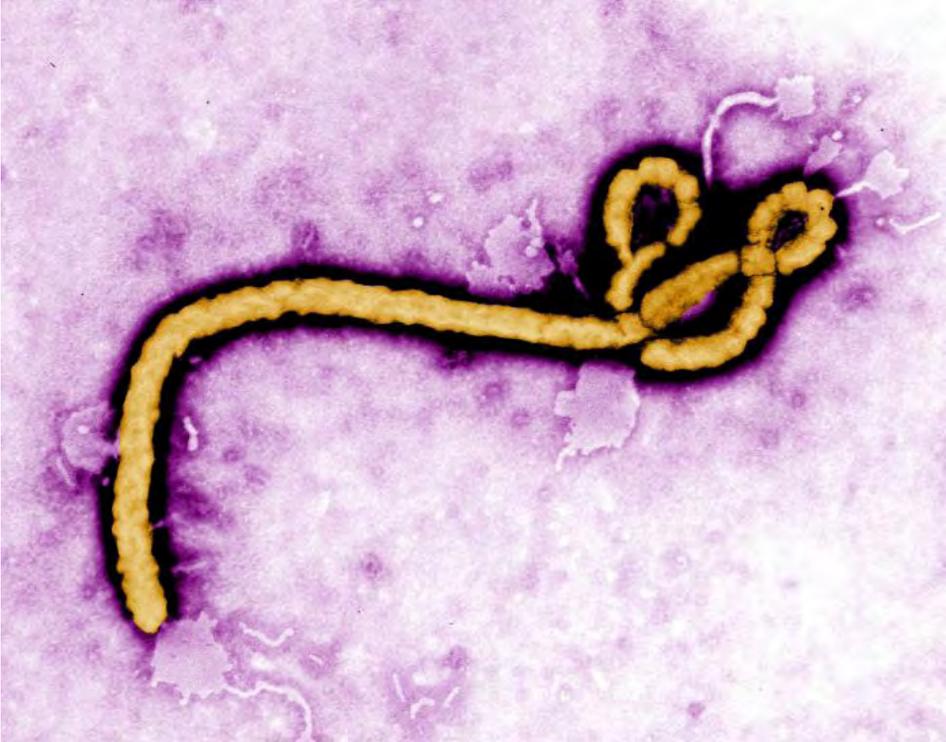


Figure 15. Ebola virus (figure used with permission under Creative Commons license)

Marburg virus (MV) was first discovered in Marburg, Germany. The source of infection was traced back to African green monkeys (*Chlorocebus aethiops*) that had been imported from Uganda. During the initial outbreak, 31 people became infected, seven of whom died. To date there have been a total of 452 cases and 368 documented deaths due to Marburg virus. Initial contraction of the virus comes *via* exposure to infected animals: either a reservoir host (several bat species) or a spill-over host such as NHPs as described in the first MV outbreak. MV has an incubation period ranging from 3 to 21 days (typically 5 to 10 days), which is likely modulated by factors such as infectious dose and possibly by route of infection. The onset of illness begins with generic flu-like symptoms; a characteristic high fever, severe headache, chills, myalgia, prostration and malaise. For many patients (50–75%) this is followed by rapid debilitation characterized by gastrointestinal symptoms including anorexia, abdominal pain, severe nausea, vomiting, and watery diarrhoea. Many of the initial symptoms may persist in the early organ phase, and patients may sustain a high fever. They may additionally display neurological symptoms including encephalitis, confusion, delirium, irritability, and aggression. Patients can also develop dyspnea and abnormal vascular permeability, particularly

conjunctival injection and oedema. During the latter part of this phase, more than 75% of patients present with some form of clear haemorrhagic manifestation such as petechiae, mucosal bleeding, melena, bloody diarrhoea, haematemesis, and ecchymoses. Fatalities typically occur 8-16 days following the onset of symptoms, with death usually resulting from shock and multi-organ failure. No treatments currently exist, aside from supportive care.

The arenaviruses have a somewhat reduced case-fatality rate compared to the filoviruses, but are more widely distributed, chiefly in central Africa and South America. Lassa, Machupo, and Lujo viruses are all associated with secondary person-to-person and nosocomial (healthcare setting) transmission. This occurs when a person infected by exposure to the virus from the rodent host spreads the virus to other humans. This can occur in a variety of ways. Person-to-person transmission is associated with direct contact with the blood or other bodily fluids containing the virus particles of infected individuals. Airborne transmission has also been reported in connection with certain viruses, as has contact with objects contaminated by these materials, such as medical equipment.

Category B

Category B agents are moderately easy to disseminate and have low mortality rates.

- Brucellosis (*Brucella* species);
- Food safety threats (for example, *Salmonella* species, *E. coli* O157:H7, *Shigella*, *Staphylococcus aureus*);
- Glanders (*Burkholderia mallei*);
- Melioidosis (*Burkholderia pseudomallei*);
- Psittacosis (*Chlamydia psittaci*);
- Q fever (*Coxiella burnetii*);
- Typhus (*Rickettsia prowazekii*);
- Viral encephalitis (alphaviruses, for example, Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis);
- Water supply threats (for example, *Vibrio cholerae*, *Cryptosporidium parvum*).

Category C

Category C of the highest-priority bio-agents includes emerging pathogens that could be engineered for mass dissemination. These agents are readily available, easy to produce and disseminate, and have potentially high morbidity and mortality rates, as well as a major impact on public health. The main bio-agents in this category include:

- Nipahvirus;

- Hantavirus;
- SARS;
- H1N1 (a strain of influenza);
- HIV/AIDS;
- Multi-drug-resistant tuberculosis;
- Tick-borne haemorrhagic fever viruses;
- Tick-borne encephalitis virus;
- Yellow fever virus.

Hantavirus

This bunyavirus infection is transmitted to humans from rodents and causes either a haemorrhagic fever with renal failure or hantavirus pulmonary syndrome. The disease occurs widely throughout the world. Person-to-person spread does not appear to take place. The incubation period is 2–3 weeks, followed by fever, headache, backache and injected conjunctiva and palate. Hypotension, shock and oliguric renal failure follow. The mortality rate is about 5%.

Nipah and Hendra virus

Nipah virus, a paramyxovirus, causes severe disease in humans and animals. It is found in South Asia and causes febrile encephalitis with a high mortality rate. The reservoir is probably fruit bats, with human infection from contact with bats or an intermediate animal host such as pigs. Person-to-person spread occurs. The related, rarer Hendra virus is also acquired from bats and causes an influenza-like syndrome or encephalitis.

Influenza viruses

Influenza virus is an enveloped orthomyxovirus (100 nm) that contains a negative single-stranded RNA genome divided into eight segments. This structure facilitates genetic re-assortment, which allows the virus to change its surface antigens and the influenza virus will take up genetic material from avian and pig influenza strains. The virus expresses seven proteins, three of which are responsible for RNA transcription. The nucleoprotein has three antigenic types that designate the three main virus groups, influenza A, B and C. Of the three types, influenza A and, more rarely, influenza B undergo genetic shift. The matrix protein forms a shell under the lipid envelope with haemagglutinin and neuraminidase proteins expressed as 10-nm spikes on the envelope, which interact with host cells. Virus immunity is directed against the haemagglutinin (H) and neuraminidase (N) antigens.

Yellow fever

Yellow fever virus is a flavivirus, an enveloped positive-sense RNA virus, transmitted by *Aedes aegypti*. Yellow fever is a zoonosis in which humans are an accidental host (sylvatic disease), but an urban cycle results in periodic human epidemics.

Human Immunodeficiency Virus

HIV is a spherical, enveloped RNA virus. It is a retrovirus, using reverse transcriptase to produce a DNA copy from viral RNA that is incorporated into the host nucleus to become the template for further viral RNA. Three genes are required for viral replication: gag, pol and env. HIV is classified as a lentivirus. There are two types that are pathogenic for humans: HIV-1, which is most common; and HIV-2, which is found mainly in West Africa and appears to be less virulent.

Infection with HIV has spread worldwide, transmitted by the parenteral and sexual routes. Infection is most common in individuals at high risk of sexually transmitted diseases, especially those where genital ulceration is common. In developed countries, the main risk groups are intravenous drug users and men who have sex with men; heterosexual transmission is less common but does occur. In developing countries, HIV spreads mainly by heterosexual transmission and through unscreened transfusions or use of contaminated medical equipment. Infection can be transmitted from mother to foetus.

The virus principally infects cells with a CD4 receptor (e.g. T cells and macrophages). Viral replication results in progressive T-cell depletion and diminished cell-mediated immunity. Different virus strains display varying affinities for cells that express particular chemokine receptors. Lacking T-cell help, B-cell function is also reduced. HIV causes damage to neural cells and stimulates cytokine release that may also cause neurological damage. Many of the clinical signs of AIDS are caused by secondary infections, which occur when the CD4 count falls.

Multi-drug resistance (MDR) pathogens

One of the biggest modern public health issues that could be exploited by bioterrorists is the resistance of pathogens to antibiotic therapy. The resistance of various pathogens to different antimicrobial drugs has emerged as a threat to public health all over the world at a terrifying rate. Almost all infectious agents (bacteria, fungi, viruses and parasites etc.), have developed high levels of multi-drug resistance (MDR) with enhanced morbidity and mortality. These are what are known as 'super bugs'. Antibiotic-resistant pathogens possess great potential

for use in bioterrorism attacks and have a high mortality rate. This is the single most common source of hospital infections, and in 2007 it was estimated that 94,360 people in the U.S. were affected, 18,650 of whom died.

1.8.2. Genetic modified pathogens

The new era of bio-terrorism is also part of the development of new, simpler methods of genetic engineering. Some pathogens can also be subjected to special proliferation procedures, followed by genetic manipulation, thereby exacerbating their virulence. Existing bioengineering techniques can be used to improve existing non-pathogenic biological agents, making them excellent biological weapons. These technologies allow genetic material to be added, removed, or altered at particular locations in the genome. One example of this type of technique is CRISPR-Cas9 (Clustered Regularly Interspaced Short Palindromic Repeats). CRISPR-Cas9 is a unique technology that enables geneticists and medical researchers to edit parts of the genome by removing, adding or altering sections of the DNA sequence.

CRISPR-Cas9 is a unique technology that enables to edit parts of the genome by removing, adding or altering sections of the DNA sequence. It is currently the simplest, most versatile and precise method of genetic manipulation and is therefore causing a buzz in the science world. The CRISPR-Cas9 system consists of two key molecules that introduce a change into the DNA. The first is enzyme Cas9, which acts as a pair of 'molecular scissors' that can cut the two strands of DNA at a specific location in the genome, so that bits of DNA can then be added or removed. The next part of this genetic engineering system is a small molecule of RNA (about 20 bases long), located within a longer RNA scaffold. The scaffold part binds to DNA and the pre-designed sequence 'guides' the Cas9 to the right part of the genome. This makes sure that the Cas9 enzyme cuts at the right point in the genome.

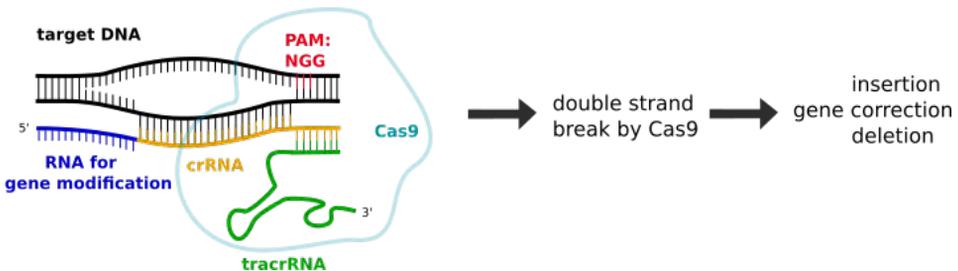


Figure 16. CRISPR-Cas9 mechanism (*figure used with permission under Creative Commons license*)

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2. TOXICOLOGY

Toxicology is a science of poisons (Greek *toxicon* – poison, *logos* – science). The term **poison** applies to substances and their combinations whose minor quantities cause severe impairment of body functions or even death (poisoning). Poisons can act instantly or accumulate in the body causing chronic poisoning. The group of poisons covers toxic substances of natural origin (venoms, animal toxins or plant toxins) and synthetic chemical toxins.

Paracelsus, who lived in 1493–1541, was a physician and naturalist, called the father of contemporary medicine and also considered the father of toxicology. He was the author of the quotation used as the foundations of hormesis: “*What is a poison? Everything is poisonous and nothing is poisonous, it’s all a matter of dose*”. Paracelsus hence claimed that it is a dose and not the kind that makes a substance poisonous. Hormesis is a phenomenon involving a beneficial effect of a harmful factor on the body, if exposed to low instead of high doses of the factor.

Toxic action of a chemical substance depends then on its dose and concentration in a tissue/organ or system. **Dose** is the quantity of chemical substance administered, taken or absorbed into the body *via* a specific route, conditioning a lack or presence of specific biological effects, expressed by percentage of organisms responding to the dose. Usually the dose is expressed in weight units per body weight or area, and sometimes additionally per day. Depending on the effects caused by the absorbed substance, the following doses can be identified. One should remember, though, that the absorbed dose is not necessarily the same as the administered dose, which depends on the method of its penetration and transport of the substance.

Threshold (limit) dose – (*dosis minima, DM*) it is the minimum quantity of a substance that causes first visible biological effects, i.e. it is the lowest exposure level (lowest dose) causing changes which exceed the adaptation limits determining maintenance of the body homeostasis.

Therapeutic dose – (*dosis therapeutica, dosis curativa, DC*) it is a quantity of a substance that demonstrates a pharmacotherapeutic effect, causes functional changes within physiological limits resulting in the desired therapeutic effect and does not impair physiological processes.

Toxic dose – (*dosis toxica, DT*) it is a quantity of a substance that causes a toxic effect after being absorbed into the body.

Lethal dose – (*dosis letalis, DL*) it is a quantity of a substance causing death after single administration.

Nowadays, a number of toxic substances commonly used in industry, agriculture, everyday products, medicines, cosmetics etc. are produced as a result of civilizational progress and development of industry and different areas of chemistry. A human being is permanently exposed to the substances, while the degree of their toxicity for a living organism depends on a number of factors, first and foremost including the dose taken, which depends on the time of the body exposure to the substance.

The term **exposure** defines a physical contact of a living organism with a chemical, physical or biological factor, described by concentration or intensity and duration. A chemical substance can be taken during exposure and then absorbed, which is expressed by the absorbed dose. The presence of a chemical substance in samples of biological material (inhaled air, body fluids, tissue preparations) provides an immediate evidence of exposure, however a lack of chemical substance in the samples is not a testimony to a lack of exposure. Exposure can also be identified by a substance concentration in the air or water and expressed as the dose taken, i.e. quantity of substance introduced into the body *via* a medium such as air, potable water or ingested food.

Xenobiotics are foreign substances that a living organism is exposed to (Gr. *ksenos* – foreign, *biotikos* – applying to life). They are substances that do not occur naturally in the body. A special group of xenobiotics covers chemical compounds produced by humans by means of synthesis. Their chemical structure does not occur naturally and that is why living organisms did not adapt to their action during earlier evolution.

Toxicology is a discipline of science that developed initially on the basis of practical observations made by subsequent generations, helping to identify toxic properties of specific substances, causing poisoning and/or death in humans. Plant poisons were used to commit suicide, murder or execute a death penalty. Deepening of the knowledge of different ways to detect poisons after their application, possibilities of using the substances for medical purposes and use of potential antidotes once a poison was overdosed constituted an important research area.

As time went by, new methodologies emerged and different areas of science developed, which contributed to a dynamic and multi-directional development of toxicology.

With regard to prevalence and great diversity of xenobiotics, contemporary toxicology covers a number of issues concerning actual and potential danger related to the action of chemicals on living organisms and ecosystems.

The role of contemporary toxicology is to spread the knowledge of harmful action of chemicals and methods to control and prevent their harmful action, to carry out scientific research to prevent adverse effects of xenobiotics by limiting their toxicity, and to develop methods of poisoning diagnosis and treatment.

Toxicology has become an interdisciplinary area combining information from such fields as biology, chemistry, biochemistry, genetics, immunology, physiology, medicine, epidemiology, pathology, criminology and pharmacy. A wide range of toxicology issues can be classified within different subdisciplines of toxicology such as neurotoxicology, immunotoxicology, genetic toxicology, molecular toxicology and toxicological analysis.

The nature of toxicological hazards has changed completely in contemporary toxicology alongside with large-scale urbanisation and industrialisation as well as development of different areas of science.

Cases of chronic poisoning, resulting from long-term (not consciously intended) effect of small doses of chemical substances on a living organism, and often on large human populations e.g. in a workplace or living environment, are the main problem of contemporary toxicology.

Formation of large conurbations has become the main cause of pollution to atmosphere, surface waters and soils, and consequently of food. Industrialisation entails common use of chemicals, e.g. detergents and other cleaning agents, cosmetics, pharmaceuticals, paints and varnishes, etc. The situation is additionally aggravated by common use of stimulants, including intensive development of new forms on synthetic psychotropic substances.

Owing to complexity of issues related to contemporary toxicology, it was divided into several separate areas. Toxicology is a separate discipline of science but a detailed nature of its issues entailed the need to identify separate areas of toxicology.

They are as follows:

- **clinical toxicology** that is a scientific discipline and medical specialisation involving diagnostics, prevention and interpretation of cases of acute poisoning as well as chronic poisoning mainly as a result of industrialisation;

- **toxicology of medical drugs** that deals with adverse effects of pharmaceuticals (side effects of medical drugs administered in therapeutic doses, toxic effects of intended or incidental overdosing of medicines or when therapeutic doses are used in a wrong way), studies on possible modifications of toxic drug symptoms by different factors including age, sex, nutrition, health condition, genetic determinants, interactions with other pharmaceuticals or other toxic factors (e.g. alcohol, drugs), pre-clinical studies of potential medicines and addictions to medicines and other substances.

- **forensic toxicology** mainly used by justice, and covering lifetime and post-death chemical analysis of poisoning with xenobiotics; first and foremost it deals with detection of toxic substances in biological material collected *post mortem*, and in majority of cases applies to diagnostics of alcohol, psychotropic drugs and doping substances;

- **food toxicology** aimed at an analysis of food composition for toxicity of substances introduced during cultivation, production and processing, and to

improve the qualities and storage capacity; it also covers toxicological safety of packaging and food contamination;

– **occupational toxicology** which is related to occupational health and medicine; it covers prevention, diagnostics and treatment of occupational poisoning, chemical substances occurring in a workplace to identify hazardous factors, and acute and chronic diseases they may cause; it also determines acceptable conditions of safe use of toxic substances and actions to prevent their absorption; it identifies the acceptable exposure limit for humans in their workplace, develops standards related to the content of chemicals in the air at work stations and identifies and evaluates exposure biomarkers;

– **environmental toxicology** which covers the impact of air, water and soil contamination on human health and preventive actions and controls to avoid exceeding the standards of chemical contaminant content in different elements of the environment; environmental toxicology evaluates the impact of toxic substances on the human body but also on animals, plants and functioning of whole ecosystems; it also deals with environmental and biological monitoring, i.e. regular measurements of the concentration of toxic compounds in the environment or biological material.

Contemporary people stay in an environment in which they are permanently exposed to the action of xenobiotics, which penetrate into their bodies *via* different routes.

Physical and chemical characteristics of toxic substances (volatility, solubility in water, readiness for sorption by solids) determine their penetration route into the ecosystem and mobility in atmosphere, water or soil. Introduction of toxic substances into the ecosystem is the first step of exposure of a body in which some functions are impaired as a result of increasing modifications in tissues and organs.

Defence mechanisms of a living organism enable metabolising and removal of xenobiotics. However, if the supply of the contaminant is greater than the body's capacity to metabolise and excrete, it accumulates in the body tissues, which is called **bioaccumulation**. Poorly degradable substances are very persistent and accumulate easily. Consequently, the organisms involved can be subjected to population changes. Individuals in such populations become weak, their reproduction capacity deteriorates, ability to give birth to healthy offspring decreases and the mortality of organisms exposed to toxic substances goes up.

Interactions of organisms in an ecosystem form a complex network of relations. They include food chains and complex trophic networks. When a toxic substance enters a trophic chain, a xenobiotic concentration is observed due to a multiple increase in the substance bioconcentration i.e. its concentration in the body tissues. The phenomenon is known as **biomagnification**. When one species eats other ones, it accumulates growing quantities of the xenobiotic, a poorly degradable one in particular. A human being on the top of a trophic chain is then exposed to highly concentrated contaminations.

Poorly degradable, highly persistent and bioaccumulative xenobiotics reveal the greatest impact on the environment, ecosystems and living organisms. They are relatively mobile in the air, water and soil, and are characterised by transport and biomagnification in the chain of trophic relations.

2.1. Toxicometry

Toxicometry is an area of toxicology aimed to assess toxicity of a substance. Cases of chronic poisoning, related to long-lasting impact of small doses of chemical substances on the body, usually applying to large human populations, are among major problems of contemporary toxicology. The issues are mainly related to occupational toxicology including exposure of humans in their workplace, and environmental toxicology – industrial toxicology in this case – applying to contamination of atmosphere (gases generated during fuel combustion in power plants, emissions from industrial plants (metallurgical and chemical plants in particular), products of crude oil and petrol combustion in mechanical vehicles), surface waters and soil (food contamination).

Occupational and environmental exposure is usually of complex nature and related to a simultaneous impact of several chemicals, which entails the need for extensive toxicological analysis for a compound toxicity assessment to identify the acceptable exposure limit for humans.

Exposure is assessed by **environmental and biological monitoring**. From the point of view of toxicology, biomonitoring stands for activities aimed to assess the condition of the environment by means of **bioidentifiers (biomarkers)**, including in particular the degree of air and water pollution. It involves regular measurements of concentration of toxic compounds in the environment or biological material facilitating comparison of the values and concentrations under different conditions, and identification of trends in time.

Biological monitoring is an important tool used for assessment of exposure and intake volume of chemicals in humans. The results of environmental monitoring are used to evaluate its contamination and the related hazard for living organisms. Such studies help to introduce the right method of limiting the source of harmful factors and to eliminate them.

Assessment of environmental or occupational exposure by biological monitoring involves identification of selected biomarkers in a biological material and establishing of the actual dose taken. It helps to identify the total intake of xenobiotics by all routes – with air, food or directly through the skin (drugs, cosmetics, cleaning agents). Complex analytical strategies combined with appropriate sampling methods facilitate qualitative and quantitative assessment of environmental (occupational) pollution and often help to identify their sources.

2.1.1. Biomarkers (bioidentifiers)

A biomarker is a wide term covering identification of parameters reflecting interactions between a biological system and environmental factors. A qualitative correlation in the biological material with the quantity of absorbed xenobiotic is an essential feature which qualifies a compound as a biomarker of exposure to the reference xenobiotic. Furthermore, a substance used as a biomarker should be easy to isolate from a biological material and persistent in analysis. A biomarker is a measurable marker of changes occurring in a body following absorption of a xenobiotic, and is the evidence of a toxin absorption into the body and a valuable source of important information.

The National Institute of Health introduced a standardised definition of a biomarker in 2001. The Institute identified a biomarker as a feature that can be objectively measured and then used as an indicator in an assessment of physiological biological processes, pathological processes or a body's response to therapeutic actions.

An analysis of biomarkers provides information on:

- occurrence of a body exposure to a harmful factor (exposure biomarker);
- health effects caused in the body following exposure to a xenobiotic (effect biomarker);
- body sensitivity to a specific xenobiotic action (sensitivity biomarker).

Exposure biomarkers are substances or their metabolites or products of interaction between a xenobiotic and body molecules measurable in the biological material and exogenous for the body. If a xenobiotic metabolism in the body is known, the volume of the absorbed xenobiotic dose and the resulting risk for health can be assessed by identifying the biomarker level. Exposure biomarkers are then intended for qualitative and quantitative analysis of exposure.

Effect biomarkers provide information about changes occurring in the body following action of a toxic factor. They are measurable biochemical, physiological, structural (including molecular changes on a sub-cellular level), behavioural and other changes occurring in the body as a result of action of a xenobiotic. Their analysis is an important element of health monitoring in employees exposed to harmful substances. A body's response to penetration of a toxic substance covers a wide spectrum of changes from transient, asymptomatic to permanent clinical changes with disease manifestations. An effect biomarker is identified by assessment of new health disorders and diseases or based on total effects with the existing disorders. The indicators are useful in early detection of diseases, which is their advantage. Biomarkers can also serve scheduling of medical treatment and prognosis.

Sensitivity biomarkers are indicators of congenital (genetically conditioned) or acquired body's ability to respond once exposed to a specific xenobiotic.

They provide information if one should expect adverse health effects in a specific organism or populations in relation to a reference exposure dose volume. They are useful in identification of the probability of a disease development following exposure to a toxin.

2.1.2. Assessment of the degree of harmful effect

The objective of toxicometric studies is to detect an adverse effect of a compound and to develop its qualitative and quantitative characteristics. Toxicometry studies quantitative relationships between a xenobiotic concentration and its toxic effect on the body, namely severity of the harmful effect of different chemical substances. It is aimed to establish concentrations of toxic substances safe for the environment and humans. Characteristics of xenobiotics provides a basis for explaining the mechanisms of their toxic action and helps to develop rules to prevent poisoning: appropriate protection during production, transport, storage and distribution of the substance.

One strives to identify acceptable parameters of human and natural environment exposure, which is expressed numerically as acceptable concentration in the air in production plants, atmosphere, potable water and food. The acceptable daily doses that a human can take with no adverse effects for health are also identified.

For understandable reasons, toxicometric studies are not performed on humans but unfortunately laboratory animals are used for this purpose. That is why the results of toxicometric studies have to be extrapolated from laboratory animals to humans. It is difficult to predict the impact of a xenobiotic on a human body based on the results of tests on animals and it can suffer from a serious error. Adequate interpretation of results in reference to humans depends on selection of the right species of test animals and the right methods of extrapolation of results.

In an optimum study array the conditions are selected so that a toxic compound and exposure conditions (exposure route and time) and biological system (animal species, age and sex) were the same as the conditions of human exposure. The condition can be hardly fulfilled and that is why in order to obtain appropriate toxic effects under experimental conditions, within a specific period of time, laboratory animals are exposed to much higher doses of toxic substances than humans (during a longer period of time). To that end extrapolation of results from high to low doses is necessary. Recalculation of the dose administered to an animal per body weight of a human is the simplest but also the least precise method of extrapolation of results.

In order to assess toxic action on humans in a precise way, based on analyses carried out on laboratory animals, one should take the following facts into consideration:

- differences between species in their resistance/sensitivity to different xenobiotics;

- difficulty in recognising mild adverse effects in animals, e.g. headache;
- differences in resistance to the action of xenobiotics may result from the impact of different internal and external factors such as body nutrition and general fitness, living conditions, medical drugs taken, kind of job etc.

Toxicometric studies are finally intended to establish levels of chemical safe for humans. The identified levels are of different nature and hence applied in different ways. Based on toxicometric studies, the values of the highest acceptable chemical and dust concentrations (safe concentrations at long-lasting exposure) and safe intensities for physical factors harmful for health in the working environment are identified and expressed as follows:

- **highest acceptable concentration (HAC)** of a substance in the air (and analogically the **highest acceptable dose (HAD)** e.g. in water) measured in the plant environment to protect the staff against poisoning in the work place, stands for the weighted average concentration of a harmful factor whose impact on the employee during an 8-hour working day and the average weekly working time, specified in the Labour Code, during their working career, should not cause negative changes in the employee's health or health of his/her future generations;

- **highest acceptable momentary concentration (HAMC)** measured in the work environment stands for the mean concentration of a factor harmful for health in the air in the work environment, which should not cause negative effects on an employee's health or his/her future generations, if it occurs at a work station for up to 15 minutes and no more than twice per shift, at time interval not shorter than 1 hour;

- **highest acceptable threshold concentration (HATC)** is measured in the work environment as a concentration of a factor harmful for health, which must never be exceeded in the work environment considering a hazard for the life or health of employees;

- **highest biological concentration (HBC)** is the acceptable concentration of toxic substances or their metabolites in the blood or urine.

- Other parameters of hygienic standards used in environment and health protection include:

- **highest acceptable concentration of a substance in the atmosphere** to protect health of the population, mainly in large conurbations and near industrial plants emitting harmful pollutions;

- **allowable daily intake (ADI)** specifying the quantity of a substance that can be safely ingested by humans with food and water every day, during whole life, usually expressed in mg/kg body weight;

- **allowable weekly intake (AWI)** specifying the quantity of a substance that can be safely ingested by humans with food and water weekly, during whole life, usually expressed in mg/kg body weight.

The results obtained in toxicometric studies, developed as dose-effect and dose-response relationships are the starting point to identify the abovementioned

standards. Toxicometric studies are based on observing the relationship between a toxic effect of a substance and its dose. A biological change caused by an action of a toxic compound is called an effect. In some cases an effect can only be identified qualitatively, while in other cases – if possible – the body response to a xenobiotic is identified quantitatively.

Dose-effect relationship

A dose-effect relationship occurs when the effect is of quantitative nature – it is stronger or weaker depending on the administered dose and can be expressed in a numerical form, reflecting the changes observed in an individual (e.g. change in the activity of an enzyme). The numerical relationship is established following several measurements of the effect in different individuals and at different doses of the substance. The dose-effect relationship helps to draw two types of conclusions:

- based on a specific dose value, a conclusion concerning the effect can be drawn;
- based on the observed effect one can estimate the dose volume that caused the effect.

The results of such an analysis should be approached with care as characteristics of an individual and population as well as different external factors can affect the relationship.

Dose-response relationship

In some cases reactions of the body to xenobiotics can only be described in qualitative aspects, which means that a reaction to a xenobiotic or no reaction is observed, with no intermediate conditions. Assessment of the effect is then limited only to a “yes” or “no” statement. Examples of such relationships include a lethal effect (the animal died or not), cancerogenesis (a tumour occurred or not). Such effects are known as individual or quantal effects. They may become aggravated at higher doses but the intensity of a quantal reaction is studied in a population of animals (group) and not in an individual. Exposing populations of living organisms to increasing doses of toxic substances is a classic example of studying the dose-response relationship. The reaction intensity is expressed in per cent as a percentage of individuals in the population in which the effect of a specific toxin dose was observed. A population reaction expressed this way is called a response. The intensity stands for a dose-response relationship, which takes a form of a sigmoid curve.

Based on studies of the dose-response relationship, the concept of **mean lethal dose** (LD_{50}) was introduced. LD_{50} tends to be expressed in mg/kg of body weight and identifies the dose of a poison which causes death of a half (50%) of the population of laboratory animals which received the poison. Sometimes it is

considered a measure of the dose-response relationship and is used for qualitative comparison of acute toxicity of different xenobiotics.

An analogical volume for inhaled poisons is known as the mean **lethal concentration** (LC_{50}) and applies to the xenobiotic concentration in the inhaled air.

The assessment of the dose-response relationship for threshold effects covers identification of the risk level of using a chemical by identifying the **NOAEL and LOAEL** values while maintaining a safety margin when the results obtained for laboratory animals are extrapolated to humans. **NOAEL (No Observable Effect Level)** – the highest dose or exposure level in studies enabling identification of the dose-response relationship for which no statistically or biologically significant increase in the prevalence or aggravation of adverse effects of the substance is observed in the studied animals as compared to the control group. **LOAEL (Lowest Observed Adverse Effect Level)** – the lowest dose or exposure level in studies enabling identification of the dose-response relationship in experimental animals for which statistically or biologically significant increase in the prevalence of adverse effects of the substance is observed in the studied animals as compared to the control group.

A **non-threshold effect** is observed in the studies of the dose-response relationship assessing toxicity of xenobiotics. The phenomenon applies to genotoxic substances for which the carcinogenic effect threshold cannot be identified. The probability of elevated risk of an additional tumour occurrence is specified for the substances and T_{25} parameter is determined to that end. T_{25} is a chronically administered dose which causes tumours in 25% of the exposed animals in a specific organ, corrected by spontaneous frequency in the average life of the reference animal species. Then, the value is converted into the HT_{25} parameter applying to humans, as follows:

$$HT_{25} = T_{25} / (\text{body weight of a human} / \text{body weight of an animal})^{0.25}$$

2.1.3. Assessment of a xenobiotic toxicity

The effects of exposure to toxic substances can be local, systemic or delayed, and their severity can be acute or chronic. With regard to the poison dynamics, mechanism and effect on the body, poisoning can be divided into the following groups:

- **acute poisoning** which is characterised by rapid development of adverse changes in the body, occurring within a short time after introducing a single dose of a poison by ingestion, inhalation or application to the skin; it is generally characterised by high dynamics of clinical symptoms; symptoms of injury/damage or death occur not later than within 24 hours;
- **subacute poisoning** – when adverse effects in the body are less violent after a single or multiple dose of the substance;
- **chronic poisoning** which occurs as a result of small doses of a poison, administered for a long period of time, usually as a result of the

poison accumulation in the body; they tend to be caused by incidental poisoning, e.g. occupational poisoning is usually chronic; the concentration of toxic substances polluting human environment is normally so low that it causes chronic effect.

Based on studies of acute toxicity in animals, Hodge and Sterner (USA) classified toxic substances dividing them into 6 groups, depending on the LD_{50} .

Table 1. Poison toxicity scale according to Hodge and Sterner

Toxicity grade	Name	Oral LD_{50} (g/kg body weight); rats	Dermal LD_{50} (g/kg body weight); rabbits	Inhalation LD_{50} (ppm); rats	Probable lethal dose for an adult human in grams
1	Extremely toxic	≤ 0.001	≤ 0.005	≤ 10	≈ 0.065
2	Highly toxic	0.05	0.043	100	4
3	Moderately toxic	0.5	0.34	1000	30
4	Slightly toxic	5.0	2.81	10 000	250
5	Practically non-toxic	15.0	22.6	100 000	1000
6	Relatively harmless	> 15.0	> 22.6	$> 100 000$	> 1000

A valid EU classification of chemical substances covers 4 classes, according to the scale of their toxicity based on LD_{50} , following ingestion (Directive of the Council No. 92/32/EEC of 30 April 1992).

Table 2. Poison toxicity classes

LD_{50} range (mg/kg body weight identified with a classic method)	Toxicity class/symbol	
$LD_{50} < 25$	I	Very toxic (T+)
$25 < LD_{50} < 200$	II	Toxic (T)
$200 < LD_{50} < 2000$	III	Harmful (Xn)
$2000 < LD_{50}$	IV	Not classified (practically not harmful)

The effects of toxic substances are divided into local and systemic. They can occur in the area of:

- absorption – skin, eyes, nasal mucous membrane, lungs, alimentary tract;
- metabolic transformations – liver, lungs, kidneys, alimentary tract;
- accumulation (deposition) – kidneys, central nervous system, liver;
- excretion – liver, alimentary tract, kidneys, urine bladder;
- special sensitivity – gonads, secretory glands, haematopoietic system.

Local effects – they are pathological changes occurring in the direct contact area of a xenobiotic with the body surface, i.e. skin, mucous membrane and eyeball. Local effects are divided into irritating and sensitising.

Systemic effects – they are changes in the central and peripheral nervous system, alimentary tract, excretory system, cardiovascular system etc.

2.1.4. Relationship between biological activity of a toxic substance and its chemical structure (examples)

From the qualitative and quantitative point of view toxic effects depend on the chemical structure of a substance. There are several general rules describing the relationship between the degree of toxicity and structure of chemical compounds, including the type of a substituent added or the chain length.

- The more complex the chain branch is, the more toxic aliphatic hydrocarbons become.

- Unsaturated hydrocarbons (benzene) are more toxic than saturated ones (cyclohexane).

- Adding an -OH group to an aromatic compound increases its toxicity (phenol is more toxic than benzene) and decreases the toxicity, if added to an aliphatic compound (glycerol is less toxic than propanol).

- Substituents increasing toxicity of the following compounds: -NH₂, -NO₂, -NO, -CN, -F and -CH₃ in cyclic compounds.

- Substituents reducing toxicity of compounds include -COOH, -SO₃H, -SH, -CH₃CO, -CH₃O, -C₂H₅O and -N=N-.

- Lipophilic compounds penetrate more easily through protein and lipid membranes, and interact with receptors and accumulate in the lipid tissue quicker.

Substances are divided into the following kinds, depending on their chemical structure:

- **irritants** cause irritation of the mucous membrane and cornea; they include highly volatile liquid substances transforming into toxic gases. Poisoning with toxic gases greatly depends on their solubility in water. Highly soluble and quickly hydrolysing gases such as: NH₃, formaldehyde (HCHO), hydrogen cyanide HCN and acid pairs: HCl, HF, HNO₃ and H₂SO₄ have a toxic effect primarily on the respiratory tract; gases less soluble in water such as: F₂, Cl₂, Br₂, H₂S and SO₂ affect the mucous membrane along the whole respiratory

tract, while gases with the lowest solubility in H_2O including: nitrogen oxides, phosgene ($COCl_2$), ozone, phosphoric chlorides mainly affect the walls of the lung alveoli;

- **asphyxiant gases** whose toxic effect results only from competitive reduction of oxygen concentration in the air include: N_2 , Ar, He and Ne, while other biologically inactive gases form explosive mixtures with air before they reach suffocating concentration;

- **narcotic substances** initially stimulate the central nervous system to impede its functions afterwards; they include organic solvents (e.g. methanol) absorbed by ingestion, inhalation or dermally; the narcotic effect of aliphatic alcohols, alkenes, hydrogen chloro-derivatives, ketones and esters increases with a higher number of C atoms in the molecule (Richardson's law, 1869);

- **caustic substances** usually cause local superficial burns following a direct contact; they mainly include concentrated solutions of hydroxides: NaOH, $NH_3 \cdot xH_2O$, KOH and alkaline chlorates: NaOCl, KOCl as well as highly toxic inorganic acids: HCl, HNO_3 and H_2SO_4 ; caustic organic acids include: acetic acid, formic acid, lactic acid, oxalic acid, carbolic acid and its derivatives (e.g. cresol and Lysol);

- **phosphorus organic compounds** which are acetylcholinesterase inhibitors (AChE);

- **heavy metals** (As, Cr, Cd, Pb, Hg, Cu and Zn) metal compounds soluble in water or body fluids have a stronger toxic effect; some elements form metalorganic combinations; generally, they are characterised by specific bioaccumulation which leads to a one-off inclusion of large quantities of the accumulated metal into the metabolic pool as a result of a discharge mechanism such as e.g. stress factor or pathogen;

- aromatic and amine-derivative nitro compounds - methaemoglobin-forming compounds.

2.1.5. Objectives and directions of toxicometric studies

Studies to assess toxic effect of compounds consist of several stages. Any subsequent stage can be performed once justified by the results obtained on the previous one.

Stages of toxicometric procedure studies (and selection)

1. Assessment of physical and chemical characteristics of a compounds and determining its toxicity.

Based on an analysis of the chemical structure of a studied compound and comparing it with data concerning related compounds, its potential toxic properties can be determined. The knowledge of the relationship between the

chemical structure and biological activity plays an important role on this stage. The analysis shall take into account the impact of physical and chemical characteristics on the toxicity of related compounds e.g. chain length or substituent type.

2. Acute toxicity assessment and identification of LD₅₀ combined with:

- assessment of irritating effect;
- assessment of sensitising effects;
- additional studies (including toxicity assessment depending on the administration route and initial studies concerning metabolism and toxicokinetics).

Acute toxicity is a strong toxic effect occurring shortly after administration of a single dose of a xenobiotic or several doses within 24 hours. Acute toxicity is expressed quantitatively by the LD₅₀ parameter, which stands for the quantity of a xenobiotic that causes death of a half of the population of the studied laboratory animals. The animals' death is the parameter monitored in acute toxicity studies. The assessment also includes major directions of toxic effect, which is useful for further studies.

Detailed guidelines concerning the acute toxicity study procedure are included in an Annex to the regulation of the Minister of Health of 2003, according to which "tests on animals shall be carried out in a humane way, following the regulations on laboratory animals and international recommendations in the area. If several equal test methods exist, the one which requires using the least number of animals shall be applied".

The Organisation for Economic Cooperation and Development (OECD) has identified four methods of studying acute toxicity of substances and identifying the LD₅₀ value. The OECD guidelines on study methods are updated periodically according to scientific progress and practical needs.

They accept the following methods:

1. classic method;
2. FD method – Fixed Dose Procedure (OECD guidelines No. 420);
3. ATC method – Acute Toxic Class Method (OECD guidelines No. 423);
4. Up-Down-Up method (OECD guidelines No. 425).

In the classic method, a xenobiotic is administered to groups of laboratory animals by ingestion or onto the skin. The clinical condition of the animals is observed for at least 14 days and all death incidents are registered. The toxic effect duration is identified, and autopsy of all animals is performed after the experiment.

Nowadays old toxicometric methods tend to be replaced by newer and more humane ones. A fixed-dose procedure is among the applied procedures. It uses doses of a toxic substance which cause a minimum (but observable) toxic effect but not the animals' death. The selected dose causing a moderate toxic reaction is administered to a group of animals for 14 days. The method only helps to identify the probable range of doses corresponding to LD₅₀. Due to humane reasons, tests are also performed on *in vitro* cell lines as well as animal and human organs.

An irritating effect of a substance is assessed through a local effect, i.e. limited to the area of direct contact of the toxic substance with the body. A chemical factor causing inflammation as a result of direct, prolonged or repeated contact with the skin, mucous membrane or other tissues is considered to be an irritating substance. A skin irritant causes reversible skin damage following a 4-hour exposure. If irreversible skin damage occurs after a 4-hour period (ulceration, bleeding, scabs, scars, epidermis and skin necrosis) it means the substance is caustic. Studies of irritating effect are conducted on glabrous skin or eye, if irritating action has not been revealed on the skin. An eye irritant causes changes fully reversible within 21 days, following application to the front area of the eye. Irreversible changes are caused by caustic substances. A sensitising effect is assessed on the skin of guinea pigs revealing relevant sensitivity to a known sensitising agent such as α -hexylcinnamaldehyde. Studies of irritating effect on animal skin should be performed when there are valid premises indicating a need for such a study, and an analysis of available data does not bring conclusions or predictions of the substance effects (also if based on the results of studies of chemically similar substances or based on *in vitro* or *ex vivo* test). Substances with $2 > \text{pH} > 11$ are not studied as their irritating effect is known in advance.

3. Assessment of subacute toxicity at repeated doses for 28 days, including assessment of cumulative effect.

4. Assessment of subchronic toxicity at repeated doses for 90 days.

Studies on subacute and subchronic toxicity are mainly intended to identify the nature of the substance effect on the body organs and systems, and establishing the maximum non-toxic dose. Studies are performed on larger groups of animals than for acute toxicity (usually 30 females and 30 males) for a longer time (usually 28 or 90 days). Different groups of animals receive different doses, whereby the lowest dose should not cause harmful effect and the highest one should have a toxic effect but not cause death of the animals. Similarly to acute toxicity studies, reactions of the animals are observed during the whole experiment and any cases of death or changes in the clinical condition are registered. The duration of toxic effect is identified and autopsy performed on all animals (which died naturally and were put down).

5. Assessment of chronic toxicity in a 2 years' (or 12 months') test.

Chronic toxicity of a chemical substance is the ability to cause a toxic effect in laboratory animals under chronic exposure. The exposure involves intermittent or continuous administration of the studied substance by different routes, five days a week for at least 12 months. The assessment of chronic toxicity can help to discover delayed effects and identify mechanisms of toxic effect (biochemical, haematological, physiological and neurological changes) as well as to identify critical organs i.e. the ones in which xenobiotics accumulate or which are damaged as a result of cumulated toxic effects. An assessment of 2 years' exposure facilitates initial identification of a potential carcinogenic effect of a compound. There are

delayed effects of exposure to toxic substances which are defined as pathological processes developing in a body following a long (or short) latency period. Delayed effects can develop in organisms directly exposed to a chemical substance (somatic cells) or in subsequent generations (as a result of gene damage). The nature of the changes varies and the changes often come as tumours (carcinogenic effect). Secondary (generation) disorders tend to be genotoxic disorders (in the genetic material), embryotoxic disorders (pathological changes in the offspring) and teratogenic disorders (pathological changes in embryos or foetuses).

The purpose of chronic toxicity studies is to assess long-term effects of using small quantities of the reference substance. They are intended to identify concentrations safe at long-term exposure, i.e. identification of the highest acceptable dose (HAD) and highest acceptable concentration (HAC), which are essential parameters for development of OHS standards.

Studies are carried out in the same way as for subchronic toxicity but a single study lasts much longer and takes 2 years (at least 12 months). Once the observation is complete, the same biological and morphological tests as in subchronic toxicity studies are performed, and the carcinogenic effect is additionally investigated to identify genotoxicity of the compound.

6. Assessment of delayed effects by:

- studying of genotoxic effect;
- studying of carcinogenic effect;
- studying the teratogenic effect;
- studying the impact on fertility, reproduction and offspring.

The purpose of a mutagenic study is to assess the ability of a chemical compound to cause mutation in human DNA, which means its carcinogenicity. Not all carcinogens are identified this way as some of them cause tumour development leaving a cell genome intact. Tests are carried out on animals, cell cultures, bacteria, fungi and plants.

A study of teratogenicity i.e. adverse effect of a substance on a foetus is performed on pregnant animals, mainly on rodents. Any potential congenital malformations caused by a xenobiotic are assessed up to three months from the end of pregnancy.

Impact on fertility and reproduction is assessed to evaluate toxic effect of a substance on the functions of the reproductive organs, insemination, delivery and feeding. A three-generation study is carried out to examine reproductive toxicity of a substance. Animals of both sexes obtain the same xenobiotic doses as in chronic toxicity studies for 2 months and then are coupled. The offspring is then observed in their lifetime for 21 days, and their behaviour and all biochemical parameters evaluated. Then, they are put down for further analysis. The parents' generation is still monitored for the impact of the studied xenobiotic and re-inseminated after a few weeks. A random group of the second litter and parents' generation is selected and the same study procedure is repeated.

7. Assessment of neurotoxic effect.

Lipophilic and phosphorous organic compounds are major compounds subjected to neurotoxic effect tests. Study methods used for neurotoxicity assessment in animals include the following tests:

- behavioural (measurement of spontaneous motor activity, testing simple unconditional reactions and conditional instrumental reactions);
- electrophysiological (electrical phenomena occurring in tissues and organs are recorded and analysed, along with measurement stimuli conduction rate and electroencephalographic tests);
- morphological (morphological changes of the nervous tissue in histopathological preparations);
- biochemical (mainly used to explain the mechanisms of toxic effect of a compound on the nervous system).

2.2. Absorption of xenobiotics

There are many ways of a living organism exposure to toxic effect of foreign substances with a potential toxic action. Most substances reveal toxic effect after penetrating into the body and reaching a relevant concentration in the most sensitive organs. Only some compounds reveal a local effect, with no need to penetrate. A toxic substance penetrating into the body, regardless of the exposure route, is distributed (transported) and subjected to biotransformation (biochemical transformations) to be finally excreted from the body.

2.2.1. Xenobiotics exposure routes

Xenobiotics can penetrate into the body *via* different routes:

- oral (**p.o.** – *per os*);
- inhalation (**inh.** – *inhalation*);
- intravenous (**i.v.** – *via intervenosa*);
- dermal (*cutaneous, dermat*);
- subcutaneous (**s.c.** – *via subcutanea*);
- topical (**p.c.** – *via percutanea*);
- intramuscular (**i.m.** – *via intramuscularia*);
- intraperitoneal (**i.p.** – *via intraperitonealis*);
- through mucosal membranes – conjunctiva, nose, anus or vagina.

Under industrial (environmental) conditions, the basic routes of xenobiotics absorption include: respiratory tract, alimentary tract and skin. The way of xenobiotics penetration into the body mainly depends on the route of exposure and the substance physical form.

Liquid or solid substances can penetrate into the body through the alimentary tract or skin. Gaseous xenobiotics, such as vapours or aerosols, are most easily absorbed in the respiratory tract, while vapours and gases can also be resorbed through the skin. Aerosols accumulate easily on hairy skin and penetrate into the body.

2.2.2. Absorption in the respiratory tract

Xenobiotics in a gaseous form (vapours and aerosols) are absorbed in the respiratory tract. Lungs are the main area of xenobiotics resorption due to their strong vascularisation and large absorbing surface (estimated as 90 m²) as well as the fact that alveoli are built of thin permeable membrane with poor selective properties. Absorption is quick and highly effective. Toxic compounds absorbed this way penetrate in an unchanged form directly into the blood stream as they avoid biotransformation by passing the hepatic circulation.

There are a number of factors affecting the resorption rate of toxic substances in the respiratory tract, including:

- concentration of xenobiotics in the air;
- lung ventilation;
- air/blood partition coefficient;
- speed of changes and poison excretion from the body;
- physical form of the substance and the size of its particles;
- solubility in water.

Based on the value of a toxic substance concentration in the air, the dose absorbed in the respiratory tract can be calculated. The following formula is used to that end:

$$D = R \times W \times C \times T$$

where: D – absorbed dose (mg), R – compound retention in the lungs expressed as a fraction, W – ventilation of the lungs in m³/h, C – concentration of the compound in the air (mg/m³), T – exposure duration (h).

The absorbed dose calculated this way is an approximate value, as all values in the formula are only estimated ones.

Breathing intensity depends on the body need for oxygen. Physical effort (intensive work, physical training) increases the demand. Greater volumes of air and hence a higher concentration of a xenobiotic are introduced into the lungs. Cardiac output rises during physical effort, which increases the rate of transporting the absorbed substance to organs and tissues with the blood. It also causes more intense xenobiotic absorption in the respiratory tract.

The quantity of toxic substance penetrating from the inhaled air into the blood depends on its solubility in the blood and other tissues, which is assessed

based on the value of the blood/air partition coefficient. Gases highly soluble in blood, i.e. having a high value of the blood/air partition coefficient, diffuse into the blood easily, which means that their absorption increases as lung ventilation becomes more intense. Gases with low value of the blood/air partition coefficient (hardly soluble in the blood) diffuse into the blood, and increased lung ventilation boosts their resorption only slightly, since the blood is fully saturated with the substance. For such substances, absorption may increase with faster blood flow in the lungs.

Absorption of toxic substances in the respiratory tract depends on the physical form of the substance. Gases and vapours are absorbed by diffusion. Resorption depends on the particle size. Large dust particles, with diameters over 5 μm deposit in the oronasal cavity and can be removed by expectoration or cleaning of the nose. The expectorated particles can be removed from the body or swallowed and absorbed in the alimentary tract. Non-expectorated particles stay in the upper sections of the respiratory tract where they are slowly resorbed. Particles with diameters ranging from 1 to 5 μm are deposited in the trachea and bronchi. Aerosols with particle sizes up to 1 μm , which penetrate into the alveoli, have the greatest toxicological importance.

Hygroscopicity of the compound and its solubility in water, which makes for over 90% of mucus covering the surface of the respiratory tract, plays a major role in resorption of liquids. Dusts of compounds hardly soluble in water (such as lead sulphates and silicates) can be absorbed in the respiratory tract through pinocytosis. Non-soluble dusts can be removed from the respiratory tract owing to ciliary transport upward the respiratory tract, which usually takes several hours. The physical half-life of non-soluble dust in the alveoli lasts much longer, up to hundreds of days.

Solubility in water greatly determines the place of vapour or gas resorption in the respiratory tract. Hydrophilic substances (such as ammonia and hydrogen chloride) dissolve in the mucilage layers already in the upper sections of the respiratory tract. After dissolving, gas particles diffuse into epithelium cells of the respiratory tract and further into the capillary vessels located below epithelial cells to be transported to the tissues with the blood. Particles of lipophilic gas (soluble in lipids) (such as nitrogen oxides and phosgene) can dissolve in the respiratory tract secretion only slightly and that is why the majority of them stays in the respiratory tract and travels down the respiratory system where they can be absorbed by the large surface of alveoli and penetrate into the capillary blood.

2.2.3. Absorption from the alimentary tract

The alimentary tract can be divided into three major sections differing for the speed, efficiency and specific nature of absorption. They are the oral cavity with oesophagus, stomach and intestines.

Toxic compounds highly soluble in water (e.g. cyanides, nicotine, nitroglycerine, strychnine, phenol and alcohols) are absorbed in the oral cavity owing to good vascularisation of the mucous membrane. They stay in the oral cavity only for a short time which is not sufficient for the quantity of the absorbed substance to be significant. On the other hand, substances absorbed from the oral cavity are not subjected to the action of digestive enzymes in the alimentary tract and are absorbed bypassing the hepatic circulation, which means they are not metabolised in the liver and stay in a biologically active, unchanged form in the body for a longer time.

In the stomach chemical substances mix with food, gastric acids, digestive enzymes and bacteria, which significantly affects biological activity of the compound and can affect its toxicity. For instance nitrates, which are practically non-toxic, are transformed into highly toxic nitrites by microorganisms in the alimentary tract. The presence of chyme in the stomach affects substance toxicity by impacting its absorption. Absorption from the alimentary tract is weakened by formation of complexes with proteins. Presence of lipids or alcohol in the chyme can intensify absorption of such toxic substances as phosphorus or pesticides. A low pH value of gastric acid (about 1) contributes to absorption of substances of low acidity (slightly dissociated) and alkaline ones in the stomach. Non-dissociated forms are highly soluble in lipids and penetrate through biological barriers easily by diffusion. Alcohols are examples of substances fairly well absorbed in the stomach.

Intestines have the highest absorption capacity. Xenobiotics are absorbed in intestines *via* different transport mechanisms such as passive diffusion, diffusion through pores, facilitated transport, active transport and pinocytosis in the intestinal villi. Efficient absorption in the intestines is fostered by a very large absorptive surface of the mucosa, estimated as 200–300 m² in humans, and high variability of the intestinal chyme reaction from low acidic in the small intestine to low alkaline in the final sections. There are mechanisms which can improve the process of a xenobiotic absorption in the intestines. Proteins in the intestinal mucous membrane act as active carriers of elements essential for the body such as apoferritin which transports Fe. They can also transport elements with similar physical and chemical characteristics, including Co, Ni and Mn. Molecules with diameters of several nanometres can be absorbed through pinocytosis in the intestine villi and penetrate into the circulatory system *via* the lymphatic system and bypassing the hepatic circulation. Large molecules such as azodyes or polystyrene can be absorbed through phagocytosis by macrophages in the intestine walls.

Substances absorbed from the stomach and intestines, before entering the peripheral blood circulation, get to the liver *via* the portal venous system, where they are subjected to biotransformation processes reducing or increasing their toxicity. Some of them come back with the bile to the alimentary tract

to be removed with faeces afterwards. Motor functions of the alimentary tract determine direct contact of the poison with the absorptive surface. If constipation occurs, poison absorption is higher as it stays in the intestines for a long time, while increased peristalsis (diarrhoea) makes the poison contact time with the alimentary tract membrane short, which limits the poison absorption.

2.2.4. Absorption through the skin

Skin is a protective barrier separating the body from the environment. Owing to the barrier, the body is able to resist exposure to xenobiotic doses as much as 1000 times higher than in the case of toxin ingestion or inhalation. Nevertheless, xenobiotic can penetrate into the body through intact skin, and toxic substances getting into the blood through the skin may cause poisoning of the whole body or even death. Dermal penetration is a long-lasting process depending on time. The time of exposure to a xenobiotic is the most important factor. Quick removal of a substance from the skin surface can prevent penetration of a large quantity of a xenobiotic.

Vapours and gases are first and foremost resorbed through the skin. They penetrate much easier than liquids or substances solved in them that can also penetrate through the skin tissue. Aerosols deposit easily on hairy skin, which facilitates their getting into the body.

Skin consists of the following three layers: epidermis (external layer), dermis (well-vascularised middle layer) and internal subdermal layer called hypodermis (composed of adipose tissue and connective tissue). Furthermore, skin anatomy identifies other skin appendages being potential spots of xenobiotic penetration, including hair follicles and sebaceous and sweat glands and ducts.

Toxic substances are absorbed through the skin by means of the following two mechanisms.

Transepidermal transport occurs directly through the epidermal cells and with regard to a large absorption area it is the main route of poison penetration into the body. Substances are absorbed in this form of transport *via* passive diffusion, going through a number of epidermal cell layers, while penetration through the stratum corneum built of tightly packed keratinocyte cells is a stage which limits the speed of the whole process. Organic compounds with a high oil/water partition coefficient and low ionisation degree such as aromatic and aliphatic hydrocarbons, aromatic amines and nitric compounds, phenols, phosphorous organic insecticides, carbon disulphate, carbon tetrachloride and tetraethyl lead are best absorbed *via* this route. Non-polar lipid compounds easily penetrate lipid areas of the membranes, while polar substances get inside the cells assisted by protein elements.

A number of factors affect the efficiency of absorption through the skin, including injury of the epidermis and skin condition, temperature, humidity

and chemicals. Higher humidity and hydration of the cornified epidermis layer facilitate transport of polar compounds. Solids which do not penetrate through the skin can dissolve on the skin surface and be absorbed as dissolved substances. Chemicals acting directly on the epidermis and causing its damage or removal of the surface lipids and a change in its structure can contribute to a greater speed of xenobiotics penetration through the skin. They include acids, alkalis, detergents and organic solvents such as dimethyl sulfoxide (DMSO), methanol, ethanol, hexane, acetone and - primarily - a mixture of chloroform and methanol.

Transfollicular transport is a xenobiotic diffusion through hair follicles, and sweat and sebaceous glands and ducts which are an easy way of penetration. Transport through the skin appendages has a low significance for absorption of toxic substances as they cover only up to 1% of the skin surface. It is the absorption route of electrolytes, heavy metals and their organic complexes of low solubility.

2.3. Distribution of toxic substances in the body

Distribution is a wide term covering the xenobiotic route from absorption through to penetration through internal barriers of the body, transport, binding with plasma proteins, getting to the tissues and organs with the blood and selective cumulation.

The circulatory system plays the most important role in the distribution of toxins. Blood collects xenobiotics from their absorption spot, distributes them all over the body and participates in their excretion. The substance distribution rate in different organs depends on the cardiac output and vascularisation of the organ. Within a few minutes from absorption, the toxic substance reaches the organs with the highest blood flow (heart, lungs, liver, kidneys and brain). It takes more time (from several dozen minutes to several hours) for xenobiotics to penetrate into poorly vascularised organs such as the skin, skeletal muscles, connective tissue and adipose tissue.

Binding with plasma proteins, especially albumins (barbiturates, sulphonamides, ascorbic acid, Cu, Zn, Ca and histamine) and globulins (cyanocobalamin, polychlorinated insecticides, vitamins A, D, E and K) makes an important element of a xenobiotic transport with the blood. It is a non-specific and reversible bond, and its strength depends on the compound affinity to the protein. With regard to a limited quantity of protein molecules and their binding spots, the degree of binding depends on the protein and xenobiotic concentration and occurrence of competitive substances with greater affinity. The K_A association constant (also called a binding constant) identifies the affinity of a xenobiotic binding with a protein. Bonds essential for a xenobiotic distribution are characterised by the association constant value of at least 1×10^4 . The association constant is calculated according to the formula:

$$KA = [XB]/[X] [B],$$

where: KA – association constant; X – free xenobiotic concentration; B – free protein concentration; XB – xenobiotic-protein xenobiotic concentration.

Binding of xenobiotics with proteins depends on the plasma pH, which determines a protein ionisation degree; for instance a reduced quantity of barbiturates bonded with proteins is observed in acidosis.

Binding of a xenobiotic with plasma proteins weakens its action and affects elimination. Substances bonded with proteins do not penetrate through the walls of capillary vessels, which prevents their getting to the target organs and glomerular filtration. Binding with proteins does not limit tubular secretion or biotransformation.

Xenobiotic-binding proteins can be found in some organs, which contributes to a random deposition of toxins. Ligandin, a simple protein with high affinity to organic acids and corticosteroids, is present in the liver. Methallotioneins are proteins occurring in the liver, kidneys and mucous membrane, responsible for binding of such metals as Cd, Cu, Zn and Hg.

Selective accumulation of xenobiotics can be important for selective cumulation of toxins. Toxins (or their metabolites) are accumulated in the body when a new dose of a xenobiotic is absorbed before the previous one has been excreted. Compounds with long half-life (which is the time necessary to reduce the substance concentration by half as compared to the initial value), taken for a long time, even in small doses tend to accumulate more often than other ones. The cumulation mainly applies to lipophilic substances (e.g. organochlorine insecticides and polychlorated biphenyls) which are stored in the adipose tissue. A sudden xenobiotic release from the reserve adipose tissue as a result of its instant metabolism e.g. in case of hunger or disease, may cause poisoning. Bone tissue is another example of a tissue prone to accumulate xenobiotics in a selective manner. It mainly accumulates lead, strontium, fluorides and radium. The elements are released in ionic exchange processes and following osteolytic activity. An increase in their concentration in the blood, and consequently in major organs (liver, kidneys and brain), may cause poisoning.

2.3.1. Biological barriers

A substance passing from its contact area with the body to the blood stream and its transport to different tissues require crossing of various barriers in a form of biological (cellular and subcellular) membranes. A semi-permeable protein and lipid membrane surrounding every cell in the body is a key control element of penetration of toxic substances into the tissues of different organs.

The substances are transported through the plasma membrane *via* a number of mechanisms, depending on the physical and chemical properties of a xenobiotic.

Passive diffusion is the simplest transport method involving free penetration of a substance through the semi-permeable membrane, according to the concentration gradient (from higher to lower concentration) and its rate increases with greater difference in the substance concentration on both sides of the membrane. Small, non-dissociated particles of compounds soluble in lipids (high oil/water partition coefficient) are prone for passive diffusion.

Transport through the pores facilitates penetration of fine molecules (up to 1 nm) of hydrophilic substances and ions. They are transported with water and the transport rate depends on the difference in the hydrostatic or osmotic pressure on both sides of the semi-permeable membrane.

Facilitated diffusion is a form of transport similar to simple diffusion but it is supported by integral membrane proteins being selective carriers for individual ions and nearly all small organic molecules, except for lipophilic molecules penetrating the membrane by simple diffusion. Diffusion facilitating proteins are carriers which temporarily change their conformation, or protein canals forming hydrophilic canals for selected inorganic ions (mainly Na^+ , K^+ , Cl^- and Ca^{2+}) inside the hydrophobic membrane. This kind of transport does not require additional energy supply because it takes place according to the concentration gradient.

Active transport takes place against the concentration gradient, is assisted by membrane pumps, and requires supply of energy, mainly from high-energy ATP (adenosine triphosphate) bonds. A sodium-potassium pump (Na^+/K^+ -ATPase) is the most typical example of an ionic pump.

Endocytosis involves penetration of a substance embedded inside a bubble formed from a fragment of a cellular membrane. The phenomenon is important in toxicology for aerosol absorption in the airways. If endocytosis applies to substances dissolved in the body fluids it is called **pinocytosis**, while endocytosis of solids is known as **phagocytosis**.

Intraorganic barriers being areas with limited permeability significantly reduce transport of toxic substances in the body. The blood-brain barrier which is a typical protein-lipid membrane constitutes such a protection in the central nervous system. It permeates small molecules of lipophilic toxic substances quite easily but forms a barrier for high molecular weight, ionised and hydrophilic compounds. A placenta is another example of such a barrier; it consists of metabolically active tissues forming a complex barrier between the mother's blood stream and the foetus. Placenta limits penetration of high-molecular compounds and ionised compounds not soluble in lipids.

2.4. Metabolism and excretion of xenobiotics

2.4.1. Biotransformation of xenobiotics

Biotransformation of xenobiotics occurs in different tissues and organs, including intestines, kidneys, lungs and skin but first and foremost the reaction take place in hepatic microsomes. Enzymatic complexes related to the membranes of the smooth endoplasmic reticulum of hepatocytes, called microsomal monooxygenases, hydroxylases or oxidases of mixed functions, play a special role in biotransformation of xenobiotics by the liver. Other enzymes participating in biotransformation are located in the mitochondria and lysosomes. They include alcohol dehydrogenase and aldehyde dehydrogenase (which oxidise aliphatic and aromatic alcohols), carboxylesterases and amidases (hydrolysing esters, thioesters and amides).

The following three main components have been identified in the microsomal monooxygenase system:

- haemoprotein – cytochrome P-450;
- flavoprotein – cytochrome P-450 reductase;
- phospholipids.

The term cytochrome P-450 stands for a complex of different haemoproteins (haem is a prosthetic group in the enzyme) with a similar but not identical structure of amino acids catalysing molecular oxygen activation and its embedding in the structure of xenobiotics or substances of endogenic origin.

Spectrophotometric, immunological, electrophoretic and chromatographic tests revealed presence of many molecular forms of cytochrome P-450. Each form indicates organ specificity (e.g. CYP: 2A1, 2A2 and 2B3 occurring only in liver, and 2A3 only in lungs), and also partly different specificity of substrates. Presence of some forms of cytochrome P-450 depends on sex or age. Many chemical compounds cause an increase in expression of different forms of the cytochrome. They include polycyclic aromatic hydrocarbons, barbiturates, steroids, macrolide antibiotics, ethanol and acetone.

The purpose of biotransformation is to transform xenobiotics into more polar compounds which are better soluble in water and hence easier removed from the body. If the metabolites formed are less toxic than the original substance, the process is called **detoxication**, and if they become more active than the xenobiotic introduced, the process is called **activation**. Reactions catalysed by cytochrome P-450 may also produce free oxygen radicals which cause metabolic disorders in the hepatocytes together with reactive metabolites, resulting in hepatic steatosis, necrosis or neoplastic transformation of the cells.

Some xenobiotics do not undergo biotransformation, though. They include highly lipophilic compounds stored in the adipose tissue in an unchanged form. Similarly, highly volatile compounds such as ethyl ether and strongly polar compounds including phthalic acid are not subjected to biodegradation.

Metabolic reactions of xenobiotics in the body occur in 2 phases:

– Phase I covers the following reactions: hydroxylation, oxidation, reduction and hydrolysis aimed to introduce polar groups (-OH, -COOH, -SH and -NH₂) into the lipophilic compound structure or to remove alkyl groups;

– Phase II – coupling; the previously formed metabolite with a polar group is coupled with endogenic compounds of very good solubility in water, e.g. glucuronic acid, sulphuric acid or glutathione, which results in formation of non-active compounds with better solubility in water, higher molecular weight and then excreted with urine or bile.

Reactions of phase I

1. Hydroxylation – substitution of a hydroxyl group to the side chains of aromatic hydrocarbons (e.g. toluene transformation into benzoic acid) and barbiturates.

2. Epoxidation – binding of an oxygen atom to a double bond with epoxide formation. Epoxy metabolites are non-durable and react with macromolecules such as DNA, and hence may cause mutagenic or carcinogenic effect. Epoxy hydratase is an enzyme which transforms toxic epoxies and arene oxides, formed by oxidation of aliphatic and aromatic double bonds, to non-active diols.

3. Oxidative deamination – removal of an amine group and amine oxidation to ketone (e.g. amphetamine to phenyl acetone) under the influence of amine oxidase in the presence of HADPH cofactor and molecular oxygen.

4. Dealkylation – removal of alkyl groups.

5. Desulphurisation – oxygen substitution instead of sulphur; phosphorous organic insecticides and thiobarbiturates are subjected to biotransformation, usually to form more toxic metabolites.

6. S-oxidation – oxygen connection to sulphur, e.g. aliphatic and heterocyclic thioethers are transformed into sulphates and sulphoxides.

7. N-oxidation – oxidation of secondary and tertiary amines to N-oxides by flavoprotein monooxygenase (oxidase of amines).

8. N-hydroxylation – primary and secondary aromatic amines are transformed into alkanolamines or nitric compounds (e.g. aniline → nitrobenzene).

9. Reduction of nitric and nitrogen compounds (nitrobenzene, chloramphenicol) to primary amines.

10. Reductive dehalogenation e.g. dichlorodiphenyltrichloroethane (DDT) → DDE.

11. Single-electron oxidation and reduction result in formation of free radicals (reactive ones, having unpaired electron) e.g. single-electron oxidation (removal of 1 electron from a benzene ring causes formation of a cationic benzene radical), a single-electron reduction (electron binding to the benzene ring results in formation of an anionic benzene radical).

Reactions of phase II

1. Glucuronidation – plays a major role in neutralisation of toxic substances; glucuronide residue from active glucuronic acid (UDP-glucuronic acid) with participation of enzymes – glucuronyl transferases – is bound by oxygen, nitrogen or sulphur group with substances having hydroxyl, carboxyl, amine or sulfhydryl groups. Many compounds including alcohols, phenols, sterols, alanine, aliphatic amines, carboxylic acids and benzoic acid are excreted as glucuronides.

2. Sulphatation – coupling with active sulphuric acid (3'-phosphoadenosine 5'-phosphosulphate); it applies mainly to phenols but also to aliphatic and aromatic amine compounds. Sulphur esters are formed after being coupled with sulphate, while hydrogen cyanide and cyanides become rhodanates (thiocyanates), while some metals form sulphides.

3. Coupling with glutathione (cysteine tripeptide – glycine – glutamic acid) by an active SH cysteine group. Epoxies of aliphatic and aromatic hydrocarbons, halogen aliphatic and aromatic hydrocarbons and halogen nitro compounds are coupled.

4. Methylation – coupling with a methyl group coming from active methionine (S-adenosylmethionine – SAM) formed as a result of activation by ATP. The process covers phenols, alcohols, aliphatic and aromatic amines, mercury, arsenic and selenium.

5. Acetylation – coupling with acetic acid. Acetyl coenzyme A, i.e. active acetate (acetyl group activation in the presence of ATP) combined with coenzyme A is the source of acetyl group. Aromatic amines are subjected to the process.

6. Coupling with amino acids – applies to compounds containing hydroxyl groups. Many amino acids participate in the process (glutamine, serine, arginine, lysine) while glycine is the most important one.

There are various endo- and exogenous factors conditioning the speed and efficiency of biotransformation processes. General condition of the body, diseases, diet, age, sex and environmental factors affect the speed of the processes. Biotransformation is slower in newborns, elderly people and women, particularly in pregnant ones. Good nutrition and balanced diet determine the correct speed of biodegradation of toxins.

2.4.2. Excretion of xenobiotics

Toxic substances are removed from the body *via* different routes: through the respiratory tract (with exhaled air), excretory system (with urine), alimentary tract (with faeces and bile), while minor quantities of toxins are removed with sweat, saliva (actually in the alimentary tract), mammary glands and placenta.

Excretion through the respiratory tract

Volatile substances including essential oils, ether, cyanides, nitrogen oxide, benzene, nitrobenzene and phenol are removed with the exhaled air. Excretion occurs by passive diffusion while its speed depends on the following factors:

- partial gas pressure in the alveoli and blood;
- degree of lung ventilation;
- cardiac output and blood flow rate through the lungs;
- value of the blood/air partition coefficient, where substances soluble in the blood (alcohol) are poorly removed with the air.

Excretion by the alimentary tract

Toxic substances absorbed in the alimentary tract get with the blood to the liver *via* the portal vein, where they are metabolised in the hepatocytes surrounding the bile ducts. Then they get into the bile or are returned to the blood and eliminated by the kidneys. They reach the lumen of the alimentary tract and can be excreted with faeces. Some substances excreted with the bile to the duodenum can be reabsorbed into the blood in the intestines. It applies to non-dissociated substances soluble in lipids. They come back to the liver and then to the bile until a polar metabolite is formed following metabolic transformations. Polar compounds and substances with high molecular weight are excreted with the bile. They are polycyclic aromatic hydrocarbons, dioxins, bile salts, glucuronides and bilirubin.

Excretion by the urinary tract

This is the most common elimination route of toxic substances; they are metabolites of organic and inorganic compounds with low molecular weight and soluble in water. The excretion process consists of the following three stages:

- glomerular filtration (passive filtration) in glomeruli capsules;
- passive tubular diffusion (reabsorption);
- active tubular transport (intratubular excretion).

Nearly all toxic compounds and their metabolites (both hydrophilic and hydrophobic ones) occurring in a free form in the plasma, i.e. the ones not linked to proteins, penetrate into the glomerular filtrate under passive diffusion. The filtration rate depends on the blood flow through the kidneys and increases with higher volumes of liquids drunk. With regard to low molecular weight, most drugs – except for albumin-related drugs – easily penetrate into the primary filtrate. The substances from the glomerule get into the tubule, where they can be reabsorbed or eliminated with urine.

About 99% of the primary filtrate volume is reabsorbed passively. Strongly ionised compounds and compounds not soluble in lipids are reabsorbed, while

non-ionised lipophilic compounds are reabsorbed into the blood. That is why substances soluble in lipids are slowly removed from the body. For weaker acids or alkalis, the pH of urine is of key importance for the mechanisms. Urine alkalinisation (administration of sodium bicarbonate or sodium lactate) increases elimination of acidic substances, while urine acidification (administration of ammonium chloride) boosts elimination of alkaline compounds.

Intratubular excretion is a process reverse to tubular absorption. Secretion of compounds to the renal tubule lumen occurs *via* active transport and only covers ionised substances non-soluble in lipids and protein-related compounds. Since the compounds are transported to the tubule lumen by means of active transport (against the concentration gradient), active intratubular excretion is the most effective mechanism of metabolites elimination by the kidneys – a compound concentration in the blood can be reduced to values close to zero. Nevertheless, a large group of compounds can use the same transport system, which may result in a competitive inhibition. The phenomenon can be used to prolong the substance action in the body (e.g. a drug) by administering a compound which inhibits drug elimination but it has adverse implications if prolonged action of toxins occurs.

Mechanisms of active transport are poorly developed in newborns and babies, which causes their high sensitivity to poisoning with toxic substances, in combination with low efficiency of the coupling processes (biotransformation phase II).

Renal clearance

The term “clearance” is used to identify the speed of substance elimination from the blood by the kidneys. It is the efficiency of renal function to clean the blood of the reference substance. The value is a resultant of the speed of all excretion processes and reabsorption in the kidneys. Clearance is expressed as the volume of blood completely cleaned of the substance in a unit of time; it is calculated according to the formula: $Cl = (C_m \times V_m) / C_o$, where: Cl – clearance; C_m – substance concentration in urine; V_m – urine volume; C_o – substance concentration in the blood. For instance, the compound clearance value of 10 ml/min means that 10 ml of blood is completely purified of the compound.

The average blood flow rate in an adult is about 600 ml/min, whereby 20% is filtrated in the glomeruli, which is about 120 ml/min. Therefore:

- renal clearance of substances excreted only by glomerular filtration and not reabsorbed in the renal tubules (inulin) is about 120 ml/min;
- renal clearance of substances excreted by glomerular filtration and active excretion in the tubules, not reabsorbed in the tubules (p-aminohippuric acid) is about 600 ml/min;
- renal clearance of substances reabsorbed in the tubules will be low, i.e. less than 120 ml/min.

2.5. Selected issues of detailed toxicology

2.5.1. Mechanisms of toxic effect

Detailed toxicology deals with systematic studies and description of poisons, covering their division into groups based on similar chemical structure, properties or functional application. Mechanisms of action of only some toxic substances have been discovered so far. A mechanism of toxic action is a general term covering acute and chronic effects and sometimes also the delayed effects. The effects of toxic action of xenobiotics may apply to specific organs or system (neurotoxic, nephrotoxic and hepatotoxic effect). All these effects can be triggered by one or more mechanisms. The mechanisms of toxic action can be divided as follows:

1. Physical action;
2. Tissue hypoxia;
3. Inhibition of enzymatic reactions;
4. Blocking of receptors;
5. Active metabolites: covalent bonds;
6. Toxic action of free radicals;
7. Chemical carcinogenesis;
8. Immunological effect.

Physical action stands for the effect caused only by the poison presence when it does not react chemically with the biological system components. Chemical action is observed when the toxic effect depends on the occurrence of a chemical reaction between the poison and a specific biological system of the body.

1. Physical action is observed in lipophilic substances which penetrate the blood-brain barrier easily, causing disorders of the central nervous system and giving symptoms of a narcotic effect. Such xenobiotics deposit on the surface and inside the neural cell (neuron) membrane, impairing its correct contact with the external environment. Once the critical concentration of a xenobiotic has been exceeded, the neuron loses its ability to transmit nerve impulses, rendering a narcotic effect.

2. Tissue hypoxia. Appropriate quantities of oxygen need to be supplied to the tissues to reach the right value of the partial pressure of oxygen in the arterial blood. Under normal composition of breathing air (21% oxygen) and normal atmospheric pressure (760 mm Hg) partial pressure of oxygen in the arterial blood amounts to ca. 100 mm Hg. Relatively minor differences only cause discomfort but when the pressure drops below 70 mm Hg, it results in a loss of consciousness. Tissues in which intensive energy transformation takes place (brain and heart) are most vulnerable to the effects of hypoxia. Some toxic compounds (e.g. arsine, phenylhydrazine and chloronitrobenzene) may cause haemolysis of red blood

cells hence reducing the quantity of active haemoglobin responsible for oxygen binding and transport. It mainly applies to acute poisoning. Impeded haemoglobin synthesis, which is a multi-stage process, can be another cause of a toxic decrease in the haemoglobin concentration. That is why the mechanism usually occurs in chronic poisoning caused for instance by lead. The haemoglobin level may also drop as a result of marrow cell damage. Such effect is attributed to benzene, cytostatic drugs and antimetabolites (which are compounds with a structure similar to a natural metabolite and impairing biochemical processes). Hypoxia may also result from haemoglobin inactivation. It can be caused by a toxic action of carbon oxide, nitrates (III), aromatic amines and other oxidants.

Carbon oxide reveals 200–300 times greater affinity to haemoglobin than oxygen so it can eliminate oxygen from combinations with haemoglobin even if its concentration in the air is low. Low concentration of CO in the exhaled air (over 0.1%) may block over 50–60% haemoglobin, resulting in a coma. A further increase in the level of carboxyhaemoglobin (HbCO) poses a risk of the respiratory centre paralysis; prolonged toxic hypoxia caused by carbon oxide may cause irreversible changes in the brain.

Oxidising substances such as sodium nitrate (III) and metabolites of aromatic amines oxidise haemoglobin to methaemoglobin. The oxidised form of haemoglobin (methaemoglobin, MetHb(Fe^{3+})) is not able to transport oxygen, while oxidation of about 60% of haemoglobin results in death.

Carbon oxide, cyanides and azide inhibit cellular breathing, which occurs mainly in the mitochondria of cells using oxygen supplied to the tissues with the blood.

3. Inhibition of enzymatic reactions. Most enzymes are protein molecules whose structure includes an area with a specific arrangement of amino acids (active centre) conditioning catalyzation of biochemical reactions.

Toxic substances may impede enzyme activity by competitive inhibition, where they compete with the reaction substrate to bind with the enzyme in the active centre. The spatial structure of such inhibitors is similar to the natural substrate. The inhibition degree depends on the affinity of the substrate and inhibitor to the enzyme.

Many inhibitors bind with an enzyme or enzyme-substrate complex by connecting in a different place than the active centre (non-competitive inhibition). Some toxins are able to block functional groups of coenzymes participating in enzymatic reactions.

Esters and phosphoric acid derivatives, being inhibitors of acetylcholine esterase, are examples of toxic inhibitors of enzymes. Acetylcholine (ACh) is a neuromediator conducting impulses in the synapses of peripheral nerves and in the central nervous system. The ACh release to the synaptic space between two neurons transfers a nerve impulse between them. ACh is also released in the motor end-plate, i.e. between a neuron and muscle. ACh binding with

a cholinergic receptor present on a post-synaptic cell causes an effector reaction such as a muscle contraction. The reaction comes to an end owing to a quick decomposition of excess acetylcholine by the enzyme – acetylcholine esterase (AChE). Esters and phosphoric acid derivatives (e.g. phosphorous organic pesticides) are able to form complexes with AChE, causing its inhibition. At high degrees of AChE inhibition, poisoning occurs following accumulation of acetylcholines in synapses and nerve ends. It also includes disorders of the central nervous system or even death as a result of the respiratory centre paralysis.

4. Blocking of receptors. Cellular receptors communicate information from the external environment into the cell. It takes place *via* signal molecules which bind with relevant receptors. They include hormones being neurotransmitters. The abovementioned acetylcholine released from a neurone into the synaptic space is an information carrier between a nerve cell and effector cell (muscle cell). ACh impacts the muscle cell *via* special receptors in the cellular membrane. There are two types of cholinergic receptors: muscarine and nicotine ones. Their name is related to selective stimulation by muscarine and nicotine, respectively. Acetylcholine stimulates both types of receptors as it is able to take different conformational forms. Muscarine is a simple alkaloid with a structure similar to acetylcholine, present in different species of toadstools, mainly in *Amanita muscaria*. Several minutes after eating such a mushroom, symptoms of stimulation of cholinergic muscarine receptors of smooth muscles and exocrine glands can be observed (lacrimation, salivation, sweating, dyspnoea, abdominal pain, diarrhoea, lower heart rate, cardiovascular collapse and coma).

Nicotine has an analogical stimulating effect on cholinergic nicotine receptors of the motor-end plate (causing contraction of striated muscles) and ganglia.

Chemical substances binding with a cellular receptor can also play a role of inhibitors, impeding cell functions. This way the receptors are blocked in a non-active form preventing nerve impulse transfer. Such a mechanism of action can be observed in compounds being antagonists of the cholinergic nicotine receptor e.g. tubocurarine, an active substance present in a plant poison known as “currara” used by Indians to poison arrows, and nowadays used as a drug paralyzing motor nerve ends. Bungarotoxin, a toxin present in snake venom, causing paralysis and respiratory insufficiency which may cause death, is among the abovementioned compounds.

A toxic substance can bind permanently with a presynaptic neurone membrane, blocking the canals which release ACh to the synaptic space. This is a mechanism of action characteristic of one of the strongest poisons – botulin (botulinus toxin) released by anaerobic bacteria *Clostridium botulinum*, developing in food products. Binding of the toxin in nerve ends is irreversible; muscles paralysed by the toxin behave as if they were deprived of neurination.

5. Active metabolites: covalent bonds. High doses of some toxins cause necrotic changes in the liver parenchyma, which is caused by covalent bond of reactive metabolites with hepatocyte proteins, including enzymatic proteins. Active metabolites can also bind with molecules of nucleic acids and lipids. Acetaminophen and bromobenzene reveal toxic action based on covalent bonding mechanism.

6. Toxic effect of free radicals. Free radicals are particles containing at least one unpaired electron. Free radicals are formed in the body under physiological conditions e.g. in the mitochondrion respiratory chain. If the conditions are favourable, despite their high reactivity the molecules do not pose any hazard for biochemical processes, since the body can use antioxidating mechanisms helping to maintain the red-ox balance. They include anti-oxidating enzymes (superoxide dismutase (SOD)), catalase, glutathione peroxidase, ceruloplasmin) and low molecular weight antioxidants such as glutathione, lipoic acid and uric acid. SOD catalyses the reaction of superoxide anion radical dismutation to hydrogen peroxide and molecular oxygen. Catalase assists in hydrogen peroxide decomposition. Glutathione peroxidase catalyses decomposition of hydrogen peroxide and lipid peroxides. A toxic effect is observed at twisted balance when the quantity of generated radicals exceeds the capacity of defence mechanisms. Under inflammation, in the case of injuries or different diseases, an uncontrolled increase in production of free radicals is observed. Toxic effect of free radicals is related to their ability to interact with the body molecules (proteins, nucleic acids, lipids and carbohydrates). Consequently, proteins lose their natural biological functions and properties (enzymatic, regulatory, building), the genetic material is subjected to mutations, while unsaturated fatty acids, which are particularly susceptible to oxidation, are fragmented to form toxic aldehydes. Carbon tetrachloride (CCl_4) is an example of a xenobiotic whose mechanism of action is based on a reaction of the formed radicals with fatty acids. Its active metabolites: trichlormethyl radical ($^*\text{CCl}_3$) and trichlormethylperoxide radical (Cl_3COO^*) are responsible for formation of covalently linked adducts and peroxidation of lipids. Hepatic necrosis is among their effects.

7. Chemical carcinogenesis. Carcinogenesis is a complex, multi-stage process of neoplastic transformation, beginning with the process initiation and followed by tumour promotion and progression. Carcinogenic factors may act on each of the stages. Initiation involves changes (mutation) in the genetic material of the cell. The changes most often apply to genes controlling the cellular cycle and make the right cells transform into potential neoplastic cells. Initiating factors may include genotoxic compounds acting directly or through active metabolites. Active forms of chemicals created as a result of biotransformation are strongly electrophilic, which determines their high reactivity with DNA; this way covalently linked adducts are formed. The adducts cause DNA damage and permanent mutations occur unless the damage is repaired. It depends on the

damage type and quantity and efficiency of the repair system. Genetic changes acquired by the cell can be latent for a long time, while the impact of genotoxic factors may reduce the time. Mutation becomes fixed, if the damage in the genetic material is transferred to the daughter cell. The second stage of a tumour development is promotion, which involves proliferation of cells in which the initial genetic changes were fixed. Growth factors, hormones and non-genotoxic chemicals can act as proliferation promoters. Tumour progression is the third stage characterised by increased invasiveness and capability to cause metastases, related e.g. to formation of subsequent mutations, mainly in the suppressor genes responsible for inhibition of cell proliferation.

Genotoxic carcinogens are divided into compounds directly reacting with the DNA (e.g. ethyleneimine, 1,2,3,4-butadiene epoxide, sulphur yperite and N-methyl-nitrosourea) and indirect compounds whose metabolites are direct carcinogens reacting with the DNA (e.g. vinyl chloride, benzo(a)pyrene, 2-naphthylamine, dimethylnitrosoamine and aflatoxin B1).

Another group of cancerogenic compounds covers epigenetic (non-genotoxic) carcinogens:

- metal ions (Ni, Be, Cr, Pb, Co, Mg and Ti);
- hormones (including synthetic ones) – first and foremost they cause hormonal imbalance, and tend to act as proliferation promoters;
- immunosuppressive drugs – stimulate development of virus-induced tumours, transplanted tumours or metastatic foci (antilymphocyte serum, azapurine, 6-mercaptopurin);
- co-carcinogens – act together with direct or indirect carcinogens and can modify transformation of indirect carcinogens into direct ones (e.g. n-dodecane, SO₂, ethanol, phorbol esters, asbestos);
- promoters: act when exposure to carcinogens ends and they intensify their effects (e.g. phorbol esters, phenol, bile acids, saccharin, o-sulphobenzoic acid imide).

8. Immunological effect. Chemical substances may cause allergic reaction and contribute to formation of antibodies.

2.5.2. Toxicity of selected heavy metals and chemicals

Toxicology of heavy metals

ARSENIC

In the past arsenic compounds would be used intentionally as a poison. Such practices were significantly limited owing to a method of arsenic detection, developed by Marsh in 1836. Nowadays, cases of acute poisoning with arsenic compounds are rare. However, the element is among top most dangerous environmental and industrial poisons.

Arsenic is a metalloid which forms inorganic and organic compounds in oxidation states -3, +3 and +5. Organic arsenic compounds are at least by one magnitude order less toxic than inorganic arsenic compounds and they are quickly eliminated from the body in an unchanged form. Inorganic trivalent As (arsenic trioxide; As_2O_3) is more toxic than the pentavalent one. Arsenic in a solution is more toxic than arsenic taken in a solid form or with food, most probably due to better absorption.

Arsenic is mainly obtained as arsenic trioxide, being a side product of copper ore, lead, nickel and gold melt, 80% of which is used for production of pesticides and 10% for glass production. It is also used in tanning and wood impregnation to protect it against insects and fungi. Arsenic compounds as semi-conductors find their application in electronics in production of microprocessors. Phenyl derivatives of arsenic accelerate the growth of animals and they are added to poultry and pig feed. Arsenic is an ingredient of many drugs, which are now being withdrawn and replaced with other ones.

Potable water and food of marine origin constitute a major environmental hazard. Inorganic arsenic compounds are absorbed from the alimentary tract with 55–95% efficiency, while absorption of compounds hardly soluble in water is lower. Organic arsenic compounds in the food of marine origin are absorbed with 75–85% efficiency. Polluted air near steelworks and power plants combusting coal with a high admixture of arsenic can be the source of the substance. Inhalation is the main route of absorption in occupational exposure, where arsenic in the air occurs primarily as trioxide. Its absorption efficiency in the lungs amounts to at least 40–60%. According to the results of post-mortem, arsenic concentration in the lungs was as much as 8 times higher in employees of steelworks than in people not exposed to arsenic in their workplace, which can directly contribute to a higher incidence of lung cancer in the case of occupational exposure to arsenic.

Arsenic in the blood is mainly bound by erythrocytes, where its concentration is about 10 times higher than in the plasma. It also reveals high affinity to keratin in the skin and its appendages (hair, nails), where 20 times higher concentrations is observed.

Following biotransformation, arsenic compounds undergo red-ox reactions and methylation with participation of methyltransferases and S-adenosylmethionine or non-enzymatically. It is regarded as a kind of detoxication because organic arsenic compounds are less toxic, less reactive and excreted faster.

Arsenic is mainly excreted with urine. The assessment of exposure to arsenic is based on the measurement of As concentration in the urine. Arsenic is not cumulated in the human body. Elimination of arsenic bound in some tissues (e.g. skin) lasts much longer than in the case of general systemic arsenic. Considering the fact that the daily hair growth is about 0.1–0.4 mm, the time of poisoning with arsenic can be estimated based on the distance of its accumulation spot from the hair root.

Acute poisoning – the lethal dose of As_2O_3 for humans is 70–180 mg (1–3 mg/kg body weight). Poisoning by ingestion is revealed by stomach and intestine injury, severe vomiting and diarrhoea (often with rectal bleeding). Dehydration of the body may result in a shock. Other symptoms include muscle spasms, swelling of the face and cardiac dysfunction.

Chronic toxicity applies to occupational exposure, mainly in high inhalation doses (concentration of several hundred $\mu\text{g}/\text{m}^3$). The symptoms include a characteristic garlic breath, perforation of the nasal septum, pharyngitis, laryngitis and bronchitis, cardiac dysfunction and Raynaud's syndrome. In people exposed in their workplace or taking As in water and food, the most typical symptoms of poisoning include skin hyperpigmentation and hyperkeratosis of the palms and soles. Long-term exposure causes necrosis of the limbs following peripheral circulation impairment (known as blackfoot disease).

Delayed effects – the carcinogenic effect of arsenic in humans is well documented and it is known that long-lasting exposure causes cancer of the skin, lungs, urine bladder, kidneys and liver. The valid HAC value for arsenic and its inorganic compounds amounts to 0.01 mg/ m^3 as per arsenic. It does not eliminate the risk of lung cancer incidence. The WHO has identified As concentration in potable water as 10 $\mu\text{g}/\text{l}$. The risk of liver, lung, kidney or urine bladder cancer for people drinking water containing 50 $\mu\text{g As}/\text{l}$ for their whole life is 13 per 1,000 people, while for water containing 500 $\mu\text{g}/\text{l}$ of arsenic it is 13 cases per 100 people.

CADMIUM

Cadmium is a chemical element from the group of transition metals, occurring in minor quantities in the rocks and soil. It can be found in zinc, lead and copper ores. Fossil fuels such as hard coal contain large quantities of Cd. Owing to valuable physical and chemical, and physical properties such as high resistance to corrosion, low melting point and good electric conduction it is widely used in industry, e.g. for production of batteries, coating of metals, in electronics, lithography, production of dyestuffs, metal alloys and in control rods of nuclear reactors. Apart from inappropriate storage and processing of battery cells, the main emission sources of harmful cadmium compounds include treatment of metal ores, combustion of coal and other fossil fuels and using phosphorus-based fertilisers.

Cd can be absorbed by inhalation or ingestion. Food is the main source of cadmium for the general population (except for smokers). It is assumed that after smoking one pack of cigarettes a human body absorbs about 5 $\mu\text{g Cd}$. Inhalation exposure has a high significance in occupational exposure.

Inhaled or ingested cadmium is bound by plasma proteins to be quickly transferred to the liver and kidneys. Cd absorption from the alimentary tract in humans is quite low and amounts to about 5%. Data obtained in autopsies

revealed that the highest quantities of Cd absorbed for many years accumulate in the initial section of the small intestine. Cadmium in the liver and kidneys is bound by a cellular protein of low molecular weight – metallothionein and in this form it is not toxic for cells. Cadmothionein released from the liver to the blood is also reabsorbed in the kidneys. Cadmium half-life in the body is very long – about 20 years. It means that cadmium is accumulated in the body for the majority of life. Excretion with urine is the main route of Cd elimination from the body. Cd concentration in the urine increases with age and results from the element concentration in the kidneys. Cadmium excretion increases dramatically when Cd concentration in the kidneys exceeds the critical level and renal tubules are damaged.

Cadmium is much more toxic than arsenic. It affects the kidneys by damaging their tubules and glomeruli, causes anaemia and disease of the bones (osteoporosis). Furthermore, it reduces insulin secretion and increases oxidation of lipids (which causes higher production of free radicals). It has an adverse effect of the circulatory system.

The symptoms of acute poisoning following inhalation include fever, headache, sore throat and pain in the chest, respiratory dysfunction, cough and conjunctivitis. The resulting pulmonary oedema may cause death and once it does not persist, interstitial pneumonia develops. The symptoms of ingestion include vomiting, diarrhoea and strong pain in the abdomen.

Chronic toxicity. Under occupational exposure and at extremely high environmental exposure (Japan), cadmium contribution to bone diseases seems to be evidenced. In the province of Toyama, Japan, where rice was grown in fields fertilised by wastewater from an industrial plant containing high volumes of cadmium, symptoms described as “Itai-Itai” disease were reported. They included strong pain of the joints and bones, mainly the backbone and lower extremities as well as a characteristic waddling gait. Osteomalation was among the observed lesions in the bones – it stands for softening of the bones as a result of insufficient mineralisation, while osteoporosis developed following a loss of the bone mass. Consequently, there were numerous cases of bone fractures and deformations.

Delayed effects. Cadmium and its compounds are considered to be human carcinogens and are listed by the experts from the IARC (International Agency for Research on Cancer) in the first group of factors with epidemiologically proven carcinogenic effect. The HAC value for cadmium and its inorganic compounds (fumes and vapours) valid in Poland is 0.01 Cd/m^3 as per cadmium.

LEAD

Lead forms connections at +2 and +4 oxidation degree. Occupational exposure to lead and its compound is mainly observed in steelworks, where lead is molten and processed. Atmospheric air can be a significant source of exposure for a general population, particularly in areas with heavy vehicle traffic as lead

is a fuel additive. Fortunately the use of the additive has been greatly limited in recent years. Lead can also be found in paints and chemical dyestuffs, whereas minor quantities of lead are used for production of soldering alloys, printing types, brass, shields protecting against ionising radiation and bullets.

About 40% of lead from the air is captured in the respiratory tract. Pb ingested with food is absorbed in the alimentary tract with up to 10% efficiency in adults and up to 50% in children. Iron, calcium and phosphates in food reduce the efficiency of absorption from the alimentary tract, while ascorbic acid increases Pb absorption.

Lead absorbed into the blood binds with erythrocyte membranes, which reduces their survival, to be later distributed in parenchymatous organs. Transfer to the muscle tissue and skin takes place a bit more slowly, while it is the slowest into the teeth and bones. Pb is accumulated in the bone tissue during our whole life, and the teeth and bones contain about 90% of the total lead present in the body. Permanent embedding of Pb into the bone tissue involves replacement of calcium ions in a hydroxyapatite crystal $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$. Lead penetrates placenta easily. It is mainly excreted in the urinary tract and in a limited extent also with faeces.

The haematopoietic and peripheral nervous system are critical systems in adults in case of lead poisoning, while in children it is the central nervous system. Lead inhibits activity of many enzymes causing impairment of processes mainly in the haematopoietic system. In the nervous system in turn it causes demyelination and degenerative changes of axons. Besides people exposed in their work, children are particularly sensitive to lead poisoning.

Acute poisoning with lead occurs relatively rarely. The lethal dose of lead salt (acetate, carbonate) administered orally amounts to 20–30 g. Lead colic is the most common symptom of acute poisoning with lead. It is demonstrated by a sweet taste in the mouth, lack of appetite, dyspepsia, abdominal pain, low blood pressure and body temperature, pale skin and bradycardia. Lead poisoning may cause renal failure which is demonstrated by haematuria, albinuria and oliguria. Lead poisoning may also result in arrhythmia or myocardial infarction. In children, and sometimes in adults, lead encephalopathy causing stupefaction, irritability, headache, muscle tremor, hallucinations, memory disorders and lack of concentration can be observed. Further stages include convulsions, paralysis and coma. Immediate medical help is necessary if acute poisoning with lead occurs. Oral or intravenous chelation with EDTA is applied. If indicated so, gastric lavage can be performed using 3% sodium sulphate with a large quantity of activated carbon. If encephalopathy or acute or chronic renal failure occur, haemodialysis is indicated. Opioids (e.g. codeine) are administered in cases of intestinal colic.

Chronic poisoning, when lead gets accumulated in the body for a long time, is mainly characterised by damage to the nervous system, skin, smooth muscles, kidneys and bone marrow. Lead colic can also occur in the course of chronic lead poisoning as acute manifestation of chronic poisoning.

Delayed effects of lead poisoning reveal its mutagenic action as well as inhibition of DNA synthesis and repair. Mutagenic action of lead was observed in experimental animals (kidney and brain cancer). Lead was classified by the International Agency for Research on Cancer (IARC) as 2B factor (probably carcinogenic to humans).

Lead level in the blood is the basic parameter used in the assessment of exposure to lead. According to the WHO recommendations, the value should be less than 200 $\mu\text{g}/\text{l}$ in 98% of population, while the permissible concentration in children is 100 $\mu\text{g}/\text{l}$. The highest permissible concentration in the workplace amounts to 0.05 mg/m^3 for Pb and its inorganic compounds as per lead. Pregnant and breastfeeding women must not do jobs entailing contact with lead, regardless of its concentration in the work environment.

MERCURY

It comes as organic and inorganic compounds. Occupational exposure to mercury applies mainly to mercury vapours and occurs first and foremost in the mining sector, in chemical plants producing chlorine by mercury electrolysis and for production of dyestuffs and fungicides. Mercury is mainly used in electrical engineering (alkaline batteries, production of measurement equipment). Exposure of the general population to mercury is related to consumption of organic mercury compounds in the fish. Metallic mercury (Hg^0) in a vapour form is mainly absorbed in the respiratory tract, and in minor quantities also through the skin and after absorption into the blood it is oxidised enzymatically to Hg^{2+} in the presence of catalase. Hg^0 contrary to Hg^{2+} is lipophilic, which means it easily penetrates the blood/brain and blood/placenta barriers. Therefore mercury, which was not oxidised in the blood, easily penetrates into the brain and foetus tissue and it is accumulated there. Brain is a critical organ for exposure to the metal vapours. The half-life of mercury in the body is several months and for the fraction deposited in the brain it can be up to several years. Mercury is mainly excreted with urine and faeces.

Acute poisoning with metallic mercury causes the respiratory tract insufficiency. Chronic exposure mainly causes symptoms of the central nervous system (hyperexcitability, depression, headache, muscle tremor, memory deterioration, insomnia and state of anxiety). Brain damage, i.e. mercury encephalopathy, can also occur. Gastrointestinal disorders and renal damage are rarely observed.

Poisoning with inorganic mercury compounds occurs rarely and usually *via* oral exposure. They result in the alimentary tract and renal disorders – albuminuria and haematuria as well as acute renal insufficiency. Chronic poisoning occurs in combination with exposure to metallic mercury vapours. Inorganic mercury compounds are absorbed from the alimentary tract and through the skin. Inorganic mercury is mainly deposited in the renal cortex, where it is bound by

methallothionein. It is not toxic in this form and renal damage occurs only at mercury doses exceeding the capacity of its binding by the renal methallothionein.

Organic mercury compounds were used as fungicides to protect seed grain. Nowadays, cases of poisoning with mercury compounds mainly apply to eating fish. Accumulation in the food chain leads to water contamination with mercury, *via* aquatic flora and fauna into predatory fish in which the half-life of mercury is several hundred days. Absorption of organic mercury compounds from the alimentary tract in humans is very effective and the absorbed mercury binds in the blood mainly with erythrocytes, and then travels to the tissues. Organic mercury compounds demonstrate high affinity to the brain tissue. A characteristic feature of organic mercury compounds, considering their hydrophobicity, is their easy penetration through biological barriers.

CARBON OXIDE

Carbon oxide is a strongly toxic gas, common in nature. Poisoning with carbon oxide is the most common gas poisoning and among most frequent causes of lethal poisoning in general. Presence of carbon oxide tends to be difficult to be identified due to its characteristics (non-irritating, odourless and colourless, easily mixing with air at any ratio).

Carbon oxide is absorbed into the body through the respiratory tract, penetrates through the alveo-capillary barrier of the lungs and binds irreversibly with haemoglobin forming carboxyhaemoglobin (COHb), which is unable to connect and transfer oxygen to the body organs. It causes tissue hypoxia of a degree proportional to the blood saturation with COHb and tissue demand for oxygen.

About 15% of absorbed CO remains outside the circulatory system, mainly in the heart and muscles as a combination with myoglobin. Carbon oxide is excreted by the lungs in an unchanged form.

The affinity of haemoglobin and myoglobin to CO is 200–300 times greater than their affinity to oxygen. Symptoms of toxic action of CO result from hypoxia of the body and apply to many systems (cardiovascular, nervous and respiratory) and organs (liver, kidneys, muscles, organ of hearing, organ of vision and the skin), and tend to occur at COHb concentration in the blood over 20%. At concentrations over 60% death occurs within several minutes.

Chronic poisoning may be caused by continuous exposure to low concentrations of CO, which cause accumulation of microinjuries leading to permanent disorders (poor memory, permanent fatigue, appetite and sleep disorders and circulatory system malfunctions).

SULPHUR DIOXIDE

Sulphur dioxide is a colourless gas with a specific, irritating odour. As a combustion product of sulphur in the coal and exhaust fumes it significantly contaminates the air in industrial areas (smog).

Even if strongly thinned, it denatures proteins, which makes it a good disinfecting, disinfecting and fungicidal agent. Sulphur dioxide is used for cold storage purposes, for bleaching in paper-making, dyeing and textile industry, while in the food industry it is a common preservative (E220) used especially in wines.

SO₂ is absorbed through the respiratory system and skin. It is a strong respiratory tract irritant. It easily dissolves in water forming sulphuric acid, and it also dissolves in methanol, ethanol and chloroform. As a gas highly soluble in water it easily penetrates through the mucous membranes of the respiratory tract. The formed sulphuric acid is responsible for strongly irritating effect on the mucous membranes of the eyes, respiratory tract (bronchospasm or chronic bronchitis and tracheitis) and the skin.

Owing to its pungent and irritating odour SO₂ can be smelled easily and quickly and that is why cases of acute poisoning are rare. Exposure to concentrations of 20–30 mg/m³ causes severe stinging, lacrimation as well as redness and swelling of the conjunctiva. High concentrations may cause damage to the cornea. Skin irritation is demonstrated by redness, pain, burning and swelling. Poisoning by inhalation is demonstrated by cough, runny nose and sore throat but dyspnoea caused by swollen glottis, laryngospasm or (rarely) pulmonary oedema can also occur. Other symptoms may also include nausea, vomiting, diarrhoea, abdominal pain, headache, dizziness and anxiety.

Chronic poisoning related to long-term exposure to low concentrations of SO₂ is often accompanied by the upper respiratory tract infection, abundant mucous secretion and conjunctivitis.

HYDROGEN CYANIDE

Hydrogen cyanide is a colourless liquid with a smell of bitter almonds. It dissolves easily in water, ethanol and ether. It occurs naturally as amygdalin in leaves and seeds of stone fruit (almonds, peaches, apricots, plums and cherries). HCN is used as a disinfecting and rodent control agent, and its salts – cyanides – are used in many technological processes.

Hydrogen cyanide is quickly absorbed *via* different routes: skin, respiratory tract and alimentary tract. Poisoning is revealed by a characteristic smell of bitter almonds in the exhaled air. HCN is metabolised in the body under the influence of thiosulfate sulfotransferase to thiocyanates (rhodanates) – compounds which are about 200 times less toxic than cyanide and excreted with urine.

After getting into the body, HCN dissociates into H⁺ ion and cyanogen ions (CN⁻), which reveal affinity to Fe³⁺ ions of cytochrome oxidase, and hence block the action of the respiratory chain and cellular respiration. Nervous system and cardiac muscle cells are particularly sensitive to hypoxia and that is why high doses of HCN cause an immediate loss of consciousness as a result of paralysis of the respiratory centre and cardiac arrest. Respiratory and circulatory insufficiency is

preceded by a scratchy feeling in the throat and burning and bitter taste in the mouth, sialosis, numbness of the lips and larynx, muscular weakness, difficulty in speaking, headache and dizziness, nausea, vomiting, accelerated breath, palpitation and heaviness in the heart area, low pulse, mydriasis, exophthalmos and convulsions.

ALIPHATIC HYDROCARBONS (INGREDIENTS OF CRUDE OIL AND NATURAL GAS)

Aliphatic hydrocarbons in high concentrations have a narcotic effect and the power of their action increases for longer carbon chains. Short aliphatic hydrocarbons with up to 4 carbon atoms are gases which cause hypoxia and general anaesthesia, while the ones with a higher molecular mass are neurotoxic liquids. Aliphatic hydrocarbons with a branched chain and unsaturated aliphatic hydrocarbons have the strongest action. Nevertheless, the greatest hazard related to high concentrations of hydrocarbons is related to a risk of explosion.

A mixture of aliphatic hydrocarbons in a form of crude oil vapour is absorbed through the lungs and skin. Liquid crude oil is in turn absorbed from the alimentary tract.

Metabolites produced in biotransformation processes are mainly eliminated *via* kidneys, coupled with sulphuric acid, while crude oil compounds not subjected to biotransformation are eliminated with the exhaled air.

Hygiene standards for crude oil have not been developed but it is classified in Poland as a potential human carcinogen (i.e. category 2). Crude oil is a skin irritant and the lesions formed in about 1% of people turn into a skin cancer.

CRUDE OIL DISTILLATION PRODUCTS (PETROL AND KEROSENE) USED MAINLY AS FUEL AND SOLVENTS

Crude oil distillation products are easily absorbed from the respiratory tract, through the skin and alimentary tract. They have an irritating effect on the skin, mucous membranes of the eyes, alimentary tract and respiratory tract.

Aliphatic hydrocarbons are poorly metabolised in the body and that is why a significant part of the absorbed vapours of petrol and kerosene is eliminated through the lungs in an unchanged form.

Crude oil distillation products are lipophilic compounds with high affinity to the adipose tissue and cellular membranes, which facilitates penetration through the blood-brain barrier and leads to dysfunction of the nervous system.

Some fractions of crude oil distillates (e.g. paraffin-based distillates) are classified as category 1 carcinogens, i.e. substances with proven carcinogenic effect in humans. Extraction naphtha and mineral spirits (painter's naphtha) were classified in Poland as category 2 carcinogens.

CHLORINE DERIVATIVES

As the molecular mass goes up, toxicity of chlorine derivatives of aliphatic hydrocarbons increases but their volatility decreases. Higher homologues with a higher content of chlorine are more dangerous at elevated temperatures.

The compounds are mainly liquid hydrophobic substances with a specific odour, which are used in the industry as solvents of organic compounds. Halogen derivatives of an aliphatic series are characterised by high chemical activity and are used for obtaining other derivatives. Halogen derivatives are chemical compounds used as semi-finished products to obtain important chemical compounds. Vinyl chloride is a gas applied in production of polyvinyl chloride.

First and foremost they constitute a hazard in occupational exposure. Chloroform is a hazard for the general population since it is formed during potable water conditioning using chlorine (the permissible dose of CHCl_3 in potable water is 0.03 mg/l). Under humidity, air and light it decomposes into strongly poisoning phosgene and hydrogen chloride. Chloroform is also known as an agent used for general anaesthesia but nowadays it is not popular for the purpose.

Chlorine derivatives of aliphatic hydrocarbons are absorbed in the respiratory tract, from the alimentary tract, through undamaged skin and mucous membranes (vinyl chloride is absorbed through the respiratory tract in a gaseous form). They quickly penetrate into the blood and other tissues and reach the highest concentrations in tissues rich in lipids, from which they are removed most slowly. They are mainly metabolised in the liver and their metabolites are primarily responsible for the toxic effect of the chlorine derivatives. For chloroform and carbon tetrachloride it is phosgene, for vinyl chlorine it is epoxy, which is highly reactive and in water solutions it is transformed into chloroacetaldehyde and glycol aldehyde, which are chemically active metabolites.

Acute poisoning with chlorine derivatives of the aliphatic order cause non-specific narcotic effects whose intensity depends on the kind and concentration of the substances in the exhaled air. Initial symptoms may include irritation of the mucous membranes and conjunctiva, vomiting and headache. A period of excitation (anxiety, high arterial pressure, and fast heartbeat) is followed by a depressive effect on the central nervous system and loss of consciousness. Vinyl chloride causes a characteristic oversensitivity to cold, numbness and tingling of the hands and Raynaud's syndrome. Further consequences of acute or chronic poisoning with the compounds include lesions of body organs, e.g. fatty degeneration of the liver, renal insufficiency and cardiac muscle damage due to hypoxia.

They are carcinogens for laboratory animals. Most probably the compounds may cause delayed effects in humans but so far such an effect was only proven for vinyl chloride. IARC classified CHCl_3 and CCl_4 as 2B class, i.e. probably carcinogenic to humans. Vinyl chloride was classified as a proven human carcinogen (category 1).

POLYCYCLIC AROMATIC HYDROCARBONS (PAHS)

PAHs are a group of over 200 known compounds containing from 2 to 13 condensed benzene rings in a particle. PAHs are among major group of chemicals polluting the environments. PAHs mixed with water steam particles form smog. They are formed during incomplete combustion of all hydrocarbons except for methane. They occur in industrial raw materials and their products including coal tar, mineral oils, asphalt, carbon black and tar. Therefore occupational exposure to PAHs applies to such industry sectors as coke engineering, metallurgy, founding and petrochemical industry. They are mainly released to the environment during combustion of engine fuels (exhaust fumes), by abrasion of car tyres and asphalt, and during operation of coke ovens (heating in buildings) and waste incineration. Smoking of cigarettes and tobacco is a source of emission (and exposure) of the compounds. Natural factors such as volcano eruptions or great fires of forests also contribute to PAH formation (PAHs are emitted when wood from coniferous trees is burnt).

PAHs are formed in food during its thermal processing e.g. frying, baking, smoking, grilling and roasting. The higher the temperature and the longer the process duration are, the higher the content of PAHs in the finished food product is. Moreover, food additives used to improve organoleptic quality of smoked products are also a source of PAHs. Roasting of coffee beans causes formation of PAHs, too. PAHs may occur in food products as a result of cultivation or animal breeding (fishing in contaminated water) near areas with highly polluted environment.

The majority of PAHs occur in the air as vapour and aerosols, and some of them can also deposit on the dust. In such a form they can penetrate into the body through the respiratory tract and depending on the particle size they can deposit in different sections of the respiratory tract. PAHs can get into the body through the alimentary tract on contact with food and tap water. Under occupational exposure, direct absorption through the skin is possible following a direct contact.

After penetrating into the body (regardless of the absorption route), they are quickly transferred with the blood into the tissues and organs. Owing to their hydrophobic properties they easily penetrate through the cellular membranes and accumulate in the adipose tissue.

The compounds are characterised by chronic toxicity, which means that even a high single dose is not harmful but only a long-lasting and regular exposure to minor doses can become a cause of diseases. The most toxic PAHs include benzo(a)pyrene, dibenzo(a,h)anthracene and benzo(b)fluoranthene. Epidemiological studies revealed a relationship between occupational exposure to PAHs and higher prevalence of cancers.

Studies on several PAHs metabolites revealed that they are mutagenic and carcinogenic substances. The mechanism of tumour formation under the influence of PAHs involves covalent bonding of metabolites with a cell's DNA or RNA.

Benzo(a)pyrene is the most studied hydrocarbon in the PAH group. In relation to the severity of its carcinogenic effect and common occurrence in the environment it was considered as an indicator for the whole PAH group. The highest acceptable concentration for PAH (as a total of 9 carcinogenic concentrations of PAHs multiplied by carcinogenicity factors) was established in Poland at 0.002 mg/m³. The highest acceptable concentration for PAH is the same as the highest acceptable concentration for benzo(a)pyrene itself.

DIOXINS

A group of organic compounds being oxantrene derivatives is commonly known as dioxins. They are polychlorinated dibenzo-p-dioxins (PCDD) composed of two benzene rings linked by two oxygen atoms and from one to eight chlorine atoms connected to benzene rings. Polychlorinated dibenzofurans (PCDF) are similar compounds, also included in the group of dioxins. 7 out of 75 PCDDs are highly toxic. 2,3,7,8-tetrachlorodibenzodioxin (TCDD) is the most dangerous compound.

Dioxins are hydrophobic compounds, easily soluble in organic solvents. They are most toxic among all synthetic substances. They are formed as a side product in production of organic chlorine compounds (pesticides, herbicides and chlorophenol derivative), during wood combustion (forest fires) and any organic compounds containing chlorine (combustion of municipal and hospital waste). With regard to low solubility of dioxins in water, they are absorbed onto the surface of particles suspended in water and fall with particles on the bottom. Studying the degree of water contamination is based on identifying concentration of dioxins in the bottoms. Plant protection products are also a source of water and soil contamination with dioxins. PCCD accumulation in plants is low but dioxins accumulate in large concentrations in aquatic animals such as snails and fish where their concentrations exceed the concentration identified in water by 1×10^3 times. Hence eating fish (oily fish in particular) from contaminated water reservoirs is a significant exposure factor.

Dioxins are mainly absorbed through the alimentary tract but also through the respiratory tract with dust. With regard to their hydrophobic character they travel with blood plasma lipoproteins and easily penetrate into cells. In the body they accumulate in the liver and adipose tissue. Metabolic transformations cause dechlorination and formation of hydroxy derivatives and coupled compounds, excreted with bile and faeces. The mean half-life is about 10 years and it depends on the adipose tissue weight.

Characteristic symptoms of toxic effect of dioxins on humans include:

- skin lesions (chlorine acne, proliferation and cornification of epidermis and formation of corneous-sebaceous cysts);
- impaired hormone balance, including changes in the thyroid hormone balance, elevated TSH level, decreased level of male sex hormones, impaired function of male and female gonads;

- immune system malfunction;
- embryotoxic and teratogenic action.

IARC classifies TCDD as group 1, i.e. human carcinogens.

PESTICIDES

They are substances of synthetic or natural origin used to protect plants against pests, diseases and weeds but also to combat parasites in breeding animals, harmful rodents etc. The use of chemical pesticides to protect plants is based on employing the toxic properties of chemical substances against biological species regarded as harmful.

It can be inferred that pesticides are intended only to destroy organisms considered by humans as unfavourable and harmful. But complete selectivity of pesticide action cannot be achieved in practice, which means that there is always a risk of toxic effects. Unfortunately, despite great variety of applied pesticides, they all reveal a harmful impact on living organisms. Exposure to pesticides occurs already during their production but first and foremost it is related to their common use in agriculture.

With regard to high number and heterogenic nature of the group of compounds called pesticides, it is not possible to characterise in the study the toxicity of all substances used for plant protection. There are different divisions of pesticides and it depends on the acquired assessment criteria but the basic classification of pesticides includes the following:

1. functional classification of pesticides depending on the target species;
2. classification based on chemical structure;
3. classification based on the toxicity scale assessment;
4. division into natural and synthetic pesticides.

Classification according to species

1. **Zoocides** – agents intended to combat animal pests, including:
 - insecticides (agents used to kill insects),
 - acaricides (agents killing mites),
 - ovicides (agents designed to destroy eggs of insects and mites),
 - afficides (agents killing aphids),
 - nematocides (agents used to kill nematodes),
 - larvicides (agents targeted against larval life stage of insects),
 - molluscicides (pesticides against molluscs),
 - rodenticides (agents against rodents),
 - attractants - attracting agents,
 - repellents - repelling agents;
2. **Bactericides** – agents killing bacteria;
3. **Herbicides** – weed killing agents, including growth regulators:
 - total – damaging the whole population of plants,
 - selective – eliminating specific plant species,

- growth regulators – inhibitors, stimulants,
 - defoliants (agents used to remove plant lists),
 - desiccants (plant drying agents),
 - deflorants (agents used to remove excessive quantities of flowers);
4. **Fungicides** – fungicidal and fungistatic agents.

Classification based on chemical structure

1. Inorganic pesticides

- herbicides: ammonium sulphamidate, borax, sodium chlorate,
- fungicides: alkaline copper (II) chloride, Bordeaux mixture, sulphur,
- arsenic insecticides: Paris green $3\text{Cu}(\text{AsO}_2)_2 \cdot x\text{Cu}(\text{CH}_3\text{COO})_2$, lead arsenate PbHAsO_4 ,
- fluoride insecticides: cryolite Na_3AlF_6 , sodium fluoride NaF , sodium fluosilicate Na_2SiF_6 ;

2. Organic pesticides

- herbicides: heterocyclic cationic compounds, triazines, phenylurea derivatives, dinitroaniline derivatives, phenoxyacetic acid derivatives, phenylcarbamate derivatives,
- fungicides: metallic derivatives of ethylenebis(dithiocarbamates),
- insecticides: chlorine derivatives of hydrocarbons, pyrethroids, organophosphorus compounds and carbamates.

Classification based on toxicity scale

Based on the regulation of the Minister of Agriculture and Food Economy of 12 March 1996.

Toxicity class	Acute toxicity		
	LD ₅₀ oral mg/kg b.w.	LD ₅₀ dermal mg/kg b.w.	LD ₅₀ Inhalation mg/l/4 h exposure
I. Very toxic T+	≤ 25	≤ 50	≤ 0.25 aerosols ≤ 0.50 gases and vapours
II. Toxic T	25 < LD ₅₀ ≤ 200	50 < LD ₅₀ ≤ 400	0.25 < LD ₅₀ ≤ 1 aerosols 0.50 < LD ₅₀ ≤ 2 gases and vapours
III. Harmful Xn	200 < LD ₅₀ ≤ 2000	400 < LD ₅₀ ≤ 2000	1 < LD ₅₀ ≤ 5 aerosols 2 < LD ₅₀ ≤ 20 gases and vapours
IV. Slightly toxic	> 2000	> 2000	> 5 aerosols > 20 gases and vapours

2.5.3. Environmental exposure of selected toxins

Catastrophic exposure:

– **Mass release of dioxin** 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was released from a factory in Soveso near Milan in July 1976; ca. 16 kg of the dioxin polluted the area of 3–4 km², 28,000 people were struck by the disaster.

– **Mass release of methyl isocyanate** from Union Carbide factory in Bhopal, in December 1984 – 200,000 people were exposed and 2,500 of them died.

– Oil spill

1977 – “Ekofisk” drilling rig on the Norwegian Sea – 12,000 tons of crude oil were spilt.

16 March 1978 the “Amoco Cadiz” oil tanker wrecked off near the French coast – ca. 220,000 tons of crude oil were spilt.

3 June 1979 – catastrophe of the “Ixotoc” drilling rig in the Mexican Bay – ca. 1 million tons of crude oil were spilt.

24 March 1989 – “Exxon Valdez” oil tanker near Alaska – ca. 40 million litres of crude oil were spilt.

1991 – during the Persian Gulf War oil wells destroyed in Kuwait, which resulted in spilling of ca. 38 billion litres of crude oil into the Gulf waters.

Endemic exposure:

– Organic mercury compounds – “Minamata” disease

Vinyl chloride plant in Kyushu (Japan), 1953 – poisoning of the inhabitants of a cottage at the Minamata Bay after eating fish; over 700 people came down with the disease and 40% died.

Industrial wastewater, the Agano river (Japan), 1964 – over 500 people were poisoned after eating fish.

Mercury-based fungicides, Iraq, Pakistan, Guatemala, 1971-72 – eating flour from toxic grain, 6400 people, 480 died.

– Hexachlorobenzene

Eating poisoned grain, Turkey, 1956 – 3000 people, 10% died.

– Cadmium – “Itai-Itai” disease

Contamination of the Untsu river with wastewater in Japan, 1995 – irrigation of rice fields with the river water resulted in a 10-times increase in the cadmium level in the rice.

– Triorthocresol phosphate (TOCP)

In 1930 food and (mainly) alcohol were counterfeited in the USA, ca. 20,000 people were poisoned.

In 1959 oil was counterfeited in Morocco and over 10,000 people were poisoned.

- Toxic oil syndrome

Frying oil containing 50 ppm aniline and 2000 ppm acetanilide was used in Spain – 25,000 people were hospitalised and 400 died.

- Polychlorinated biphenyls (PCB)

consumption of oil from rice bran, Japan (1968) (the incident was called Yusho i.e. rice disease) – 1800 people were poisoned; a similar mass poisoning occurred in Taiwan in 1979.

- Polybrominated biphenyls (PBB)

Incidental contamination of grain and feed, USA, 1973 – 250,000 cows, 1.6 million chickens and hundreds of pigs died.

Using toxins to exterminate humans:

- During the Vietnam War, the US Air Force soldiers sprayed a herbicide called Agent Orange containing high concentration of dioxins, including TCDD – being among the most toxic dioxins. It was used to destroy plants to facilitate detecting guerrillas from a plane. The objective was also to damage the crops to deprive the guerrillas of food. The “Operation Ranch Hand”, in which rainbow herbicides were used, lasted 10 years (1961–1971). Over 75 million litres of herbicides were used not only in Vietnam but also in Laos and Cambodia. Almost 20% of forest areas were sprayed. Unfortunately, the results of the spraying operation have become a contemporary issue due to contamination of the herbicides with TCDD. The Vietnamese calculated that almost 5 million people were exposed to direct action of the agent, i.e. they were directly exposed to the sprayed agent or consumed contaminated food and water. Almost 400,000 people died or were permanently injured. The spraying caused long-term effects of permanent genetic disorders. Deformed, physically and mentally disabled children are still born in the contaminated areas. The American soldiers and pilots of the aircrafts used for spraying also experienced negative health effects. A higher incidence of cancer, skin diseases and respiratory tract diseases was observed among them.

- Ground has remained contaminated until nowadays in the areas of the former US military bases where the herbicides were stored. Dioxins penetrate into the soil and ground water as well as into the crops, and consequently the whole food chain is contaminated, which causes skin diseases, lung cancer, laryngeal cancer and prostate cancer.

- In 2004 the same dioxin – TCDD, which is among most toxic artificially obtained substances, was used to poison then-presidential candidate of Ukraine, Victor Yushenko.

- In 2002 during a siege in the Moscow Dubrovka Theatre 40 terrorists and 129 hostages died as a result of using sleeping gas containing phentanyl and 3-methylphentanyl (painkillers and anaesthetic drugs with opioid effect).

– In the war between Iraq and Iran (1980–1988) the Iraqi used chemical weapon on a mass scale (mainly chemical warfare – yperite and tabun), which caused numerous and often lethal injuries, including severe burns and blinding.

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3. GENETICS

One of the discoverers of DNA's double helical structure, Francis Crick, said that "almost all aspects of life are engineered at the molecular level, and without understanding molecules, we can only have a very sketchy understanding of life itself." Others scientists, including Mendel, discovered the existence of units of heredity that were later called genes, and associated with the movement of chromosomes during mitosis and meiosis. Without knowledge of the molecules that carry genetic information, it would be impossible to understand anything about the biochemical processes through which genes determine phenotypes and pass on instructions to subsequent generations.

3.1. The structure of DNA

At the beginning of the twentieth century, there was no information about what 'genetic material' is. After 50 years of great experimental efforts, DNA was discovered to be the prime genetic molecule, carrying all of the hereditary information within chromosomes. This structure of DNA was helpful for understanding how it carries the genetic messages that are replicated when chromosomes divide to produce two identical copies of themselves. The full chemical name of DNA is deoxyribonucleic acid, reflecting three characteristics of the substance: one of its constituents is a sugar known as deoxyribose; it is found mainly in cell nuclei, and it is acidic. DNA contains only four distinct chemical building blocks linked in a long chain (Fig. 1). The four individual chemicals belong to a class of compounds known as nucleotides; the bonds joining one nucleotide to another are covalent phosphodiester bonds, and the linked chain of building block subunits is a type of polymer.

Now, over 50 years after the discovery of the double helix, this simple description of genetic material remains true and has not had to be appreciably altered to accommodate new findings. Nevertheless, we have come to realize that the structure of DNA is not quite as uniform as was first thought. The chromosomes of some small viruses have single-stranded, not double-stranded, molecules. Some DNA sequences even permit the double helix to twist in the left-handed sense (Z-DNA structure), as opposed to the right-handed sense originally formulated for DNA's general structure. Some DNA molecules are linear, whereas others are circular. Additionally, the structure of DNA varies and

these DNA variations arise from the unique physical, chemical, and topological properties of the polynucleotide chain.

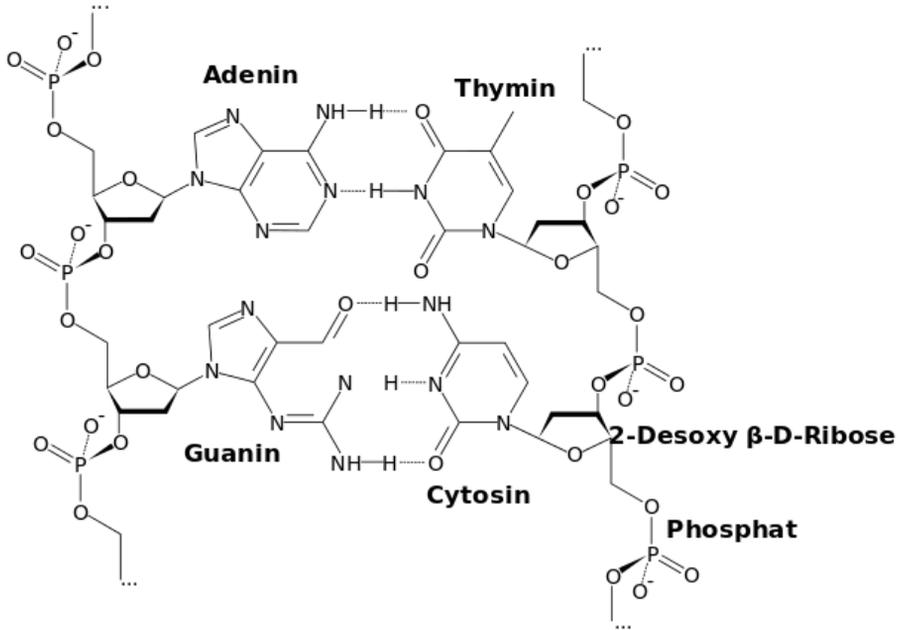


Figure 1. Chemical structure of DNA (*figure used with permission under Creative Commons license*)

DNA is composed of two polynucleotide chains twisted around each other in the form of a double helix. Figure 2 presents the structure of the double helix in a schematic form. The backbone of each strand of the helix is composed of alternating sugar and phosphate residues; the bases project inward but are accessible through the major and minor grooves (Fig. 3). The nucleotide, the fundamental building block of DNA, consists of a phosphate joined to a sugar, known as 2'-deoxyribose, to which a base is attached. The phosphate and the sugar have structures as shown in Figure 4. The sugar is called 2'-deoxyribose because there is no hydroxyl at position 2' (two hydrogens). The sugar and base alone are called a nucleoside and addition of a phosphate to a nucleoside creates a nucleotide. Making a glycosidic bond between the base and the sugar, and also making a phosphoester bond between the sugar and the phosphoric acid, creates a nucleotide. Moreover, nucleotides are joined to each other in polynucleotide chains through the 3'-hydroxyl of 2',-deoxyribose of one nucleotide and the phosphate attached to the 5',-hydroxyl of another nucleotide.

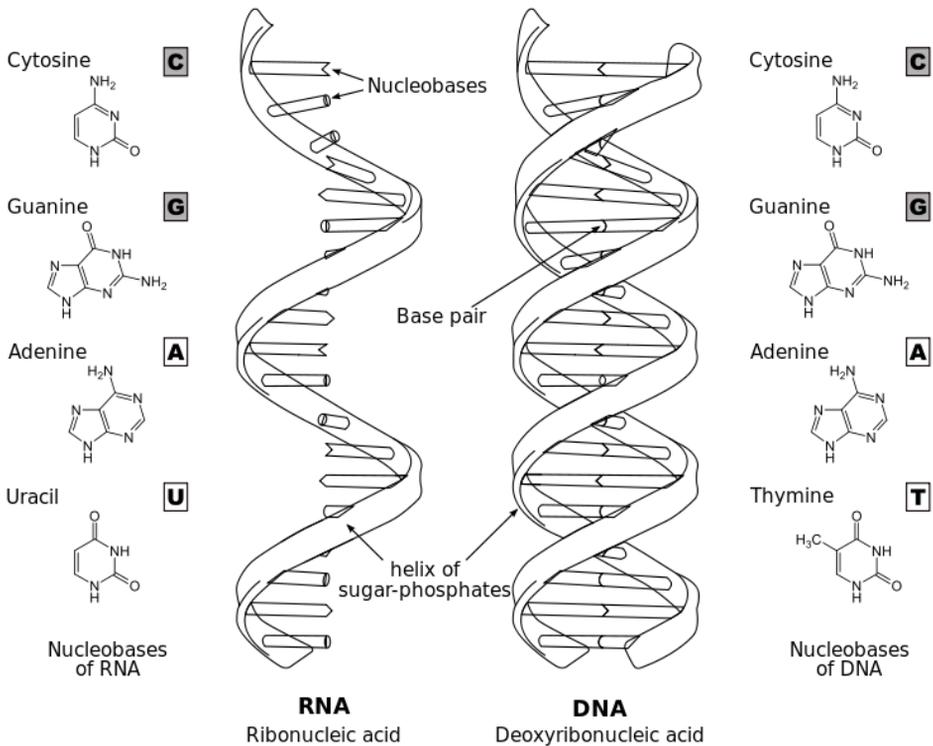


Figure 2. Structural differences between DNA and RNA (*figure used with permission under Creative Commons license*)

The double helix consists of two polynucleotide chains that are aligned in opposite orientation. The two chains have the same helical geometry but have opposite 5, to 3, orientations. That is, the 5' to 3' orientation of one chain is antiparallel to the 5' to 3' orientation of the other strand, as shown in Figures 2 and 3. The two chains interact with each other by pairing between the bases, with adenine (A) on one chain pairing with thymine (T) on the other chain and, likewise, guanine (G) pairing with cytosine (C) in anti-parallel manner. Despite large variations in the relative amounts of the bases, the ratio of A to T is not significantly different from 1:1, and the ratio of G to C is the same in every organism.

The hydrogen bonds between complementary bases are a fundamental feature of the double helix, contributing to the thermodynamic stability of the helix and the specificity of base pairing. The double helix is stabilized by hydrogen bonds and by stacking interactions between the bases. The stacked bases are attracted to each other by transient, van der Waals interactions.

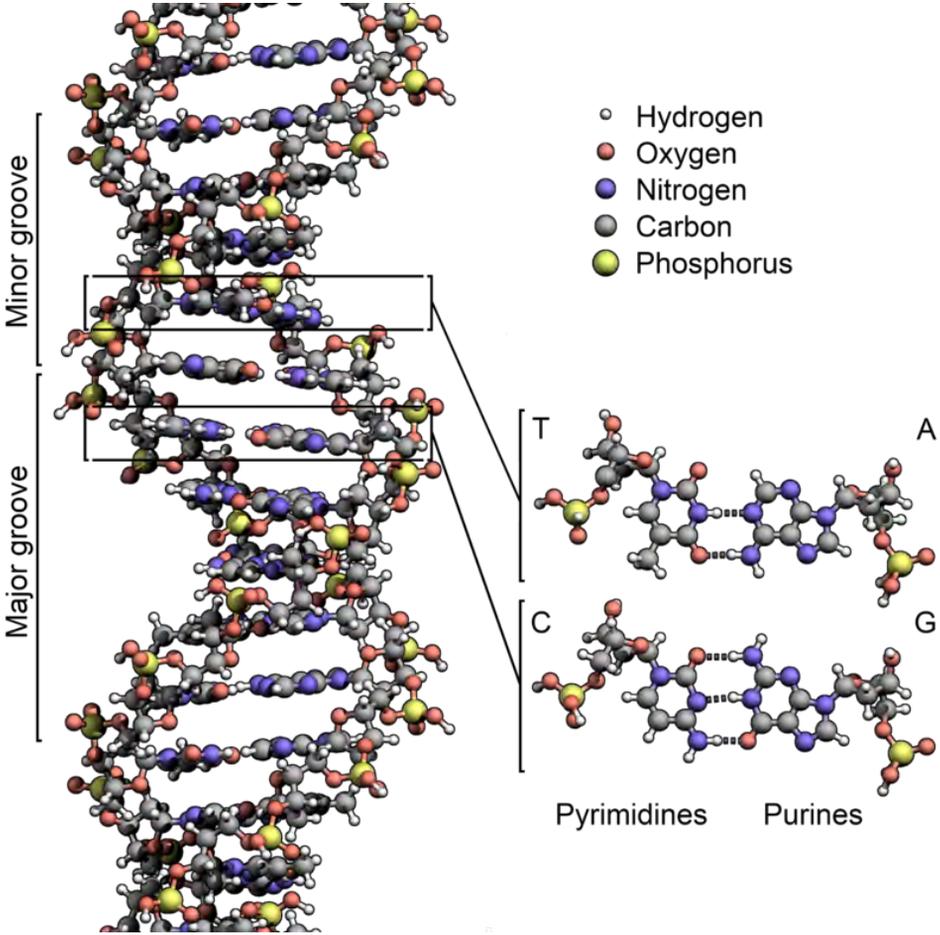


Figure 3. DNA structure (figure used with permission under Creative Commons license)

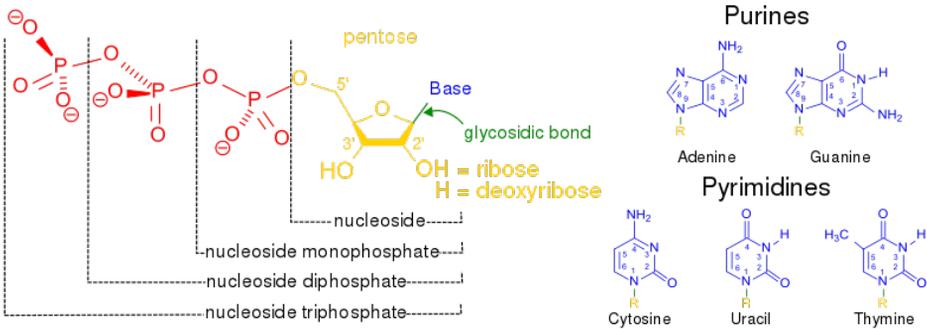


Figure 4. Structure of nucleotides (figure used with permission under Creative Commons license)

Each of the polynucleotide chains in the double helix is right-handed. As a result of the double-helical structure of the two chains, the DNA molecule is a long, extended polymer with two grooves that are not equal in size to each other. These different grooves are a simple consequence of the geometry of the base pair (the angle between the glycosidic bonds different for the narrow and wide angle). The two strands of the double helix are held together by relatively non-covalent (weak) forces, and can be easily separated, when a solution of DNA is heated above physiological temperatures ($\sim 100^{\circ}\text{C}$), or under conditions of high pH, and this process is known as denaturation, a reversible process. Additionally, when heated solutions of denatured DNA are slowly cooled, single strands often re-form regular double helices. This feature can be used in forming artificial hybrid DNA molecules by slowly cooling mixtures of denatured DNA from two different sources. It can also be made between complementary strands of DNA and RNA. This feature, called hybridization, is the basis for several indispensable techniques in molecular biology, such as Southern blot hybridization and DNA microarray analysis. If we plot the optical density of DNA as a function of temperature, we observe that the increase in absorption occurs abruptly over a relatively narrow temperature range. The midpoint of this transition is the melting point, or T_m (Fig. 5).

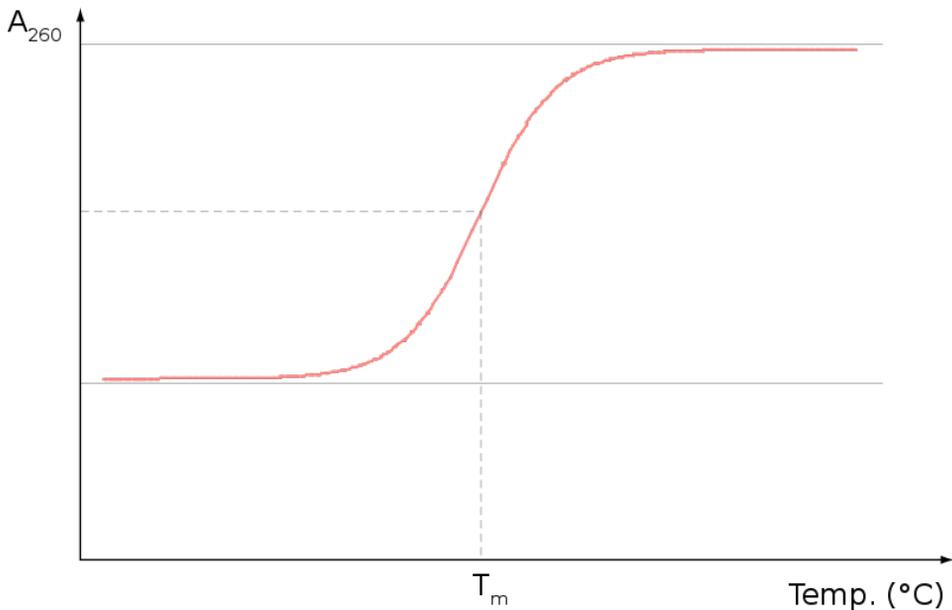


Figure 5. Hyperchromicity of DNA (*figure used with permission under Creative Commons license*)

DNA melts because of the transition from a highly ordered double-helical structure to a much less ordered structure of individual strands. Renaturation occurs by a slow nucleation process in which a relatively small stretch of bases on one strand finds and pairs with their complement on the complementary strand. T_m of DNA is largely determined by the G:C content of the DNA and the ionic strength of the solution. A larger amount of the percent of G:C base pairs in the DNA than A:T pairs (three hydrogen bonds versus two) gives the higher melting point. Likewise, the higher the salt concentration of the solution, the greater is the temperature at which the DNA denatures. Additionally, it happens because the stacking interactions of G:C base pairs with adjacent base pairs are more favourable than the corresponding interactions of A:T base pairs with their neighbouring base pairs. Moreover, another fundamental feature of DNA is the effect of ionic strength when the backbones of DNA strands contain negative charged phosphoryl groups, which are close enough across the two strands that, if not shielded, they tend to cause the strands to repel each other, facilitating their separation. At high ionic strength, the negative charges, shielded by cations, stabilize the helix, and at low ionic strength the unshielded negative charges make the helix less stable.

The majority of naturally-occurring DNA molecules have the Watson and Crick configuration, known as B-form DNA, right-handed. DNA structure also exists in left-handed form, known as a Z form, in which the helix spirals to the left and the backbone takes on a zigzag shape.

The information content of DNA resides in the sequence of its four bases which are like the letters of an alphabet and different sequences of them spell out different 'words', which possess their own phenotype effect, for example, GGTCCA and ATTGCCA mean different things. In all cellular forms of life as Prokaryotes and Eukaryotes and many viruses, DNA carries the genetic information. By contrast, some viruses, including retroviruses such as HIV, use RNA as their genetic material.

3.2. The structure and function of RNA

RNA is principally found as a single-stranded molecule. An intrastrand base pairing, RNA exhibits extensive double-helical character and is capable of folding into a wealth of diverse tertiary structures. Most remarkable of all, some RNA molecules are enzymes, one of which performs a reaction that is at the core of information transfer from nucleic acid to protein and hence is of profound evolutionary significance.

There are three major chemical differences between RNA and DNA: First, RNA possesses sugar ribose instead of deoxyribose found in DNA, (Fig. 2), second, RNA contains the base uracil (U) instead of the base thymine (T); U,

like T, base pairs with A (Fig. 6) and third, RNA molecules mostly are single-stranded and they are much shorter than the very long DNA molecules found in nuclear chromosomes. Within a single-stranded RNA molecule, can form a short double-stranded, base paired stretch. RNA, in comparison to double-helical shape of a DNA molecule has a complicated structure of short double-stranded segments interspersed with single-stranded loops. RNA has the same ability as DNA to carry information in the sequence such as in some viruses, but is much less stable than DNA. Additionally, RNA fulfils several vital functions in all cells, such as gene expression and protein synthesis and plays a significant role in DNA replication.

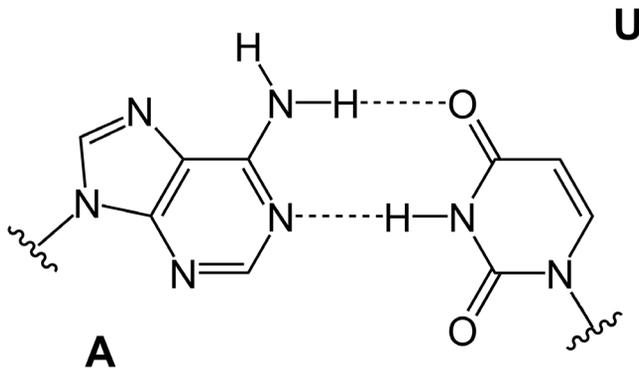


Figure 6. Base pair A:U (*figure used with permission under Creative Commons license*)

RNA functions as the intermediate, the messenger RNA (mRNA), between the gene and the protein-synthesizing machinery. Another function of RNA is as an adaptor, the transfer RNA (tRNA), between the codons in the mRNA and amino acids. RNA can also play a structural role, as in the case of the RNA components of the ribosome (rRNA). Finally, some RNAs are enzymes that catalyse essential reactions in the cell. RNA is capable of forming long double helices, but these are very rare in nature. Despite being single-stranded, RNA molecules can form a double-helix form, the two stretches of complementary sequence are near each other, the RNA may form a stem-loop structure, internal loops (unpaired nucleotides on either side of the stem), bulges (an unpaired nucleotide on one side of the bulge), or junctions.

The central dogma of molecular biology states that DNA maintains the information to encode all of our proteins, and that three different types of RNA convert this code into polypeptides. But with the advent of new techniques, scientists have revealed that RNA does much more than simply play a role in

protein synthesis, such as catalytic reactions. Generally, this type of RNSs are named non-coding RNAs and play complex regulatory roles in cells.

In eukaryotes, non-coding RNA comes in several varieties, such as tRNA and rRNA, involved in the translation of mRNA to proteins, but there are also a lot of non-coding RNAs assist in many essential functions in eukaryotic cells and new ones are still being discovery. Various regulatory non-coding RNAs exert their effects through a combination of complementary base pairing, complexing with proteins, and their own enzymatic activities, such as small nuclear RNAs, microRNAs, small interfering RNAs, small nucleolar RNAs or riboswitches.

This complexity and diversity of RNA forms and activities in cells lends credence to the so-called 'RNA world' hypothesis, which states that RNA may have evolved prior to DNA and protein, and it may have played the roles of both of these molecules in the earliest life-forms. This hypothesis is supported by example of existing RNAs having both coding and catalytic capacity. Nevertheless, the full contribution of RNA to the life of the cell may still be unknown.

3.3. The gene expression process as a flow of information from DNA through RNA to protein

The Central Dogma maintains that genetic information flows in two distinct stages (Fig. 7). The information of genes written in the language of nucleic acids is subsequently transcribed to the same instructions written in the RNA dialect. The conversion of DNA to RNA is called transcription. In the next stage of gene expression, the cellular machinery translates mRNA into its polypeptide equivalent in the language of amino acids. This decoding of nucleotide information to a sequence of amino acids is known as translation and is done using ribosomes, which are composed of proteins and rRNAs. Decoding depends on the genetic code that defines each amino acid in terms of specific sequences of three nucleotides, as well as on tRNAs, small RNA adaptor molecules that place specific amino acids in the correct position in a growing polypeptide chain. The Central Dogma does not explain the behaviour of all genes, when a large subset of genes is transcribed into RNAs that are never translated into proteins, because they encode rRNAs and tRNAs.

In addition, scientists later found that certain viruses contain an enzyme that can reverse the DNA-to-RNA flow of information by copying RNA to DNA in a process called reverse transcription. The language of nucleic acids is written in four nucleotides – A, G, C, and T in the DNA dialect; A, G, C, and U in the RNA dialect – while the language of proteins is written in amino acids. Now we know the list of the 20 amino acids that are genetically encoded by DNA or RNA.

Central Dogma

- The direction of the **flow of genetic information** is from DNA to RNA to polypeptide.

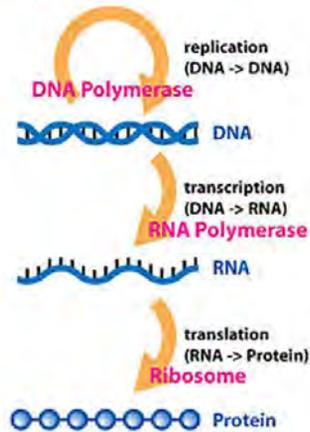


Figure 7. The central dogma of molecular biology (*figure used with permission under Creative Commons license*)

3.4. Transcription: From DNA to RNA

Transcription is the process by which the polymerization of ribonucleotides guided by complementary base pairing produces an RNA transcript of a gene and is catalysed by RNA polymerase. The template for the RNA transcript is one strand of that portion of the DNA double helix that composes the gene. This process is divided into three phases: initiation, elongation, and termination. Transcription is begun by RNA polymerase at the DNA sequences near the beginning of genes, called promoters. After that, RNA polymerase adds nucleotides to the growing RNA polymer in the 5' to 3' direction. Transcription uses ribonucleotide triphosphates (ATP, CTP, GTP, and UTP) instead of deoxyribonucleotide triphosphates. Transcription is terminated at the sequences in the RNA products, known as terminators, where RNA polymerase stops this process. In eukaryotic organisms most primary transcripts undergo processing in the nucleus before they migrate to the cytoplasm to direct protein synthesis, and this process plays a fundamental role in the evolution of complex organisms. Some RNA processing in eukaryotes modifies only the 5' or 3' ends of the primary transcript, leaving the information content of the rest of the mRNA untouched. Other processing deletes information from the middle of the primary transcript, leaving the content of the mature mRNA related, but not identical, to the complete set of DNA nucleotide pairs in the original gene. Enzymes known

as methyl transferases add methyl ($-\text{CH}_3$) groups to G nucleotide at the 5' end of a eukaryotic mRNA, forming a so-called methylated cap. Like the 5' methylated cap, the 3' end of most eukaryotic mRNAs is not encoded directly by the gene and in a large majority of eukaryotic mRNAs, the 3' end consists of 100–200 base pairs, referred to as a poly-A tail. Both the methylated cap and the poly-A tail are critical for the efficient translation of the mRNA into protein.

3.5. Translation: From mRNA to Protein

Translation is the process by which the sequence of nucleotides in a messenger RNA directs the assembly of the correct sequence of amino acids in the corresponding polypeptide and takes place on ribosomes that coordinate the movements of transfer RNAs carrying specific amino acids with the genetic instructions of an mRNA. Ribosomes facilitate polypeptide synthesis by recognizing mRNA features that signal the start of translation: helping to ensure accurate interpretation of the genetic code by stabilizing the interactions between tRNAs and mRNAs *via* codon-anticodon recognition; supplying the enzymatic activity that links the amino acids in a growing polypeptide chain, and helping end polypeptide synthesis by dissociating both from the mRNA directing polypeptide construction and from the polypeptide product itself. The small subunit is the part of the ribosome that initially binds to mRNA and the larger subunit contributes an enzyme known as peptidyl transferase, which catalyses formation of the peptide bonds joining adjacent amino acids (Fig. 8). Both the small and the large subunits contribute to three distinct tRNA binding areas known as the aminoacyl (A) site, the peptidyl (P) site, and the exit (E) site. This process is divided into three phases: initiation, elongation, and termination.

AUG is the first (initiation) codon to be translated at the 5' end of the gene's reading frame, and is recognized by initiating tRNAs carrying a modified form of methionine called N-formylmethionine (fMet). The ribosome moves along the mRNA in the 5' to 3' direction. At each step of translation, the polypeptide grows by the addition of the next amino acid in the chain to its C terminus. Translation terminates when the ribosome reaches a UAA, UAG, or UGA nonsense codon at the 3' end of the gene's reading frame.

Protein structure is not irrevocably fixed upon completion of translation (Fig. 9). Several different processes may subsequently modify a polypeptide's structure, such as cleavage and removal of amino acids, such as the N-terminal fMet, from a polypeptide, or generation of smaller polypeptides from one larger product. The addition of chemical constituents, such as phosphate groups, methyl groups, or carbohydrates, to specific amino acids can also modify a polypeptide after translation and this process is referred to as post-translational modifications, very important or biochemical function of many enzymes.

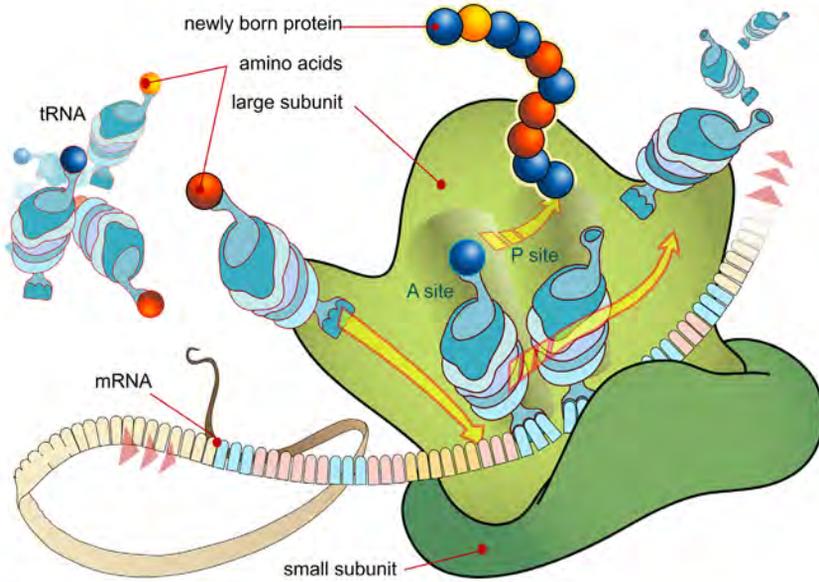


Figure 8. Translation process (figure used with permission under Creative Commons license)

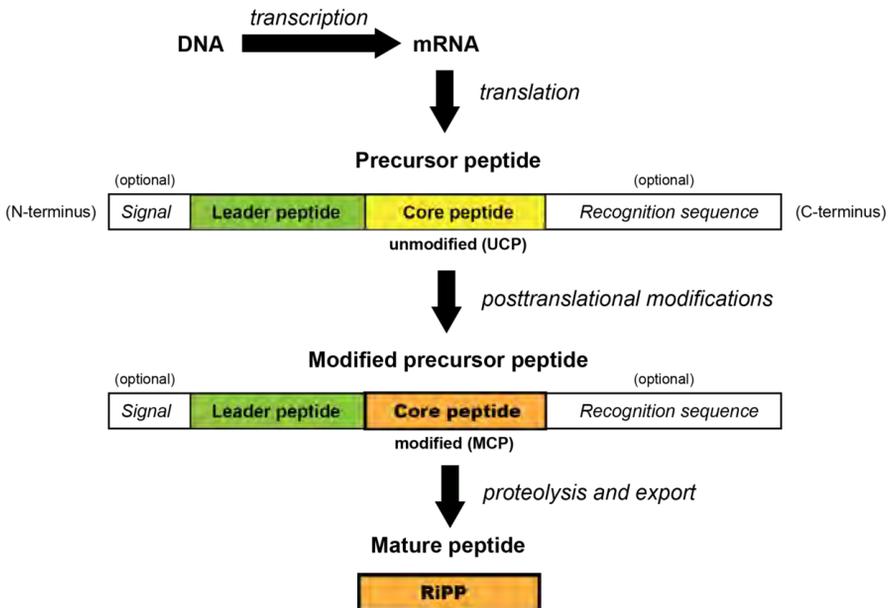


Figure 9. Biosynthesis of protein (figure used with permission under Creative Commons license)

3.6. Prokaryotic and Organelle Genetics

The advent of recombinant DNA technology in the 1970s and '80s facilitated an understanding of genes, chromosomes, and restriction enzymes in bacteria. The bacterial chromosome is the essential component of a typical bacterial genome and is a single molecule of double-helical DNA arranged in a circle with 4–5 Mb long in most of the commonly studied species. Inside the cell, the long, circular DNA molecule condenses by supercoiling and looping into a densely packed nucleoid body.

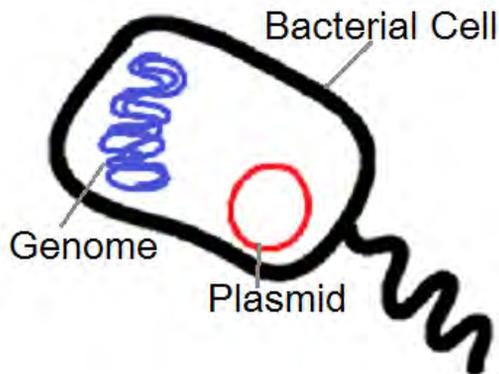


Figure 10. Genetic information within bacterial cell (*figure used with permission under Creative Commons license*)

Bacteria carry the genes necessary for their growth and reproduction in their large circular chromosome. In addition, some bacteria carry genes not needed for growth and reproduction under normal conditions in smaller circles of double-stranded DNA known as plasmids. These elements carry genes that protect their hosts against toxic metals, encoding resistance to antibiotics or contribute to their pathogenicity.

3.7. Analysis of molecular information

3.7.1. Sequence-specific DNA Fragmentation

A restriction enzyme recognizes a specific sequence of bases anywhere within the genome and then severs two covalent bonds (one in each strand) in the sugar-phosphate backbone at particular positions within or near that sequence. This digestion process generates well-defined restriction fragments

suitable for manipulation and characterization. Restriction enzymes originate in and can be purified from bacterial cells and protect them from viral infection by digesting viral DNA. Bacteria shield their DNA from digestion by their own restriction enzymes through the selective addition of methyl groups to the restriction recognition sites in their DNA. Restriction enzymes recognize target sequences of 4–8 bp in DNA isolated from any other organism and cut the DNA at or near these sites. For the majority of these enzymes, the recognition site contains 4–6 base pairs and exhibits a kind of palindromic symmetry in which the base sequences of each of the two DNA strands are identical when read in the 5'-to-3' direction. Most enzymes make their cuts in one of two ways: either straight through both DNA strands right at the line of symmetry to produce fragments with blunt ends, or displaced equally in opposite directions from the line of symmetry by one or more bases to generate fragments with single-stranded ends-sticky ends (easy to ligation process, because they are free to base pair with a complementary sequence from the DNA of any organism cut by the same restriction enzyme) (Figs. 11 and 12). They are an important molecular tool to create unique DNA fragments and also as an analytic tool to create restriction maps of viral genomes and other purified DNA fragments showing the relative order and distances between multiple restriction sites, which thus act as landmarks along a DNA molecule.



Figure 11. Restriction enzyme recognition site leaving sticky ends (*figure used with permission under Creative Commons license*)



Figure 12. Restriction enzyme recognition site leaving blunt ends (*figure used with permission under Creative Commons license*)

One of the most commonly used methods for creating a restriction map involves digestion fragments made by multiple restriction enzymes, followed by gel electrophoresis to visualize the produced fragments.

Restriction enzyme digestion and standard agarose gel electrophoresis allow only for analysis of simple DNA molecules, in up to 50,000 base pairs (50 kbp). The genomes of animals, plants, and even microorganisms are far too large to be analysed in this way. Only purified and then amplified original DNA molecules can be treated with chemical and physical techniques to analyse the isolated DNA fragment.

3.7.2. Cloning Fragments of DNA

There are the two strategies to accomplish the purification and amplification of individual fragments, such as molecular cloning, which replicates individual fragments of previously uncharacterized DNA, and the polymerase chain reaction (PCR), which can purify and amplify a previously sequenced genomic region.

Molecular cloning, which consists of two basic steps, is the process that takes a complex mixture of restriction fragments and uses living cells to purify and make many exact replicas of just one fragment at a time (Fig. 13). In the first step, DNA fragments are inserted into specialized carriers called vectors necessary for the proper transport, replication, and purification of individual inserts. In the second step, vector harbouring insert is transported into living cell, where many identical copies of this molecule (DNA clones) are made (Fig. 14). These DNA clones may be purified for immediate study or stored within cells as libraries for future analysis. When the inserted DNA fragment and DNA vector are from two different origins, the spliced molecule creates a recombinant DNA.

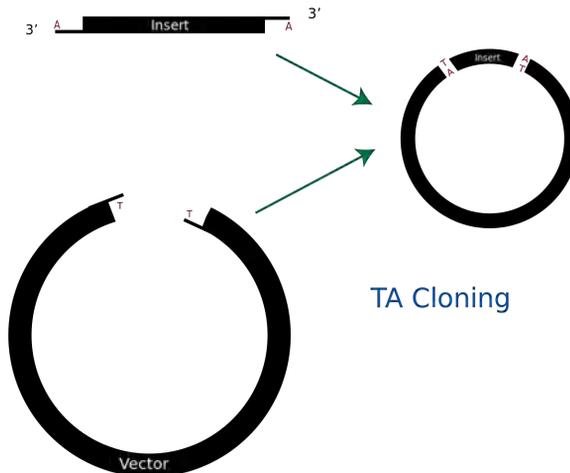


Figure 13. Introducing DNA insert into DNA vector (*figure used with permission under Creative Commons license*)

A vector must possess distinguishing features, such as size or shape, by which it can be purified away from the host cell's genome. Creating single-stranded sticky ends is a basis for the efficient production of a vector-insert recombinant. Moreover, the ends produced with the same enzyme are complementary in sequence and are available for base pairing, and no matter what the origin of the DNA. Such digested DNA mixtures in the presence of DNA ligase allows for creating completed, stable recombinant DNA molecule.

Molecular Cloning

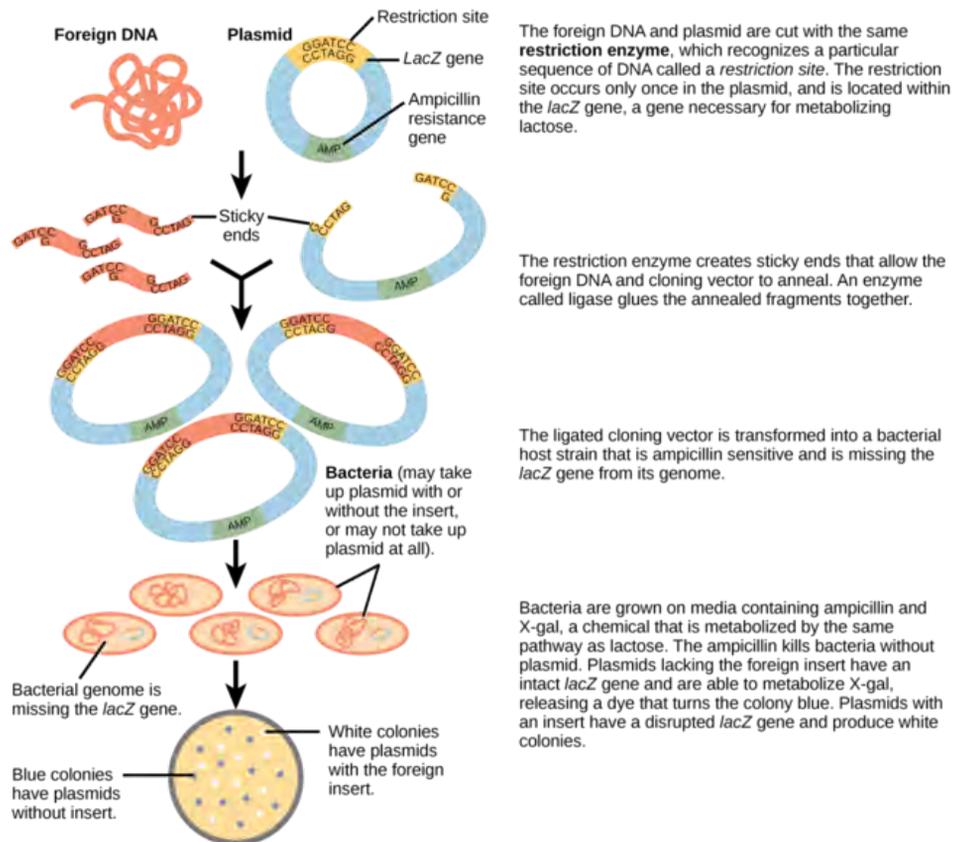


Figure 14. Molecular cloning (figure used with permission under Creative Commons license)

The simplest DNA vector is a plasmid vector. Each plasmid vector carries an origin of replication and a gene for resistance to a specific antibiotic necessary for proper selection of positive clone (cells harbouring a DNA vector). For the efficient cloning very important is distinguishing insert-containing recombinant

molecules from vectors without inserts. Figure 14 illustrates the three-part process with a plasmid vector containing an origin of replication, the gene for resistance to ampicillin (amp^R), and the *E. coli* lacZ gene, which encodes the enzyme β -galactosidase. It is possible to insert foreign DNA into the gene at that location and then use the disruption of the lacZ gene function to distinguish insert-containing recombinant molecules from vectors without inserts. Taking up a foreign DNA molecule by bacterial cell, changing the genetic characteristics of that cell is known as transformation.

3.7.3. DNA Sequence Analysis

The DNA sequence of a genome provides a lot of practical information, such as the evolution of genomes when the DNA sequences between genes are compared. Additionally, comparison of genomic and cDNA sequences immediately shows how a gene is divided into exons and introns, and can suggest whether alternative splicing of the gene's primary transcript is occurring.

The Sanger method

There are two steps to the Sanger method of sequencing. The first step is the generation of a complete series of single-stranded sub-fragments complementary to a portion of the DNA template under analysis, which differs in length by a single nucleotide from the preceding and succeeding fragments, distinguishable according to their terminal 3' base. In the second step of the sequencing process, the mixture of DNA sub-fragments through polyacrylamide gel electrophoresis is analysed, under conditions that allow the separation of DNA molecules differing in length by just a single nucleotide.

In this method the single strands are mixed in a solution with DNA polymerase, the four deoxynucleotide triphosphates, and a radioactively labelled oligonucleotide primer complementary to DNA adjacent to the 3' end of the template strand under analysis. The solution is next divided into four aliquots. To each one, a small amount of a single type of a nucleotide triphosphate lacking the 3'-hydroxyl group (dideoxynucleotide), which is critical for the formation of the phosphodiester bonds that lead to chain extension is added. Dideoxynucleotide comes in four forms: ddTTP, ddATP, ddGTP, or ddCTP. In each sample reaction tube, the oligonucleotide primer hybridizes at the same location on the template DNA strand. As a primer, it will supply a free 3' end for DNA chain extension by DNA polymerase by adding the nucleotides to the growing strand that are complementary to those of the sample's template strand. The addition of nucleotides continues until, by chance, a dideoxynucleotide is incorporated instead of a normal nucleotide. The absence of a 3'-hydroxyl group in the dideoxynucleotide prevents the DNA

polymerase from forming a phosphodiester bond with any other nucleotide, ending the polymerization for that new strand of DNA. After enough time for the polymerization of all molecules to reach completion, templates may be released from the newly synthesized strands by denaturing the DNA at high temperature. Each sample tube now holds a whole collection of single-stranded radioactive DNA chains as well as the nonradioactive single strands of the template DNA. The lengths of the radioactive chains reflect the distance from the 5' end of the oligonucleotide primer to the position in the sequence at which the specific dideoxynucleotide present in that particular tube was incorporated into the growing chain. The samples in the four tubes are now electrophoresed in adjacent lanes on a polyacrylamide gel, and the gel is subjected to a system that detects the presence of the radioactive label. Each band represents a chain that is one nucleotide longer than the chain of the band below.

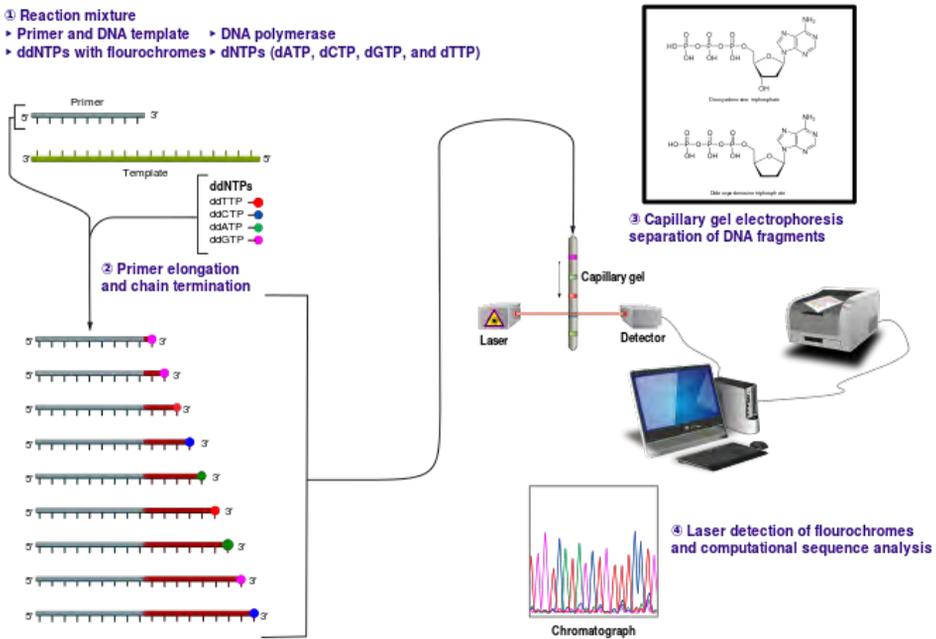


Figure 15. Automated Sanger sequencing (figure used with permission under Creative Commons license)

To automate the DNA sequencing process, the method of labelling the newly formed complementary DNA strands has changed; each of the four chain-terminating dideoxynucleotides is labelled with a different colour fluorescent dye. A DNA sequencing machine follows the DNA chains of each

length in the ascending series through a special detector that can distinguish the different colours associated with each terminating dideoxynucleotide (Fig. 15).

High-throughput sequencing instruments can analyse up to 384 DNA fragments at a time. New technologies and strategies allow the sequencing of 100 billion bp in 96 hours using highly-parallel simultaneous analyses of millions of DNA fragments, and facilitate sequencing of individual human genomes.

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4. BIOLOGICAL TOXINS

Biological toxins are substances produced by living organisms – bacteria, plants and animals – for defence or predation. One dictionary defines biological toxins as “Chemicals produced by living organisms that have toxic properties for another organism”. Biological toxins have been used by humans for a long time. The first information on natural poisons and some guidelines on their preparation can be found in the Ebers Papyrus, which dates back to about 1,500 B.C. This paper looks at many of the plants containing poisonous substances. There are many ways of classifying biological toxins. The first is by their action, into A and B categories. Category A includes neurotoxins that affect the functions of the nervous system with effects that are temporary and theoretically reversible. Category B includes toxins that directly damage cells and whose effect causes functional disorders of tissues and organs. Tissues and organs are damaged indirectly or directly by release of secondary mediators. The effects of such toxins are often irreversible and cause permanent health damage. A biological toxin’s lethal potential is measured in terms of the amount of material required to kill 50% of a group of test animals (usually rats or mice). This is written as LD_{50} . $LD_{50} < 25$ mg/kg means that a substance is very toxic; $LD_{50} < 25$ mg/kg to 200 mg/kg is toxic; $LD_{50} < 200$ mg/kg to 2,000 mg/kg $< LD_{50}$ is harmful, while substances of $LD_{50} > 2,000$ mg/kg are not classified as toxic agents. The most potent biological toxins are produced by bacteria, plants and fungi, and some animal toxins also have deadly potential.

Biological toxins are very attractive to terrorists, for use in acts of bioterrorism. The first reason is that many biological toxins can be obtained very easily. Simple bacterial culturing systems and extraction equipment dedicated to plant toxins are cheap and easily available, and can even be constructed at home. Additionally, the actual toxins are easy to obtain and in most cases not restricted by law. Many toxins affect the nervous systems of mammals by interfering with the transmission of nerve impulses, which gives them their high potential in bioterrorist attacks. Others are responsible for blockage of main cellular metabolism, causing cellular death. Moreover, most toxins act very quickly and are lethal in low doses ($LD_{50} < 25$ mg/kg), which are very often lower than chemical warfare agents.

Table 1. Comparison of biological toxins toxicity with chemical warfare agents

Agent	LD ₅₀ parameter (µg/ml)	Molecular weight
Botulin toxin	0.001–0.002	150,000 (Protein)
Shiga toxin	0.002	55,000 (Protein)
Tetanus toxin	0.002–0.003	150,000 (Protein)
Abrin	0.01–0.04	65,000 (Protein)
Ricin	0.1–1	65,000 (Protein)
Clostridium perfringens toxins	0.1–5	35,000–40,000 (Proteins)
VX	15	267
Staphylococcal enterotoxin B	27	25,000 (Protein)
Soman	64	182
Sarin	100	140
Aconitine	100	647
T-2 mycotoxin	1,210	466

The potential of biological toxins in bio warfare was presented in a report of the Iraq Biological Warfare Program, prepared by the UN Special Commission in 1995. The report concluded that Iraq had 19,000 litres of botulinum toxin and 2,400 litres of aflatoxin at its disposition.

4.1. Bacterial toxins

Bacterial toxins are substances produced and released by bacterial cells to destroy other bacteria and surrounding cells. Most bacterial toxins are proteins, encoded by bacterial chromosomal genes, plasmids and phages. Bacterial toxins can be classified as either exotoxins or endotoxins. Exotoxins are generated and actively secreted; endotoxins remain part of the bacteria. Endotoxins are usually part of the bacterial outer membrane, and are released by lysis of the bacterial cell. The response of an organism to an endotoxin can include severe inflammation. In general, the inflammation process is usually considered beneficial to the infected host, but if the reaction is severe enough it can lead to sepsis. Exotoxins are usually secreted by bacteria and act at a site removed from bacterial growth. Exotoxins are usually proteins, minimally polypeptides, that act enzymatically or through direct action with host cells and stimulate a variety of host responses.

4.1.1. Botulin toxin

Botulin toxin is a neurotoxin produced by the spore-forming, Gram positive, anaerobic bacteria *Clostridium botulinum*. Spores are resistant to heat, chemical substances, radiation and aerobic conditions. One disease caused by the toxin

is botulism. Botulin toxin has a protein construction (with a mass of 150 kDa) and it belongs to a group of metal proteins (zinc-dependent endopeptidases). It is built of two polypeptide chains: a heavy chain with a mass of 100 kDa and a lightweight chain (50 kDa), connected by a disulphide bond. This toxin is not resistant to chemical and physical agents. It is depredated at 85°C in 5 minutes, and is destroyed by sunlight within 1-3 hours. Additionally, it is immediately decontaminated by chloride or H₂O₂.

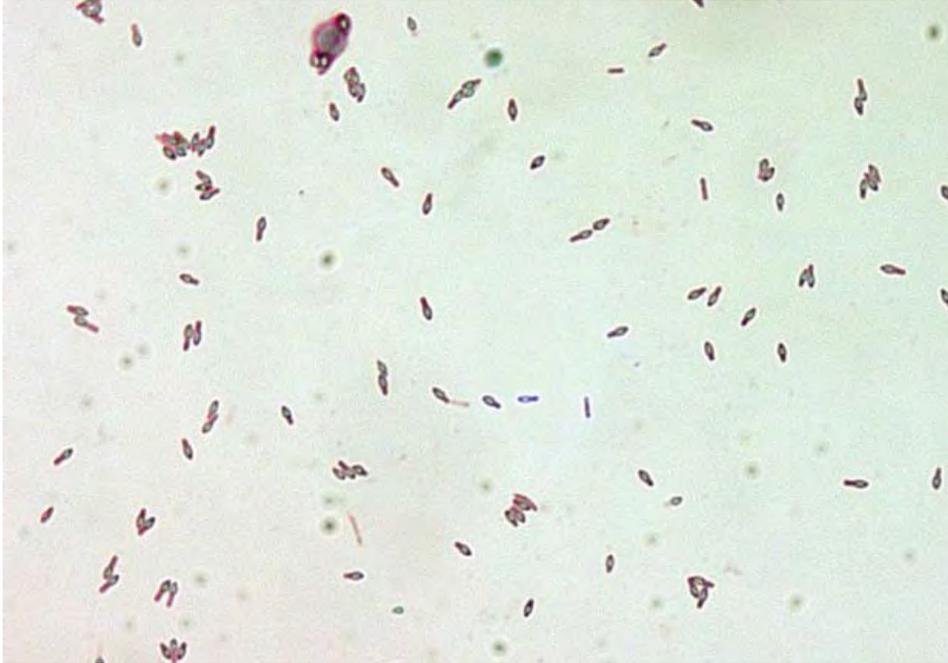


Figure 1. *Clostridium botulinum* spores (figure used with permission under Creative Commons license)

The protein has three domains responsible for its biological functions: a binding domain which binds the toxin to the receptor located on the surface of the target cells of the cellular membrane; a translocation domain that transports the toxin through the plasma membrane into the cell, and an enzymatic domain with a proteolytic activity that causes defragmentation of the SNAP-25 protein, which is responsible for merging synaptic bubbles with a cellular membrane and releasing neurotransmitters. The binding and translocation domains are located within the heavy chain, while the domain with enzymatic activity is within the lightweight chain. The mechanism of the toxic action of botulin toxin inhibits the release of neurotransmitters, including acetylcholine, within neuromuscular junctions, resulting in de-contraction and relaxation of skeletal muscles.

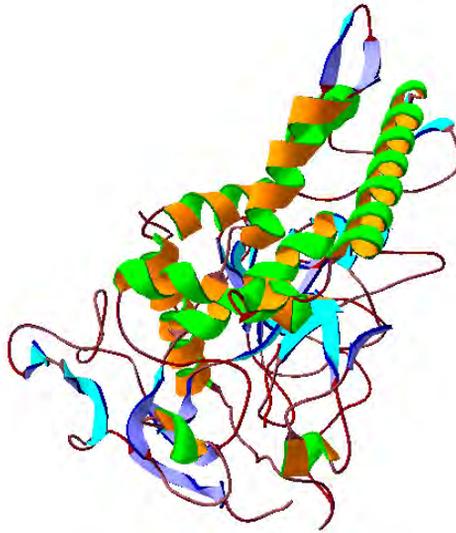


Figure 2. Botulin toxin structure

Botulin toxin is the most toxic substance in the known world, with an LD_{50} of 1-2 ng/kg. As the only biological toxin, it has been classified by the Centre for Disease Control and Prevention (CDC) in Atlanta as a Category A bio agent. The lethal dose for a human weighing about 70 kg is 0.7–0.9 μ g of inhaled toxin, or 70 μ g of poison ingested with food.

There are 7 kinds of botulin neurotoxin: A, B, C, D, E, F and G. Types A, B, E and F are dangerous to humans, with type A being the most dangerous and most toxic. It can be detected in soil samples from China, South America and the western territories of the United States. Type B toxins can be isolated from the soil of the eastern United States and Europe. Type E can be found in the sea and lake sediments in the northern hemisphere. Types C and D are dangerous to animals, although no cases of disease as a result of poisoning with type G have been reported so far. *Clostridium botulinum* spores are also capable of developing in poorly prepared and stored food. This applies to meat, fish and vegetable preserves.

In nature there are 4 sources of botulism: from food, wounds, animals and in infants. Regardless of where it grows, similar clinical symptoms of botulin poisoning are observed, and all occur within several hours of the toxin's penetration into the body. Initially, the symptoms include difficulty speaking and swallowing, double or unclear vision, anuria, and cessation of saliva and tears. Next, control of one's body is lost, along with the gag reflex. Paralysis of the respiratory muscles causes respiratory insufficiency and this is the main cause of death of infected people. Consciousness and physical sensation are maintained throughout all of the symptoms, which aggravates the suffering. Foodborne botulism occurs after eating poorly preserved

food. Wound botulism is caused by the *Clostridium botulinum* bacteria penetrating the body through broken skin, e.g. wounds suffered in accidents. Most cases of wound botulism are observed in drug addicts taking heroin subcutaneously. Infant botulism occurs through the ingestion of honey or powdered milk contaminated with *Clostridium botulinum* spores. An analysis of global infant botulism between 1996 and 2008 revealed 524 cases in 26 countries. Botulism in animals is a result of eating feed contaminated with bacteria spores. Horses, cattle, birds and minks are particularly sensitive to botulin toxin.

Treatment of botulism is based on administering botulin antitoxin as quickly as possible. Its role is to neutralise the toxin. Additionally, in most cases mechanically-assisted respiration and supportive treatment (with a return to self-reliance in up to 2–3 months), is also needed.

In a bioterrorist attack, botulin toxin can be used in the form of an aerosol or can be added to food and water sources. The neurotoxin's effectiveness is equally high, regardless of the route of exposure, due to the nearly identical symptoms. Initial symptoms can be mistaken for other symptoms of chemical warfare agents, such as sarin or VX, and this can additionally impede rescue actions. The mortality rate in such attacks is estimated as being nearly 100%. For this reason, the first responders should be prepared to recognize poisoning with this toxin.



Figure 3. Typical symptom of botulin poisoning – ptosis (figure used with permission under Creative Commons license)

Table 2. Comparison of typical symptoms of botulin toxin and nerve gas poisoning

Botulin toxin	Nerve agent
paralysis	convulsions
no change in heart rate	bradycardia
drooping eyelids (ptosis)	constricted pupils (mioz)
increased secretory functions	lack of secretory functions

4.1.2. Tetanus toxin

Tetanus toxin is another potent neurotoxin produced by the vegetative cells of *Clostridium tetani* in anaerobic conditions, which causes tetanus. Similarly to *Clostridium botulinum*, this bacteria also forms very resistant spores. In nature, *Clostridium tetani* is found in soil, especially heavily-manured soils, and in the intestinal tracts and faeces of various animals. The toxin is produced during cell growth, sporulation and lysis. The LD₅₀ of this toxin has been estimated to be approximately 2–3 ng/kg, making it second only to botulinum toxin as the deadliest toxin in the world.

The bacterium synthesizes the tetanus toxin as a 150 kDa polypeptide chain, which is composed of 2 subunits: A (50 kDa), and B (100 kDa). Similar to botulin toxin, it also has zinc-dependent endopeptidases with similar physical and chemical properties. The B-chain binds to disialogangliosides on the neuronal membrane and contains a translocation domain which aids the movement of the protein across that membrane and into the neuron, while the A-chain cleaves the synaptobrevin. This protein is necessary for vesicle fusion to membranes. Cleavage of the synaptobrevin inhibits neurotransmitter exocytosis in the inhibitory interneurons. The effect of the toxin is to block the release of inhibitory neurotransmitters (glycine and gamma-amino butyric acid (GABA)) across the synaptic cleft, which is required in checking of the nervous impulse. Blocking the release of GABA, which is mainly a neurotransmitter that inhibits motor neurons, causes a violent spastic paralysis which is a characteristic symptom of this toxin.

The routes of exposure are similar to those in botulin toxin, however tetanus toxin has a clinical picture of poisoning. The first symptom of poisoning is a slack jaw, followed by the so-called sardonic smile, stiffness of the neck, difficulty swallowing and muscle contractions in the torso and limbs, with a characteristic bent back (opisthotonos). Next, the cramps become stronger (even breaking the long bones and spine), and are very painful and exhausting.

Treatment is based on administration of anti-tetanus immunoglobulin (antitoxin) that bind the toxin while circulating in the blood. Adjuvant therapy consists of painkillers and relieving the cramps.

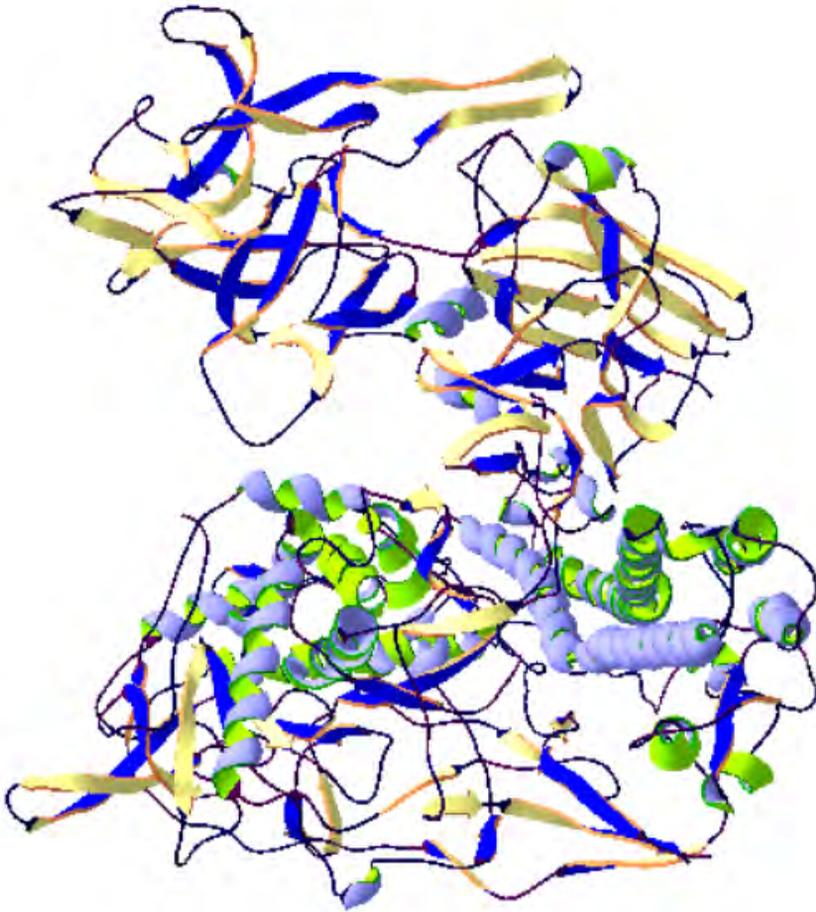


Figure 4. Tetanus toxin structure

4.1.3. Enterotoxins

Enterotoxins are a kind of exotoxin that is secreted by some pathogens. In nature, this group is mostly responsible for food poisoning. Enterotoxins include staphylococcal enterotoxins and AB5 group enterotoxins (i.e. cholera toxins), Shiga toxins and heat-labile enterotoxins produced by *Escherichia coli*.

Staphylococcal enterotoxins are a super antigen able to activate many T lymphocytes, causing overproduction of cytokines, which are responsible for inflammation. Enterotoxins from the AB5 group bind with protein G and cause the transport of sodium and water ions outside the cell. The LD_{50} at oral administration of staphylococcal enterotoxin is about 25 $\mu\text{g}/\text{kg}$ body weight; the

LD₅₀ of the Shiga toxin is 0.5–20 µg/kg body weight, and the LD₅₀ of cholera toxin is 260 µg/kg body weight.

Staphylococcus aureus, which is responsible for toxin production, is a very rapidly spreading bacterium. Usually, the bacteria are present in the throat, nasal cavity, and crotch/anus. There is no vaccine for staphylococcus, and the disease itself is resistant to antibiotics. *Staphylococcus aureus* is well developed in meat and dairy products. Its enterotoxins are mainly produced by *Staphylococcus* microorganisms, which are also present in food and cause food poisoning in humans. However, it is rare that they cause death. The symptoms persist for one or two days and mainly include abdominal pain, nausea, diarrhoea and vomiting. They contain 21 serotypes: A, B, C1, C2, C3, D, E, G, G2, H, I, J, K, L, M, N, O, P, Q, R, S, T, U, U2 and V. Serotypes A, B, C1, C2, C3, D and E are known as 'classic enterotoxins', while the others are 'new types'. Staphylococcal enterotoxins are proteins with a mass of 26–35 kDa, having single polypeptide chains. These proteins are highly soluble in both water and salt solutions. Staphylococcal enterotoxins are highly resistant to proteolytic enzymes (pepsin, trypsin), exhibiting unchanged biological activity in the gastrointestinal tract. These toxins are also resistant to gamma radiation, a wide pH range (2 < pH < 12), and dehydration. High thermal stability is characteristic of staphylococcal enterotoxins, which makes them potential food poisoning agents.

As superantigens they have a non-specific way of binding with Class II TCR complex MHC molecules. Consequently, T lymphocytes CD4 and CD8 are activated and overproduction of cytokines occurs. T lymphocytes are subjected to apoptosis or enter a state of anergy. Natural food poisoning is usually caused by A and D staphylococcal enterotoxins, but less frequently also by the B and C types.

The most dangerous enterotoxin, with great potential for use in bioterrorism, is Staphylococcal Enterotoxin B (SEB). It is the most heat resistant toxin, and is detected even in samples of thermally sterilized food. Enterotoxin B induces toxic shock syndrome in the body, which is a very strong immune response by the human organism. Strong food poisoning occurring very quickly can lead to dehydration and death. In the case of SEB being ingested into the body other than through food, for example by inhalation, it can trigger a septic response throughout the organism. The toxicity parameters for this type of poisoning are LD₅₀ 20 µg/kg and ED₅₀ 400 ng/kg.

Treatment is mainly based on conservative treatment, while inhalation intoxication is treated through the administration of anti-inflammatory steroids.

Enterotoxins in the AB5 group include Shiga toxin, heat-labile enterotoxin and cholera toxin. They have a similar construction and consist of a heterodimeric subunit A, containing polypeptide chains A1 and A2 linked by a disulphide bridge, and a homopentameric subunit B. Subunit A2 is linked with subunits A1 and B. Subunit B is built of five monomers. They are arranged in a ring that contains binding spots for the cellular membrane receptors.

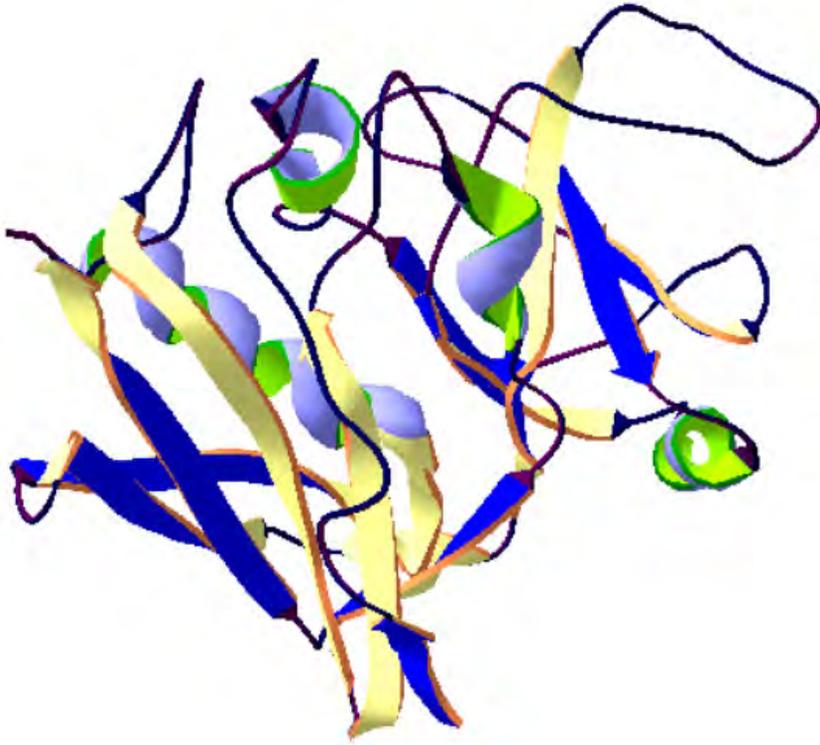


Figure 6. Enterotoxin type B structure

Shiga toxins are produced by *Shigella dysenteriae*. The Shiga family also includes toxins produced by *Escherichia coli* (O157:H7). A Shiga toxin contains a subunit A1 with a mass of 27.5 kDa, A2 with a mass of 4.5 kDa, and B with a mass of 7.7 kDa. These toxins are responsible for inhibition of protein synthesis in sensitive eukaryotic cells. Protein synthesis is blocked by the removal of adenine residue from the 28S rRNA of the 60S ribosome. Shiga toxin-mediated damage to the ribosome induces a response in cells called ribotoxic stress response, which is both pro-inflammatory and pro-apoptotic. The first symptoms occur after 6 hours of poisoning. The only viable medical intervention is supportive treatment.

4.1.4. Clostridium perfringens toxins

Clostridium perfringens is a rod-shaped, anaerobic, Gram-positive, spore-forming bacteria common in many different microbiota, and are found in the soil, marine sediment, in decaying vegetation, and in the intestinal tract of humans and other animals. This bacteria is able to produce at least 17 different

toxins. However four of them (alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX)), are the major toxins and have high toxic potential. Alpha and epsilon toxins have been classified by the CDC as Category B bioterrorism agents, which suggests their potential in these kinds of acts.



Figure 7. *Clostridium perfringens* (figure used with permission under Creative Commons license)

CPA is a 43 kDa protein containing two domains, an alpha-helical N-terminal domain harbouring the phospholipase C active site, and an alpha-sandwich C-terminal domain involved in membrane binding. This toxin is a classic example of a toxin that modifies cell membranes through enzymatic activity degrading phosphatidylcholine and sphingomyelin the components of the eukaryotic cell membranes. This damages the cell membrane which results in cell lysis. Additionally, the lipolysis of the cell membrane activates an arachidonic cascade resulting in the formation of thromboxanes, leukotrienes and prostaglandins, which activate the inflammation cascade and produce vasoconstriction. The ensuing intravascular haemolysis and capillary damage, platelet aggregation and hepatic necrosis results in multiple organ failure.

ETX is the third most potent biological toxin after botulinum toxin and tetanus toxin, with an LD_{50} of about 70–100 ng/kg. The active toxin is a protein with a molecular mass of 29 kDa, with relative resistance to proteases in the gastrointestinal tracts of mammals. This toxin is most stable at room temperature for up to a few weeks, and far longer at colder temperatures.

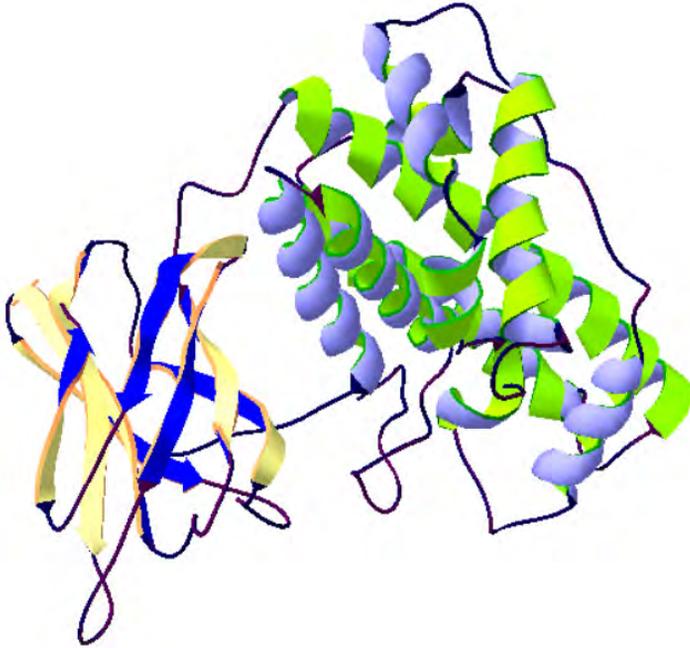


Figure 8. Alfa toxin structure

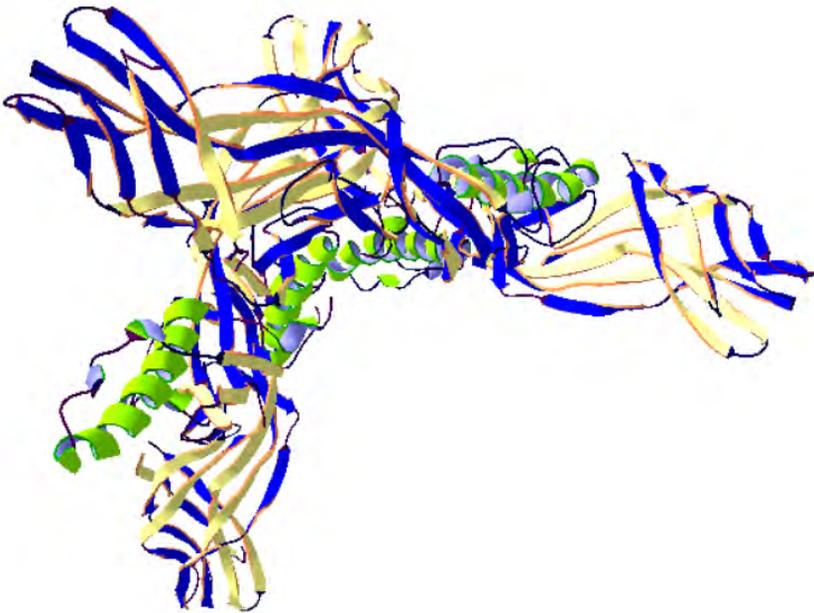


Figure 9. Epsilon toxin structure

Epsilon toxin interacts with the cellular membrane and creates pores within the membrane that modify its porousness and quickly becomes cytotoxic. Epsilon toxin induces pore formation in eukaryotic cell membranes *via* detergent-resistant, and cholesterol-rich membrane domains (lipid rafts). This damage results in very fast degenerative and necrotic changes in cells, leading to organ failure. The greatest potential for this toxin's use in bioterrorism is in an aerosolized form that can be used as a bioterrorist weapon. Additionally, this toxin can be dispersed in food intended for human consumption.

4.2. Plant toxins

Plant toxins are substances produced by plants as part of their defence mechanism. Pests and herbivores do not differentiate between particular plants. Plant toxins are additionally produced to protect the plants against various other threats, such as bacteria, fungi and insects. They are usually proteins or secondary metabolites which are not essential to the life of the plant producing them. Poisonous plants have a seed, root, leaf, stalk, fruit or juice from which even a relatively small amount, either received unwittingly or administered deliberately, can harm the human organism. In some plants, the poisonous constituents occur throughout the whole plant. In others, they are present in one or more parts. Toxins are produced by plants from every climate zone and in almost every ecosystem. Their easy accessibility has allowed them to kill people since ancient times. Human ingenuity in this area was unlimited. The pathways of plant toxins into the body can be very different, but most often are *via* inhalation or food. Also, the impact spectrum is very extensive and covers, amongst others, inhibition of the activity of cellular enzymes, actions on cell receptors (activation or inhibition), and interference with nucleic acids. The main chemical types of biological toxins are alkaloids, glycosides, tannins and lectins (a type of protein).

4.2.1. Ricin

Ricin is a toxin of plant origin, obtained from whole seeds or the waste of the castorbean plant (*Ricinus communis*), from which castor oil is pressed. The plant is endemic in eastern Africa and Asia, although nowadays it also grows in regions of subtropical and temperate climate. The yield from 1 kg of seeds is about 1 gram of pure ricin. Pure, extracted ricin is a white and yellow powder that is stable in the environment.

Ricin belongs to a group of proteins called lectins, which are proteins with high affinity to two glycoprotein chains: A and B, linked by a disulphide bridge. Chain B is a lectin, which binds with glycoprotein containing the mannose that occurs in the outer layer of the cellular membrane, facilitating endocytosis

of the toxin to the cell cytosol. Chain A has active RNA N-glycosidase, which causes decomposition of the glycoside bond in the adenine nucleotides of RNA molecules present in the large (60S) and small (28S) ribosome subunits. Chain A causes inactivation of ribosomes in the cells and blocks protein synthesis, which results in cellular death. One ricin molecule is able to deactivate as many as 2,000 ribosomes within 1 minute.

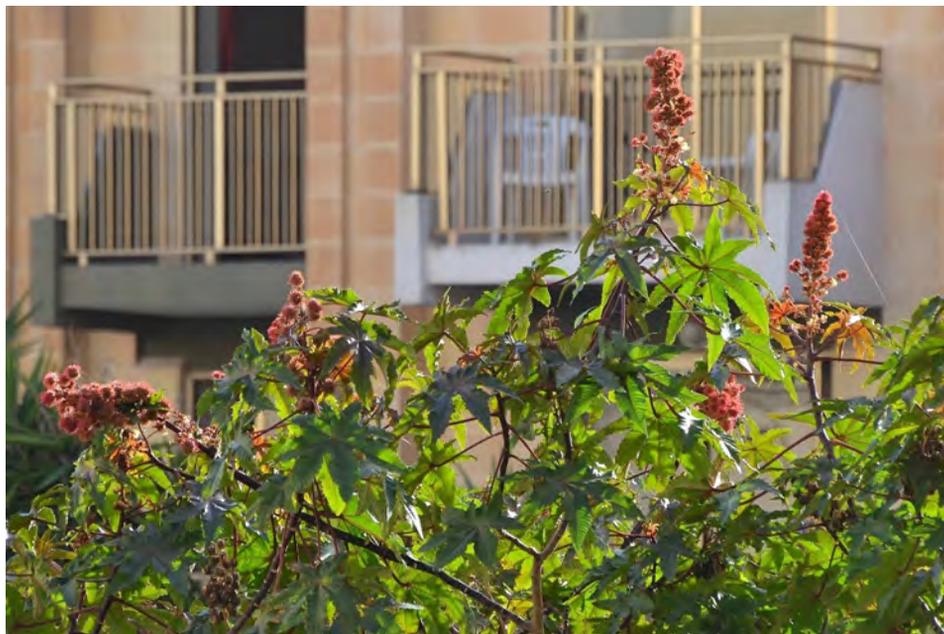


Figure 10. *Ricinus communis* in nature

Ricin's toxicity depends on the exposure route and dose. The LD₅₀ value for humans after ingestion is estimated at about 22–25 µg/kg body weight. Just 5–6 grains of castorbean plant seeds are considered a lethal dose to children, while for adults it is about 20 grains. A lethal dose following inhalation is 3 µg/kg body weight. It is estimated that if 8 tons of ricin are released as aerosol in an area of 100 km², 50% of the population in the area would die.

Following ingestion of castorbean plant seeds or food contaminated with the toxin, the alimentary tract is the first to be affected and damaged. The symptoms do not occur immediately upon ingestion but after a few days of vomiting and diarrhoea, as a result of the alimentary tract irritation. This causes bodily dehydration, which is followed by alimentary tract bleeding and necrosis of the liver, kidneys and pancreas. Vascular collapse tends to follow next. If the toxin enters *via* the respiratory tract, the symptoms that occur within several hours following inhalation include

fever, coughing and progressive respiratory insufficiency. In more severe cases, pulmonary oedema, hypotension and vascular collapse are observed. Intramuscular administration of the toxin causes oedema in the injection area and necrosis of local lymph nodes, while other typical symptoms include gastrointestinal bleeding and renal necrosis. Death as a result of ricin poisoning occurs, on average, within 36–48 hours, regardless of the route of exposure. If the patient does not die within 3–5 days, their chance of being cured and surviving is high.



Figure 11. *Ricinus communis* seeds (figure used with permission under Creative Commons license)

Victims of ricin poisoning are not dangerous to their environment. However, there are no detailed treatment methods or vaccines available yet. Even so, experimental vaccines are in development, and tests on animals have proven their effectiveness.

Considering its high toxicity and the easy separation of the toxin from *Ricinus communis* plant seeds, there is a high risk of ricin use in bioterrorist attacks. Considering the various advantages of castor oil's use in cosmetics and its low production costs, 1.5 million tons of castor oil are produced in 30 countries

around the world, which creates the opportunity to obtain large amounts of ricin toxin. A small toxic dose and many potential exposure routes also make it a very good biological agent for use in bioterrorism.

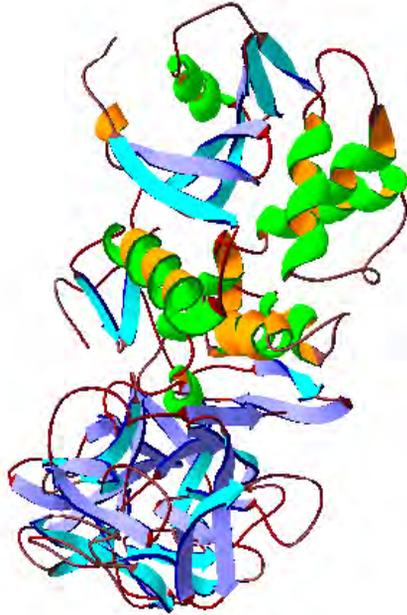


Figure 12. Ricin structure

The identification of an aerosol attack can be based on observation of lung symptoms. Lung oedema occurs much later (1–3 days after exposure) than with staphylococcus enterotoxin (about 12h), or phosgene (about 6h). Additionally, the symptoms will deepen despite antibiotic therapy, contrary to their effect on infectious agents. Patients with acute respiratory poisoning require intensive ARDS therapy: oxygen, intubation, artificial ventilation, and haemodynamic monitoring. In gastrointestinal poisoning gastric lavage should be performed, followed by laxatives. Activated carbon is not effective against ricin, which has a high molecular weight. Instead, as supportive treatment proper hydration and electrolyte status of the patient is required.

4.2.2. Abrin

Similarly to ricin, abrin is also a toxin of plant origin obtained from *Abrus precatorius*, a plant also known as the ‘rosary pea’. Originally, it used to grow in South-East Asia and in Guinea, Africa, but nowadays can be found in many tropical regions all over the world. The structure of abrin is similar to the

structure of ricin toxin. Abrin consists of two polypeptide chains: A and B, linked by a disulphide bridge. Chain B, which has the properties of lectin, plays the role of a binding domain and is supposed to connect with glycoprotein receptors on cell surfaces. Chain A is RNA N-glycosidase, as in ricin. The mechanism of abrin action involves blocking of the translation in the cells into which it has penetrated. In the cell cytosol, chain A separates the C-N bond in adenines located in a small subunit of 28S rRNA ribosome. Adenine depurination destabilises rRNA, and so consequently the synthesis of proteins is inhibited.



Figure 13. *Abrus precatorius* (figure used with permission under Creative Commons license)

Abrin is 30 times more toxic than ricin, with an LD₅₀ estimated at about 0.7 µg/kg body weight. Additionally, all parts of the *Abrus precatorius* plant are toxic but the highest concentration of the toxin occurs in the seeds. The pure, extracted form of abrin is a white and yellow powder that is stable in the environment. It can also be used in several other forms such as a mist, or dissolved in water.

A lethal dose following ingestion of the toxin amounts to 0.1–1 µg/kg body weight, which means that swallowing just 1-2 grains of rosary peas can cause death. The toxin can penetrate into the body by ingestion of the seeds, through the respiratory tract or by injection.



Figure 14. *Abrus precatorius* seeds (figure used with permission under Creative Commons license)

Poisoning with abrin by ingestion of seeds or food infected with the toxin causes severe abdominal pain, vomiting and diarrhoea. Renal insufficiency then develops. In most cases, bleeding from the alimentary tract is also observed. If abrin is absorbed by inhalation, the symptoms include pulmonary oedema, hypertension in the pulmonary arteries and haemolysis of erythrocytes. Death usually occurs 36–72 hours after exposure, depending on the route of exposure and dose. It is considered that if the patient does not die after 3–5 days, there is a chance that they will survive.

Because of their attractive appearance, the seeds of *Abrus precatorius* are used as beads for rosaries and bracelets, which causes more people to be exposed to the toxic action of abrin. Since there are no available drugs or vaccines against abrin, treatment is supportive and based on minimising the effects of the poisoning. The type of medical care administered depends on several factors, with the infection route being the most important, and includes support for the respiratory tract, intravenous administration of fluids and stabilisation of blood pressure. Possible neurological symptoms after exposure include hallucinations, reduced consciousness and convulsions.

Recognition of the poisoning as well as its medical treatment are identical to the response to ricin poisoning.



Figure 15. Abrin structure

Abrin is the strongest plant toxin, which means it can be potentially used as a biological weapon. Because only a small dose is required to cause severe poisoning, and because of the possibility of using abrin in several forms as well as its fast action, make it attractive to bioterrorists. The common occurrence of *Abrus precatorius* in tropical regions, which makes access to it quick and easy, is another advantage.

4.2.3. Aconitine

Aconitine, also known as the ‘Queen of Poisons’, is a plant toxin present in the very commonly-occurring *Aconitum napellus* Rchb. This is a species of plant belonging to the glaucoma family. It occurs naturally in the temperate climate zone of Eurasia. The name *Aconitum* may have been derived from the Greek word ‘akone’, meaning ‘rocky’ or ‘ravine’, which is where the plant usually grows. The flowers of *Aconitum* are considered to be very beautiful (with a medium to dark semi-saturated blue-purple colour), which means that this plant is often cultured in gardens as a decorative flower.

Aconitine is a secondary metabolite of the *Aconitum* plant and chemically is classified as a norditerpenoid alkaloid. Aconitine is a C19-norditerpenoid, barely

soluble in water, but very soluble in organic solvents such as chloroform or diethyl ether. Aconitine is also soluble in mixtures of alcohol and water if the concentration of alcohol is high enough. In nature it occurs mainly in the leaves, stems and roots of the *Aconitum* plant. The LD parameter is in the range 0.1–1 mg/kg of body mass. This toxin is very well absorbed by mucous membranes and skin.



Figure 16. *Aconitum napellus* in nature

The toxic action of aconitine results from its interaction with the voltage-dependent sodium-ion channels, which are proteins in the cell membranes of excitable tissues, such as cardiac and skeletal muscles and neurons. Normally, the sodium channels close very rapidly, but depolarization of the membrane potential causes the opening (activation) of potassium channels and potassium efflux, which results in repolarization of the membrane potential. Binding of aconitine increases the permeability of the membrane for sodium ions, resulting in a huge sodium influx to the cell. As a result, the membrane rapidly depolarizes. Due to the strong depolarization, the permeability of the membrane to potassium ions increases very quickly, resulting in a potassium reflux that releases the positive charge from the cell. These events result in the transmission of action potentials being suppressed, leading to non-excitable target cells or paralysis.

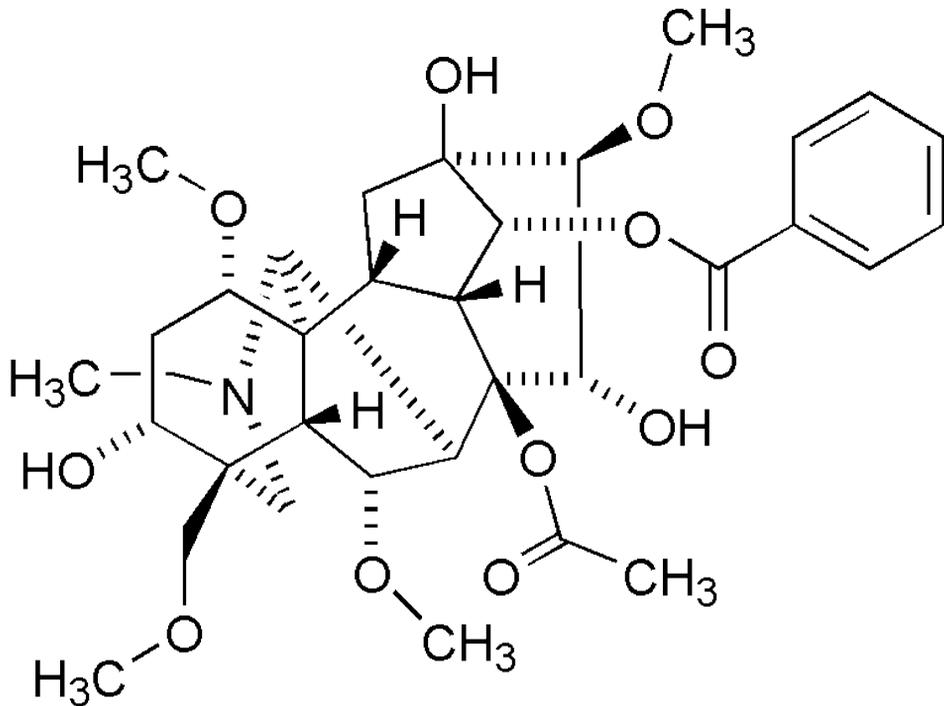


Figure 17. Aconitine chemical structure

Depending on the route of exposure, observable toxic effects include localised numbness, diarrhoea, convulsions, arrhythmia and death. After administration, aconitine initially stimulates all vegetative parasympathetic centres and subcortical nuclei, causing cardiac failure. This leads to severe vomiting, colicky diarrhoea, intense pain and then paralysis of the skeletal muscles. Cardiac failure is a cascade process beginning with cardiac arrest (irritation of the vagus nerve), followed by sinus node stimulation and accelerated cardiac function. Next comes increased sensitivity of autonomic heart ligaments, leading to hypersensitivity, dissociation of heart movements, cardiac arrhythmias, ventricular pacemakers, atrial and cardiac tremor, and cardiac arrest in diastole. Death is caused by paralysis of the centres of the extended core. Respiratory failure and cardiac arrest follow, all with full patient awareness.

The medical response should be first based on treatment with stimulatory substances – caffeine, adrenaline, amphetamine; antiarrhythmic drug should then be given (good results have been reported for lidocaine). Considering the fact that aconitine acts as an agonist of the sodium channel receptor, antiarrhythmic agents which block the sodium channel (Vaughan-Williams' Classification I) should be chosen for the acute therapy of aconitine induced arrhythmia.

4.3. Fungal toxins

Toxin-forming fungi produce mycotoxins present in feed, cereals and food products. The toxins have a wide spectrum of action and are harmful to humans, animals, plants and microorganisms. They impair various metabolic processes as well as DNA replication and transcription. This type of toxin is very easy to produce, even in homemade labs, which increases the potential for their use in bioterrorist attacks.

4.3.1. T-2 toxin

T-2 toxin is a mycotoxin synthesised by different species of *Fusarium* fungi. Concentrations of the toxin that are potentially dangerous to health occur in mouldy cereal grains and other inappropriately-stored agricultural products. Of all studied grains, the ones most prone to being infected include oat, corn, wheat and rye. The toxin can be found all over the world, particularly in the tropical regions, as heat and high humidity foster development of the *Fusarium* fungi.



Figure 18. *Fusarium* fungi (figure used with permission under Creative Commons license)

From the chemical point of view the toxin is a low-molecular organic compound with a mass of 466 Da. The compound has a tetracyclic sesquiterpene 12, 13-epoxytrichotocenic cyclic arrangement and is part of the Trichothecenes group. This group includes various toxins such as neosolaniol, saratoxin, diacetoxyscirpenol, crocetin and HT-2. However, T-2 possesses the highest potential.

The T-2 toxin is very stable and resistant to high temperatures (it is not damaged during regular food preparation), and UV radiation. Its toxic action can be neutralised by heating at 200°C–210°C for 30 to 40 minutes, or by adding sodium hypochlorite. T-2 is non-soluble in water but is soluble in chloroform, acetone, ethanol and methanol. T-2 mycotoxin can penetrate into the body with food, or as a smoke or aerosol sprayed by different dispersive systems.

The action mechanism of the compound works thanks to the presence of an epoxy ring in its structure, which is capable of intracellular reactions with nucleophilic units. It reacts with DNA and RNA molecules by inhibiting their synthesis in the cell, and with membrane phospholipids by damaging cellular structures. Additionally, the interaction of T-2 with ribosomes and enzymes participating in translation has also been demonstrated. This toxin prevents formation of peptide bonds in the centre of peptidyl transferase located in the ribosome 60S subunit, which inhibits synthesis of eukaryotic proteins.

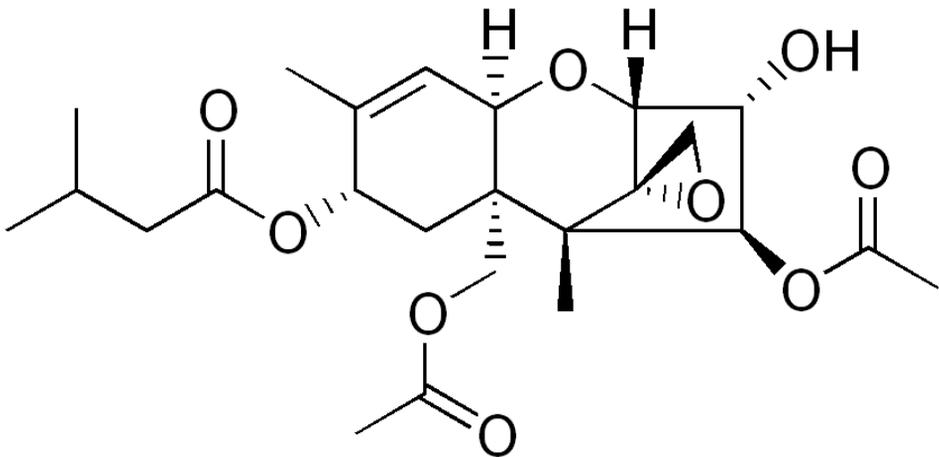


Figure 19. T-2 toxin chemical structure

The toxic action of T-2 causes apoptosis and necrosis of cells, immunosuppression and severe damage to skin tissue and other organs. Immunosuppression is an effect of high doses that damage the bone marrow, pancreas, thymus and lymph nodes, resulting in impeded functions of the immune system. Contrary to most biological toxins that do not affect the skin, T-2 is a strong skin irritant. Symptoms occur within

several minutes of exposure. Skin damage caused by the toxin is 400 times more severe than after using sulphur yperite, and include sintradermal haemorrhage and necrosis. This effect is observed even in nanograms of toxin.

Ingestion of the toxin results in abdominal pain after 15 minutes, up to one hour following ingestion. Moreover, irritation of the throat and severe diarrhoea occur.

Scientists have suggested 4 clinical stages for a bioterrorist attack using T-2 mycotoxin. The first stage would include inflammation of the alimentary tract mucous membrane, and progressive dehydration. After about 10 days, anaemia, thrombocytopenia and leukopenia would develop in the next stage. Petechiae and haemorrhages, followed by necrotic lesions in the alimentary tract or larynx leading to sepsis and death would occur in the third stage. The fourth stage would be the recovery of survivors.

At present there are no drugs counteracting poisoning with T-2 mycotoxin. Treatment involves maintaining the functions of the circulatory, respiratory and alimentary tract. These actions include administering oxygen and maintaining hydration. Polyethylene glycol is effective in removing the toxin from the skin's surface. Experiments carried out on mice revealed that T-2 toxin is strongly adsorbed by active carbon, which suggests that the same results can be obtained for humans.

T-2 toxin, considering the lack of accurate data on its action mechanisms in the human body, could become a perfect B weapon. Moreover, studies of T-2 have not gone beyond the laboratory level and information on its lethal doses or effects are estimates and based on animal reactions. Another advantage of the toxin in biological warfare is the lack of drugs counteracting it, which greatly delays or prevents recovery. Additionally, this toxin was used in biological warfare in Iraq (by Saddam Husain), as well as by Soviet forces in Kampuchea and Afghanistan (1979–81). There were also reports of "yellow rain" in Laos during the Vietnam war.

4.3.2. Aflatoxin

Aflatoxin produced by *Aspergillus flavus* and *Aspergillus parasiticus* is one of the most common fungal toxins. There are 6 identified metabolites: B1, B2, G1, G2, M1 and M2. The LD₅₀ for the toxin following oral administration is 0.003–18 mg/kg body weight. They all contain a lactone ring which is bound with a benzene ring and two furan rings. The most potent toxin of this group is aflatoxin B1.

B1 aflatoxin is oxidised with P450, taking an epoxy form in the liver. A covalent bond is then formed with guanine N-7 nitrogen. A double bond in the extreme furan ring enables tight connection of aflatoxin with DNA. The toxin causes transversions of GC alkalis into TA in the p53 protein codon. It damages the mechanism responsible for DNA repair and impairs apoptosis. Aflatoxins also affect cellular respiration paths, post-translation modification of proteins and methylation of nucleic acids and proteins, which lead to the formation of

neoplasms. For this reason, the aflatoxin B1 is a genotoxic hepatocarcinogen with its exposure strongly linked to the development of hepatocellular carcinoma. The oral LD₅₀ range of aflatoxin B1 is estimated to be 0.3–17.9 mg/kg body weight for most animal species. Symptoms of acute poisoning include anorexia, malaise, low-grade fever as well acute necrosis of liver.

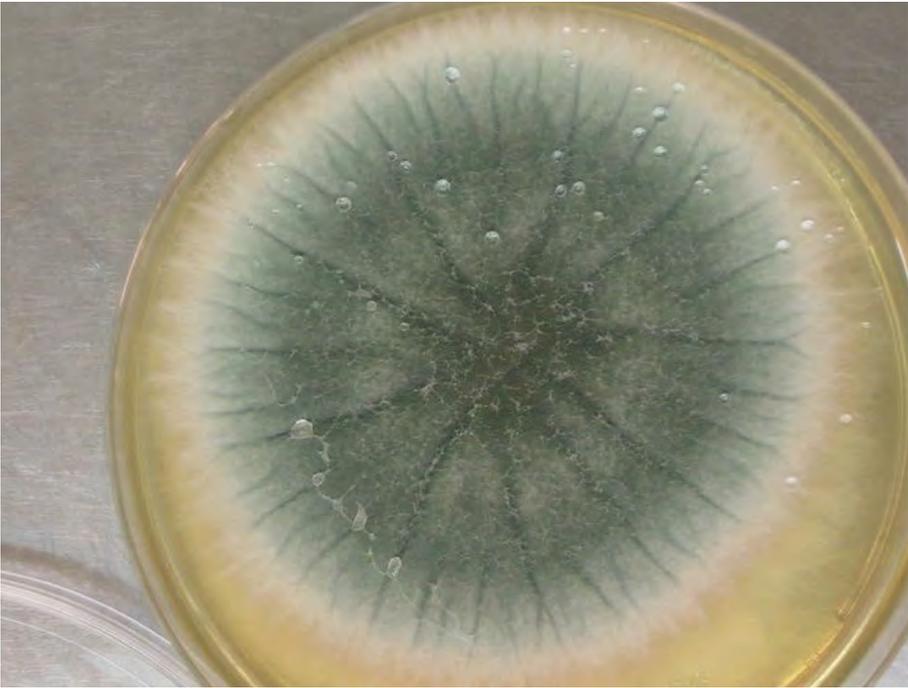


Figure 20. *Aspergillus sp* (figure used with permission under Creative Commons license)

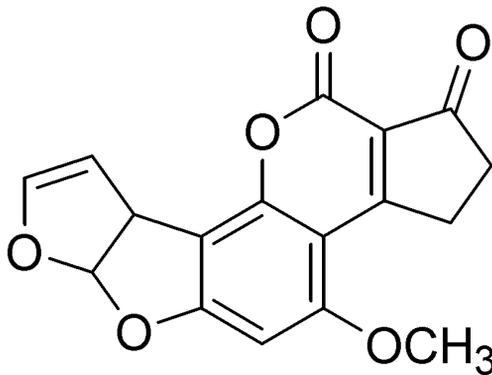


Figure 21. B1 aflatoxin chemical structure

4.3.3. Ochratoxin A

This toxin, produced by *Aspergillus ochraceus*, is a peptide of L-phenylalanine amino acid bound with iso coumaric acid *via* an amine group. Ochratoxin A contains a chlorine atom in the benzene ring. The LD₅₀ dose for ochratoxin A administered orally is 20–46 mg/kg body weight. Ochratoxin A gets into the alimentary tract. It binds with albumins of high affinity. Ochratoxin A is linked with IIA or IIIA subdomains on the human serum albumin. Organic anions transporting peptides (OAPTs) are located in the liver, while an organic anion transporter (OAT) can be found in the kidneys. These are molecular structures, whose active function is cellular capturing of ochratoxin A. Ochratoxin A is a protein synthesis inhibitor that inhibits the activity of the t-RNA synthase enzymes phenylalanine and phenylalanine hydroxylase. It reduces ATP production in the cell by impeding the activity of phosphoenolpyruvate kinase. Moreover, the toxin can bind with proteins in the mitochondria, inhibiting transport of electrons and blocking phosphorane transport. Similarly to aflatoxin, it has genotoxic properties. The toxin metabolites can bind with DNA causing mutations, and consequently neoplasms. During metabolic transformations of the toxin, overproduction of free radicals is observed, which causes oxidative damage to DNA, induces apoptosis, impairs mitosis and contributes to the instability of chromosomes. It also has a negative impact on the cellular cycle.

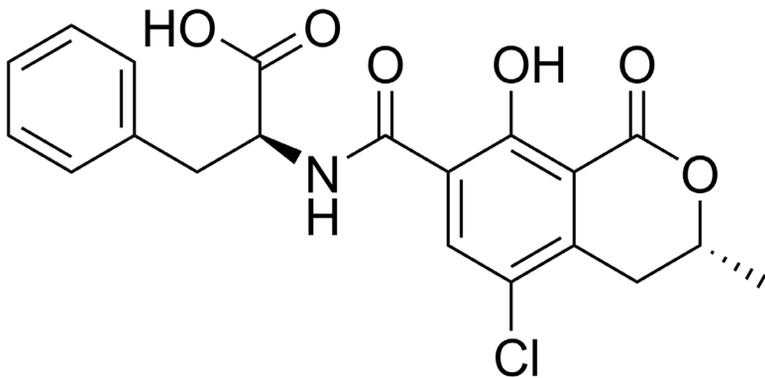


Figure 22. Ochratoxin A chemical structure

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5. RADIOBIOLOGY

Radiobiology is the science of the effects of ionizing radiation (IR) on biological tissue and living organisms. It is a combination of two scientific branches: radiation physics and biology. Ionizing radiation is defined as any type of electromagnetic or particulate radiation with sufficient energy to ionize atoms or molecules. That is, to eject one or more electrons from their outer orbitals. X-rays or γ -rays are two forms of electromagnetic radiation, while particulate radiations are α , β particles (electrons), protons, neutrons or heavy charged ions.

All living organisms consist of water and other inorganic and organic compounds. Water is the primary component of cells and accounts for about 2/3 of body mass. It is the environment of the majority of chemical reactions taking place in the body. Most of the water is in the lymph (95%), blood plasma (90%) and nervous tissue (approximately 90%), the least in the tooth enamel (0.2%). Non-organic compounds (minerals) are most commonly found in the form of water-soluble cations (e. g. K^+ , Na^+ , Cu^+ , Cu^{2+} , Ca^{2+} , Fe^{2+} , Fe^{3+} , Mg^{2+}) and anions (Cl^- , NO_2^- , NO_3^- , SO_4^{2-} , CO_3^{2-} , PO_4^{3-}). Some of these are important elements in building bones and teeth (Ca, Mg, P). Carbon, oxygen, hydrogen and nitrogen comprise the main components of these biomolecules. Organic compounds are present in the form of small molecules (glucose, nucleotides, amino acids, fatty acids), and high-molecular weight macromolecules (nucleic acids, proteins, carbohydrates and lipids).

The cell is the smallest structural and functional unit of an organism capable of carrying out all basic life processes. The three main constituents of a cell are the nucleus, the cytoplasm with its organelles (i. a. mitochondria, endoplasmic reticulum) and the cell membrane (the cytoplasmic or plasma membrane), which is selectively permeable to ions and organic molecules. The nucleus contains the genetic information (DNA), the cytoplasm supports all cellular metabolic functions, and the plasma membrane is responsible for the movement of substances in and out of the cell; it is also involved in many cellular processes, such as cellular adhesion and cell signalling.

Cells use a variety of clearly defined signalling pathways to fulfil and regulate their activity. They respond to all physical and chemical changes in their environment. Intracellular signalling pathways are responsible for transmitting information within the cell. Signal transduction pathways control all vital cellular processes, such as cell growth, differentiation, motility and

metabolism, as well as cell survival and death. Cellular responses are triggered by the recognition of extracellular signals at the cell's surface (usually in the forms of chemical signals, such as hormones and growth factors), which result in the activation of complex cytoplasmic and nuclear biochemical pathways. Many cellular-specific proteins involved in these pathways undergo activation and/or inactivation processes caused by changes in protein phosphorylation and enzymatic activity, localization, or the formation of protein-protein complexes. Signals are received by receptors, specific proteins that function as molecular antennas (sensors) located in the plasma membrane. A receptor is the integral membrane protein capable of binding its specific signal molecules – so-called ligands – with a high affinity. Ligands bind to a specific part of the receptor in a similar manner as substrate binds to an enzyme. Dysregulation of normal signalling pathways can contribute to malignant transformations in human cells, and even cellular death.

5.1. Classification of radiation in radiobiology

When IR traverses through matter, it loses energy progressively through the various interactions along the length of its path. For a particular absorber, the rate of the energy loss depends on the energy, the type of radiation and the density of the material.

The density of energy deposition in tissue is called the Linear Energy Transfer (LET) of radiation. LET is a measure of energy deposition per unit length of the 'track' that the radiation creates as it proceeds through tissue ($-dE/dx$). The unit of measurement of LET is keV/ μm . LET basically indicates the quality of different types of radiation and is important because the biological effect of radiation depends on its average LET. Charged particles generally have higher LET than electromagnetic radiation, due to the greater energy deposition along their track. Therefore, ionizing radiations are categorized into low and high LET radiations. X-rays and γ -rays are considered low LET radiations because of their sparse ionization, and are usually less damaging than high LET (densely ionizing) radiation, such as neutrons or α -particles.

The main factors determining the effects of IR on living organisms are: dose size and type of radiation, radiation conditions, and the biological properties of the irradiated system. Dose is a measure of the energy transferred by the radiation unit to the absorbent mass (absorber). In radiobiology there are several types of dose units depending on how this energy transmission is described.

The **absorbed dose** is one of the most important dosimetric quantities in radiation protection. It is the radiation energy (Joules) absorbed by unit mass of material (tissues). The (S.I.) unit is known as the gray (Gy). Historically, the unit of measurement was Radiation Absorbed Dose [rad]; 1 Gy = 100 rad.

$$1\text{Gy} = 1\text{J/kg} = 100\text{ rad}$$

To take into account the biological effects of radiation, radiation weighting factors (W_R) have been introduced (Table 1), which reflect the severity of the biological effects of different types and energies of radiation.

Table 1. Radiation weighting factors

Type and energy of radiation	W_R
Photons (X-rays, γ -rays), electrons (>5 keV)	1
Slow neutrons (<10 keV)	5
Intermediate neutrons (0.1-2 MeV)	20
Fast neutrons (2-20 MeV)	10
Protons (>2 MeV)	5
α -particles, (5 MeV); high energy ions	20

A biologically effective dose, termed **equivalent dose** (H_T) is defined as:

$$H_T [\text{Sv}] = \sum W_R \times D$$

where D is the absorbed dose averaged over a tissue or organ due to radiation; D is measured in units of grey. The equivalent dose is measured in sieverts (Sv); 1 Sv equals 1J kg^{-1} . For X-rays and γ -rays, 1Sv equals 1Gy. The earlier unit was the **radiation equivalent man** [rem]; 1Sv = 100 rem. The equivalent dose is expressed as a sum to include the effects of IR caused by more than one radiation type. This dose is used to compare the biological effectiveness of different kinds of radiation to the tissues.

The biological effect of radiation also depends on the type of tissue (organ) which has been irradiated. To quantify the total damage from the exposure of several tissues (organs), the concept of the **effective dose** (E) was introduced. This is the sum of the weighted equivalent doses in all the tissues (organs) of the human body, and is defined as:

$$E [\text{Sv}] = \sum W_R \times D \times W_T$$

where the so-called tissue weighting factor (W_T) reflects the relative contribution of this tissue (organ) to the total damage resulting from uniform irradiation of the whole body. The unit of measurement of the effective dose is Sv. Effective dose is used to estimate the risk of IR to humans.

There is also the concept of the **collective dose**, generally used for protection purposes and in calculations for population response, which is defined as the dose received per person (in sieverts) multiplied by the number of individuals exposed per year.

Table 2. Radiation weighting factors

Tissue (organ)	W_T
gonads (ovary, testis)	0.20
bone marrow, colon, lung, stomach	0.12
bladder, breast, liver, thyroid, oesophagus	0.05
skin, bone	0.01
all others	0.05
Whole body (total)	1.00

Radiation conditions differ, but mainly include dose rate, the mass of the irradiated tissues, irradiation of critical organs, and tissue oxygenation. The **dose rate** is the ratio of the absorbed dose to the time it was given (e.g. Gy/h). The influence of other radiation conditions on overall biological effects is discussed in the 'Radiosensitivity' section.

5.2. Mechanisms of radiation damage – direct and indirect radiation effects

When a high-energy photon or particle (e. g. γ -rays, α -particles) hits a human cell it produces a narrow 'track' (less than 1 μm thick) as it proceeds through the material. Radiation absorbed by cells has the potential to affect a variety of critical cellular targets (nucleic acids, proteins, lipids, carbohydrates), causing ionisation and excitation. The radiation breaks one or more chemical bonds producing free radicals – atoms and molecules with unpaired electrons. Free radicals can easily react with other molecules leading to the chain of physical and chemical events that eventually produce the biological damage. The radical formation occurs in a matter of picoseconds after the passage of the photons.

The chemical and biological effects of ionizing radiation on living matter are the result of the deposition of radiation energy directly into the target macromolecule, which is a component of the cell, and of the indirect action resulting from the excitation and decay of water molecules – radiolysis of water. It is generally accepted that DNA is the critical radiation target in the cell. Thus, the interaction of radiation directly with DNA molecules is what is called a direct effect, and this is the dominant process in the interaction between high LET radiations with biological material. The interaction of radiation with water molecules, which is the major constituent of the cell, leads to production of free radicals that in turn can attack other critical molecules. This action of radiation through an attack by free radicals is known as the indirect effect. Free radicals are able to diffuse throughout the cell, so the initial ionization event does not even have to occur close to the DNA molecule in order to cause damage. It is

estimated that only approximately 1/3 of biological damage caused by low LET radiations (γ -rays) is caused by direct effects. Importantly, the indirect action of IR can be modified by radiation protectors and by chemical sensitizers. All chemicals (natural or synthetic) that are able to react with radicals and other reactive oxygen species (ROS) can inhibit the indirect action of radiation. These chemicals are called ROS scavengers.

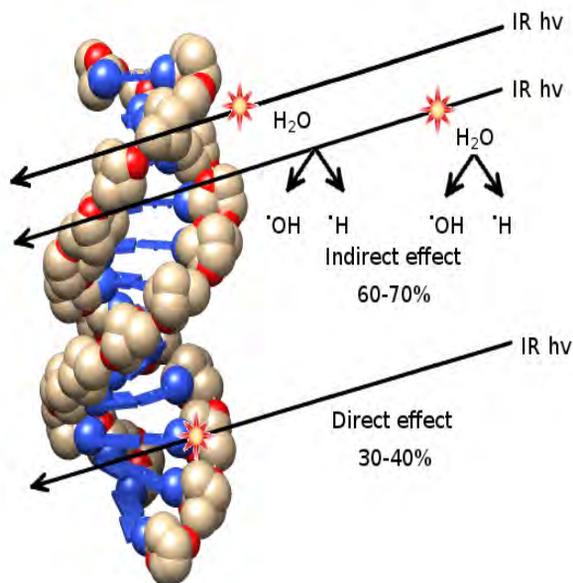


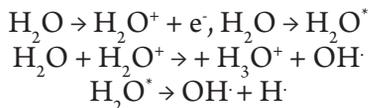
Figure 1. Direct and indirect radiation effects (Author: Michał Ponczek)

Together, the direct and indirect effects of radiation initiate a series of biochemical and molecular signalling events that can repair the damage or culminate in permanent physiological changes or cell death.

5.2.1. Radiolysis of water

Exposure of living matter (cells, tissues) to IR triggers a complex series of chemical reactions in water, which accounts for about 80% of cellular constituents. This process is called water radiolysis and leads to generation of specific radiolytic products. The radiation chemistry of water is a relatively well-known process described in several reviews. Ionization and excitation of water molecules are two basic primary processes resulting from electromagnetic interaction of IR with water. These processes produce short-lived H_2O^+ radical-cations, fast electrons and excited water molecules (H_2O^*). H_2O^+ radical cations and excited water,

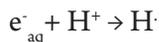
which are very unstable, decompose within 10^{-13} s to form hydroxyl radical ($\text{OH}\cdot$) and hydrogen radical ($\text{H}\cdot$):



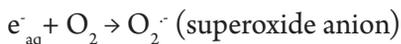
The hydroxyl radical has an unpaired electron and is a highly reactive oxidizing agent. It can diffuse some distance and react with critical target molecules, producing another radical. The ejected electrons can interact with a water molecule to produce hydroxyl ions and a hydrogen radical (hydrogen atom):



Then, the electrons undergo solvation by dielectric interactions with neighbouring water molecules to form solvated (hydrated) electrons (e^-_{aq}). A solvated electron reacts with a proton to give a hydrogen atom ($\text{H}\cdot$):



The solvated electrons and hydrogen atoms are the strongest known reducing agents at pH 7.0. In oxygenated solutions, a solvated electron can be converted to the superoxide anion ($\text{O}_2^{\cdot-}$), which in turn is a powerful oxidizing species and a precursor of hydrogen peroxide (H_2O_2):



The virtual yields of water radiolysis products depend on the LET of the radiation and the pH. The concentration of these radicals is expressed as a G value (G is defined as the number of radicals or molecules produced per 100 eV of energy absorbed in the medium).

To summarise, at physiological pH in the aerobic cellular environment, the major primary ROS includes the superoxide anion, a hydroxyl radical and hydrogen peroxide. The high energy of photons (low LET radiations) absorbed by biological matter, and the passage of fast charged particles (high LET radiations) through the matter, initiate a complex series of events that are responsible for the final radiobiological effects. The timescale of these events, divided into four, subsequent temporal phases, is as follows:

- Physical phase – (duration up to 10^{-16} s) – ionization, energy deposition, excitation and formation of very active secondary electrons;
- Physico-chemical stage – (10^{-15} - 10^{-6} s) – electrons undergo fast reorganization to form primary radicals and other molecular products of water radiolysis;

- Chemical stage (10^{-6} s) – free radicals diffuse, interact with each other and with other molecules in the cellular environment, causing changes in life-critical molecules;
- Biological stage – (from several seconds to several years) – enzymatic reactions, recognition of damage and cellular signalling dysregulations, repair of the damage. This leads to death or mutation at the cellular level, and immune responses at the system level. Hormonal changes, carcinogenesis and even death are all possible final effects of these reactions in a human body.

5.3. The molecular radiobiology

5.3.1. Radiation-induced DNA damage

Ionizing radiation passing through living tissues generates reactive free radicals and other ROS. These ROS can interact with critical macromolecules, such as DNA, proteins and cellular membranes, and can induce cell damage and, potentially, cell dysfunction and death. It is believed that DNA is the most critical radiation target in the cell. The wide range of lesions in DNA that result from ionizing radiation include: (1) chemical alteration of the nitrogen bases or sugar moieties; (2) formation of the apurinic, and to a lesser extent, apyrimidinic sites; (3) single- and double-strand breaks (SSB and DSB) of the double helix molecule, and (4) cross-linking to DNA-related matrix proteins or nucleotides in the DNA molecule itself.

Oxidative modifications to the bases caused by the attack of the hydroxyl radicals have been mostly studied *in vitro* (irradiation of the free bases, nucleotides, DNA in aqueous solutions), and this chemistry is well understood. More than two hundred IR-induced oxidative modifications of the base are known. The most common is the attack of the hydroxyl radical on the bond between the C5 and C6 in the pyrimidines and C4 and C5 in the purines (Fig. 2).

Depending on the local oxygen conditions, the resulting intermediate products may undergo further oxidation or reduction. For example, the most common product of the deoxyguanine oxidation is 8-hydroxyguanine (8-oxoguanine), which is used as one of the biomarkers of oxidative stress in an organism (Fig. 3).

If not repaired, 8-oxoguanine can pair with adenine and cause a G:C to T:A transversion. Insertion of 8-oxoguanine during DNA replication can generate double-strand breaks. Treatment of DNA with the hydroxyl radical generating system can also lead to the formation of the imidazole-opened ring derivative of guanine, such as 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FaPy) as an

abundant lesion. The damage made by IR-induced hydroxyl radicals is similar to that produced by oxidative metabolism. Moreover, the DNA repair system, called a base excision repair (BER), efficiently repairs lethal lesions, so that in the repair-capable cells, isolated base damage may be irrelevant in radiation mutagenesis.

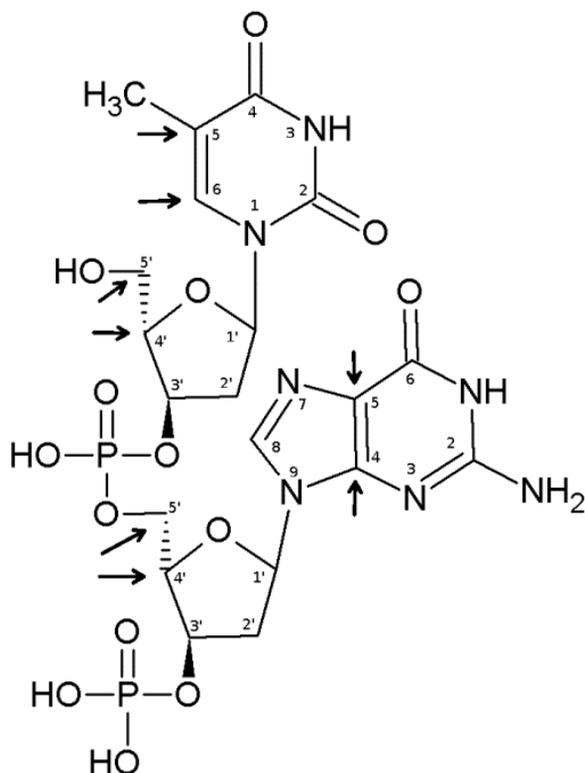


Figure 2. Positions of the hydroxyl radical attack in the DNA molecule
(Author: Michał Ponczek)

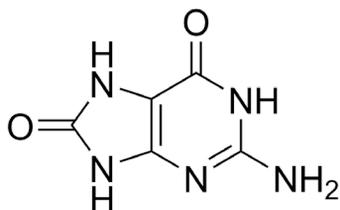


Figure 3. The product of guanine oxidation – 8-oxoguanine

The attack of the hydroxyl radical on the DNA sugar-phosphate backbone leads to the abstraction of the hydrogen atoms from the deoxyribose. As a result

of the hydrogen abstraction and subsequent reactions with oxygen molecules, several types of DNA damage can be formed: the cleavage of the phosphodiester bond and the formation of a single-strand DNA break, destabilization of the N-glycosidic bond and generation of the abasic deoxyribose residue or the opening of the deoxyribose ring, and the formation of a so-called alkali-labile site. Single-strand breaks are typically easily and rapidly repaired (DNA ligation). Double-strand breaks – that is, breaks that arise in both strands opposite to each other (or separated only a by few base pairs) – are less readily repaired and constitute much more critical IR lesions. The DSB that remains unrepaired or mis-repaired can induce chromosomal abnormalities and lead to mutations, genomic instability, and cell death. The aberrant chromosomes occur when broken ends re-join with other broken ends to form translocations, rings, dicentrics and others. Karyotyping, the micronuclei formation assay or fluorescent in-situ hybridization (FISH), can be applied to detect IR-induced unrepaired DNA damage in chromosomes. The majority of mutations do not affect cellular function as they do not alter the meaning of coded genetic information, or because they occur in differentiated cells that do not divide. Mutations in stem cells are harmful, for example haematopoietic stem cells leading to the development of blood cancers, and those that trigger divisions, such as mutations of the proto-oncogens leading to cancer.

The most important feature of radiation toxicity is that IR induces not only isolated DNA lesions, but also clusters of lesions generated within a few tens of base pairs. The clustered DNA damage sites can include DSB and tandem lesions (DSB or SSB associated with modification of the bases, or much more complex lesions, such as multiple closely scattered DSB). Cluster damage can arise from the combined indirect action of ROS, which are instantaneously produced in a very high yield during irradiation, and from direct effects induced by the track of the radiation. These processes are not yet well understood.

The broad variety of DNA lesion forms requires multiple, largely distinct DNA repair mechanisms. The majority of the DNA damage is repaired by a sequence of reactions involving specific enzymes mediated by multiple proteins. These typically include: the base excision repair (BER); nucleotide excision repair (NER); mismatch repair (MMR); repair through homological recombination (HR), and non-homologous end joining (NHEJ).

5.3.2. Radiation-induced oxidative damage to proteins and lipids

Protein oxidative modifications

Free amino acids and amino acid residues in proteins are highly susceptible to oxidation by ROS generated during exposure to IR. Exposure of proteins to these ROS can alter the physical and chemical structure of the target,

causing consequent oxidation of side-chain groups, protein scission, backbone fragmentation, cross-linking, unfolding, and/or formation of new reactive groups. The latter include oxidation of hydrophobic amino acyl residues to hydroxy and hydroperoxy derivatives, protein carbonylation, oxidation of -SH groups, dityrosine formation, and many others.

The hydroxyl radical generated during water radiolysis initiates the oxidation of the polypeptide backbone by abstraction of the hydrogen atom at the amino acid α -carbon. This results in generation of a carbon-centred radical (alkyl radical; R^*) that rapidly reacts with the oxygen molecule to form the peroxy radical (alkylperoxy radical) intermediate (ROO^*), which can give rise to the (hydro)peroxide (alkylhydroperoxide; $ROOH$), followed by the formation of an alkoxy radical (RO^*). The resulting alkoxy radical can be transformed into a hydroxylated (at the α -carbon) amino acid residue, or it can lead to the fragmentation of the polypeptide chain (Fig. 4, panel A). The alkyl, peroxy and alkoxy radicals can react with other amino acid residues of the same – or another – polypeptide chain of the protein, resulting in the formation of new, carbon-centred radicals (Fig. 4, panel Ba). In the absence of oxygen, when the formation of peroxy radicals is hampered, the alkyl radicals can react with each other, within the same or different proteins, and this leads to cross-linkages between the polypeptide chains (Fig. 4, panel Bb). The alkoxy radicals can also promote the reactions that lead to the breakdown of the polypeptide chain. Depending on the location of the cleavage at the α -carbon, two different types of breakdown products will be formed (Fig. 4, panel C) with one being the α -ketoacyl derivative ($O=C=R$) (Fig. 4, panel C, a – the α -amidation pathway).

The fragmentation of the polypeptide chain can also occur following an attack of ROS on the residues of glutamic acid, aspartic acid and proline, followed by reactions analogous to those described earlier (Fig. 4, panel A). This eventually leads to formation of a peptide fragment with the N-terminal amino acid existing as an N-pyruvyl derivative, with oxalic acid and hydrogen peroxide as the side reaction products. Abstraction of the hydrogen atom from the γ -carbon atom of glutamic acid by the hydroxyl radical leading to chain fragmentation is shown in Fig. 4 (panel D).

As a result of the oxidation of proline residues, 2-pyrrolidone is formed, concomitant with the breakdown of the polypeptide chain (Fig. 5, panel A). In turn, the hydrolysis of 2-pyrrolidone in an acidic medium leads to the formation of 4-aminobutyric acid, whose presence in the hydrolysis products of proteins treated with oxidizing agents indicates the occurrence of the mechanism of protein fragmentation by proline oxidation.

All amino acid residues found in proteins are susceptible to oxidation. The most sensitive to IR-produced ROS are cysteine, methionine, tyrosine, and tryptophan. Cysteine and methionine residues in the polypeptide side-chain can be oxidized to disulphides and methionine sulfoxide residues, respectively (Fig. 5,

panel B). The majority of biological systems can repair these types of oxidative modifications because they possess enzymes such as disulphide reductases and methionine sulfoxide reductases that are able to convert the oxidized forms back to their unmodified forms.

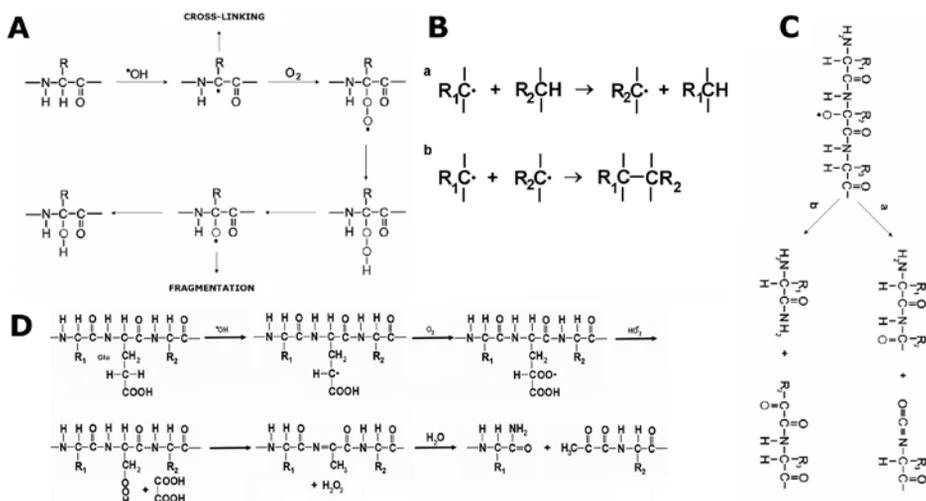


Figure 4. Oxidation of the polypeptide backbone by IR-generated hydroxyl radical: formation of the carbon-centred and peroxy radicals, hydroperoxide, alkoxy radical and a hydroxylated amino acid derivative (panel A); side reactions of radicals with other amino acid residues to form a new carbon-centred radical or formation of the protein-protein cross-linked derivative (panel B, a and b, respectively); peptide bond cleavage (panel C). Polypeptide fragmentation as a result of oxidation of the glutamyl residue (panel D) (Author: Michał Ponczek)

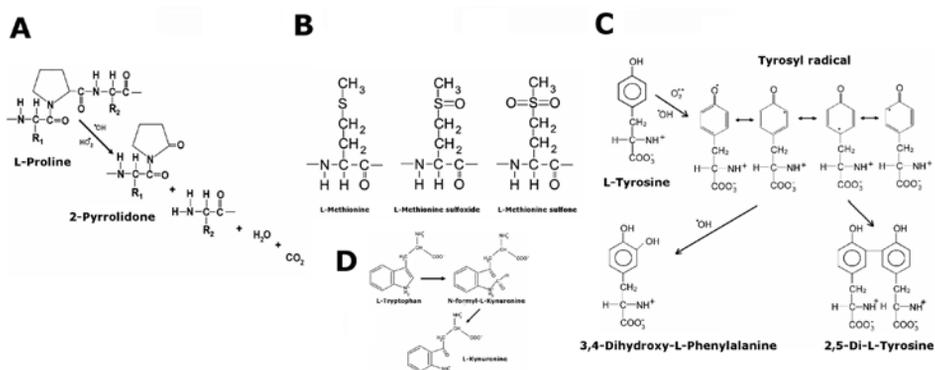


Figure 5. Oxidation of amino acid side chains: oxidation of the proline residue leading to peptide bond cleavage (panel A); products of the methionine residue oxidation (panel B); oxidation of the tyrosine residues that results in DOPA formation or cross-linking (panel C); products of the tryptophan residue oxidation (panel D) (Author: Michał Ponczek)

Oxidation of the tyrosine residues results in the generation of tyrosyl radicals, which can lead to the formation of 3,4-dihydroxyphenylalanine (DOPA) by incorporating an additional hydroxyl group into the aromatic ring. In contrast, the formation of 2,5-dithirosine leads to the cross-linking between the aromatic rings of the two molecules of the tyrosine (Fig. 5, panel C). Tryptophan residues can be oxidized to formylkynurenine and kynurenine (Fig. 5, panel D) while histidine to 2-oxohistidine, asparagine and aspartic acid.

Oxidation of amino acid residues with free amine, amide or hydroxyl groups (especially arginine, lysine, threonine) leads to the formation of carbonyl derivatives. This also applies to proline, whose ring undergoes oxidation during fragmentation. Such amino acid derivatives can react with other free amino groups of lysine residues in the same or another protein molecule, to form cross-linking. This is another mechanism of cross-linking in polypeptide chains, in addition to the reactions of carbon-centred radicals and the formation of dityrosine. Carbonyl derivatives are also produced in the reactions of amino acid residues with lipid peroxidation products and reducing sugars. The presence of carbonyl groups in proteins have been used as a stable marker of ROS-mediated protein oxidation.

The conformational changes that result from this complex of reactions lead to the decrease or loss of protein biological function.

Lipid peroxidation

The hydroxyl radical and other highly oxidizing agents produced during water radiolysis may interact with unsaturated fatty acids in biological membranes, resulting in their peroxidation. This process is initiated by the abstraction of hydrogen from the unsaturated fatty acid leading to formation a carbon-centred lipid radical (L^*), which is stabilized by a molecular rearrangement of the double bonds to produce a conjugated diene which then combines with oxygen to form a peroxy radical (LOO^*) (Fig. 6).

The peroxy radical is itself capable of abstracting a hydrogen atom from another polyunsaturated fatty acid, thus starting a chain reaction. As a result, fatty acid peroxide (lipid peroxide; $LOOH$) and another alkyl radical (L^*) are formed, which can contribute to the next peroxidation reaction. These series of reactions are termed the propagation phase, which imply that one initiating 'hit' can result in the alteration of many unsaturated fatty acids to lipid peroxides. For metals such as Fe^{+2} , in the presence of the transition, the re-initiation process can occur, in which the $ROOH$ formed can undergo a reductive cleavage producing alkoxy radical.

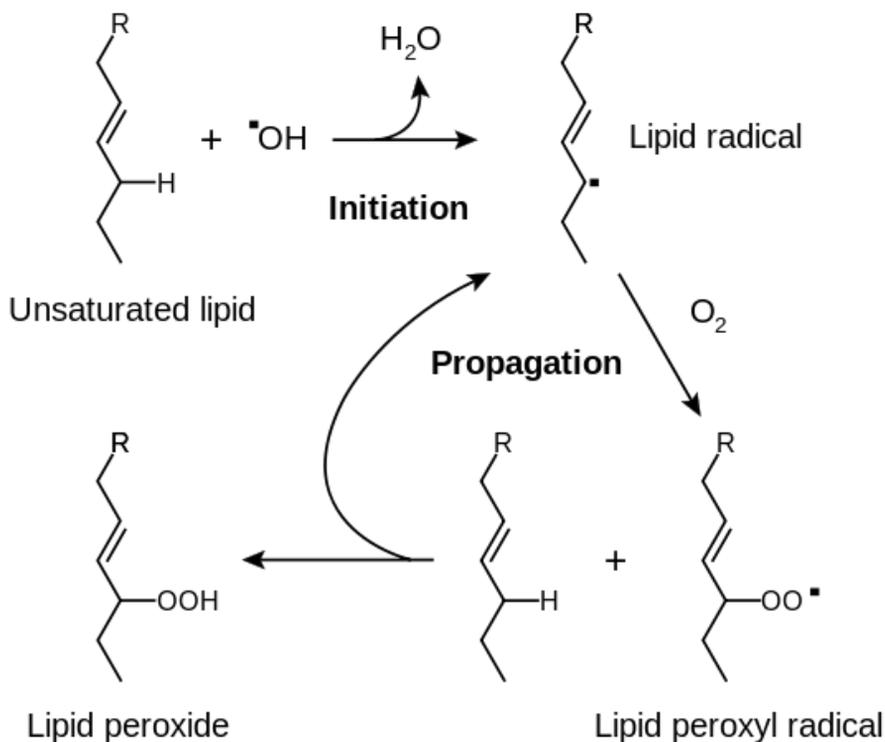
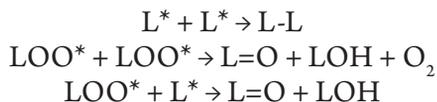


Figure 6. The initiation and propagation phase of lipid peroxidation

During the termination phase of lipid peroxidation, the reactions between radicals that lead to non-radical products dominate. Termination reactions (recombination of free radicals) can include the reaction between two alkyl radicals or two peroxy radicals or two different radicals, including:



The products of termination reactions are fatty acid dimers (in membranes – phospholipid dimers), as well as oxo and hydroxy fatty acids (these are the modified, damaged lipid molecules), which eventually undergo a breakdown (including through a β -elimination reaction) to produce many products. The most common is malondialdehyde (MDA) which is a major bioactive marker of lipid peroxidation. In addition, other aldehydes and hydroxyaldehydes are

generated, such as 4-hydroxynonenal (4-HNE), 2-propenal (acrolein), hepta-2,4-dienal, hydroxyoctanal, hydrocarbons (ethane, pentane), and many others. Increased plasma MDA has been found in persons occupationally exposed to low radiation doses. The aldehydes, MDA and 4-HNE, can cause DNA strand breaks, and are cytotoxic, mutagenic and carcinogenic agents.

5.4. Cellular responses to ionizing radiation

The molecular mechanisms of IR-induced cellular injury depend on many factors that primarily include radiation dosage as well as the cell type, cycle phase, and its transformed status. Taking into account the chemical processes occurring during irradiation, the extent of the cellular damage that results from the specific type of IR is similar in terms of the dose and the amount of DNA. However, the final IR effect is determined by the post-radiation processes such as DNA damage repair and the proliferative activity of the cell. There are somatic cells and germ cells in the human body. Cells can propagate through division; somatic cells undergo mitosis, whereas germ cells divide through meiosis. Somatic cells are usually classified into the following three groups:

1. Stem cells – self-renewing cells that exist to produce cells for a differentiated cell population (e.g. stem cells of the haematopoietic system, epidermis and mucosal lining of the intestine),
2. Transit cells – cells in movement to another population (e.g. a reticulocyte that is an immature cell, differentiating to become an erythrocyte)
3. Mature cells – entirely differentiated, and do not undergo mitosis (e.g. neural or muscle cells).

The cellular death of non-proliferating (static) mature cells can be defined as the loss of a specific function, while for stem cells and other cells capable of many divisions it is defined as the loss of reproductive integrity (reproductive death). A surviving cell that maintains its reproductive integrity and is able to proliferate is supposed to be clonogenic. Accordingly, in radiation biology, IR-induced cell death has been functionally classified into interphase or reproductive (mitotic) death. The former, observed early post-irradiation, is the death of irradiated cells before they enter mitosis. The latter, which is observed after several cycles of cell division, is the loss of the proliferative ability of the cell.

5.4.1. Radiation-induced cell cycle arrest

The mammalian cell cycle is divided into two major phases: the interphase and the mitotic phase (M) (Fig. 7).

During the M phase the cell divides its copied DNA and the cytoplasm to make two new cells. The M phase comprises two distinct division-related processes, mitosis and cytokinesis (C). The G_1 is the first gap in cellular activity

between mitosis and the S phase, in which DNA synthesis takes place, and G_2 the second gap in activity between the S phase and the next mitosis.

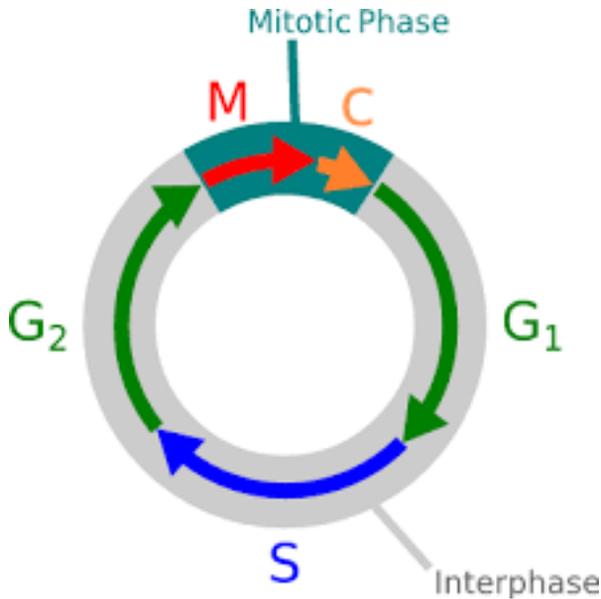


Figure. 7. The overview of the cell cycle (figure used with permission under Creative Commons license)

There are types of cells (e.g. early embryo, stem cells, cancer cells) that divide rapidly, and their daughter cells can undergo another round of cell division without delay. Other types of cells divide slowly, or do not divide; when cells stop progressing through the cycle, they can enter the resting state (G_0 phase). This can be a permanent state for some cells, while others can re-start division in response to the right signal. Big variations in radiosensitivity in different phases have been shown. Although the mechanisms responsible for this phenomenon are not fully understood, it could be related to some of the changes in chromatin organization, and with the different amounts of cell cycle phase-dependent thiols in the cell. Commonly, cells are the most radiosensitive in the M and late G_2 phases, and the most resistant in the late S phase. At the beginning of the G_1 phase, cells are relatively resistant to IR, followed by an increase in their sensitivity.

IR damages cells by altering the protein expression that affects the signalling pathways involved in damage/repair mechanisms. Several defence mechanisms exist to restore DNA integrity (Fig. 8). Damage of the cellular DNA (SSB, DSB) activates the expression of two specific kinases – ATM (ataxia-telangiectasia mutated) kinase, and ATR (ataxia-telangiectasia and Rad3 related) kinase. These are involved in the DNA damage response (DDR) pathway, which in turn

induces cell cycle arrest and activates a range of downstream targets involved in DNA repair. DDR is a network of cellular pathways that sense, signal and repair DNA lesions. The ATM gene is mutated in the autosomal recessive disease ataxia telangiectasia (AT), which is characterized by a pleiotropic phenotype including neuronal degeneration, oculocutaneous telangiectasias, immune dysfunction, and cancer predisposition.

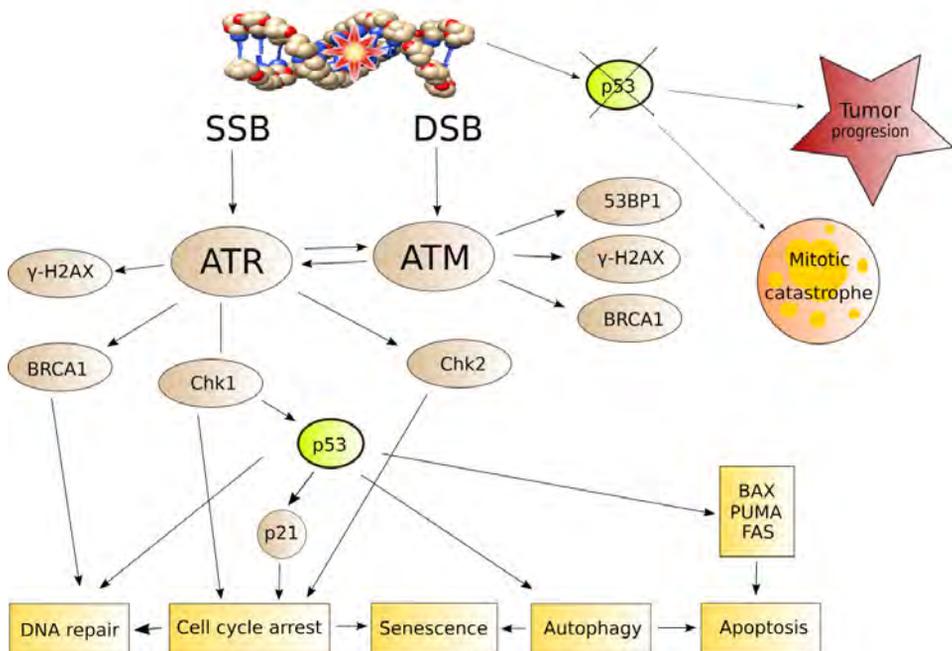


Figure 8. The DNA damage response (DDR) pathway and cellular responses to ionizing radiation (Author: Michal Ponczek)

The ability or inability of the repair mechanisms to fix the IR-induced DNA damage decides the fate of the cell – survival or death. One of the primary substrates of the ATR and ATM kinases is histon H2AX; its phosphorylation (to form γ H2AX) induces the recruitment and attachment of the subsequent proteins in DDR signalling. The most well-studied ATM/ATR targets are the protein kinases CHK1 and CHK2, which together with ATM and ATR act to reduce the activity of the cyclin-dependent kinases (CDKs) through various mechanisms. Some of these mechanisms are mediated by activation of the p53 transcription factor. Inhibition of the CDKs leads to deceleration or arrest of the cell-cycle progression. Therefore, DNA damage can lead to arrest at the cell

cycle checkpoints that exist at the boundaries between adjacent phases G1/S and G2/M. There is also an intra-S checkpoint. Cell cycle arrest is believed to increase the time available for DNA repair before the subsequent replication or mitosis. In parallel, ATM/ATR signalling enhances the repair mechanisms by inducing DNA-repair-proteins, transcriptionally or post-transcriptionally, by recruiting repair factors to the damage and by activating DNA repair proteins. The main factor involved in modulating the IR-induced cellular response is a p53 protein, which is a transcription factor and tumour suppressor gene. P53 is often denoted as the 'guardian of the genome' as it controls many target genes that can induce either arrest of the cell cycle and DNA repair, or trigger apoptosis.

To summarize, irradiation of a cell can result in one of a variety of possible outcomes (Fig. 8), such as: no effect; cell cycle arrest; DNA repair; cell death; genomic instability; mutation (wherein a cell survives but the DNA was not repaired, or was improperly repaired); transformation and tumour progression (wherein a cell survives but the mutation leads to a transformed phenotype and possibly carcinogenesis), and bystander effects (in which the irradiated cell sends signals to neighbouring un-irradiated cells and induces genetic damage in these cells). DNA damage that is hard to repair, and/or more severe damage, will induce cell death, either in the form of apoptosis, which is a p53-dependent pathway, or p53-independent mitotic catastrophe.

5.4.2. Mechanisms of cellular death after irradiation

Cells that fail to repair DNA damage caused by IR are killed. The detailed mechanisms of cellular death are still not fully understood. Cellular death, however can be caused through different molecular pathways, each representing a different mode. Several types of cellular death have been demonstrated to occur *in vivo* and *in vitro*, in response to IR. Depending on the radiation dose, type and radiosensitivity of the exposed cell, IR can induce apoptosis, necrosis, senescence, autophagy or mitotic catastrophe. Apoptosis is a major control mechanism by which cells undergo self-destruction. It is a programmed cell death, an evolutionarily maintained and highly regulated process that is required to remove damaged, infected, extraneous or transformed cells from normal tissues. The morphological signs of apoptosis include cell shrinkage, membrane blebbing, condensation of chromatin, DNA fragmentation and eventually disintegration of the cell into the membrane-bound microvesicles, or so-called apoptotic bodies (Fig. 9).

These apoptotic bodies are removed by a phagocytic system *in vivo*, which prevents the inflammation associated with cellular death. Apoptosis remains the main IR-induced death mechanism in cells from the lymphoid and myeloid lineages, while significantly less apoptosis is seen in cells of the epithelial origin. Execution of apoptosis is intimately associated with the activation of caspases, a family of cysteine-aspartic proteases. Caspases are present in cells as non-active

zymogens and become activated when the cell comes across external or internal stimuli. Caspases are divided into the initiator and effector (executioner) caspases. IR-induced apoptosis can activate a cascade of caspases *via* the extrinsic and/or intrinsic pathway (Fig. 10). Overall, these apoptotic pathways converge to activate the effector caspase-3, caspase-6 and caspase 7. Caspases cleave the vital cellular proteins and dismantle the cell.

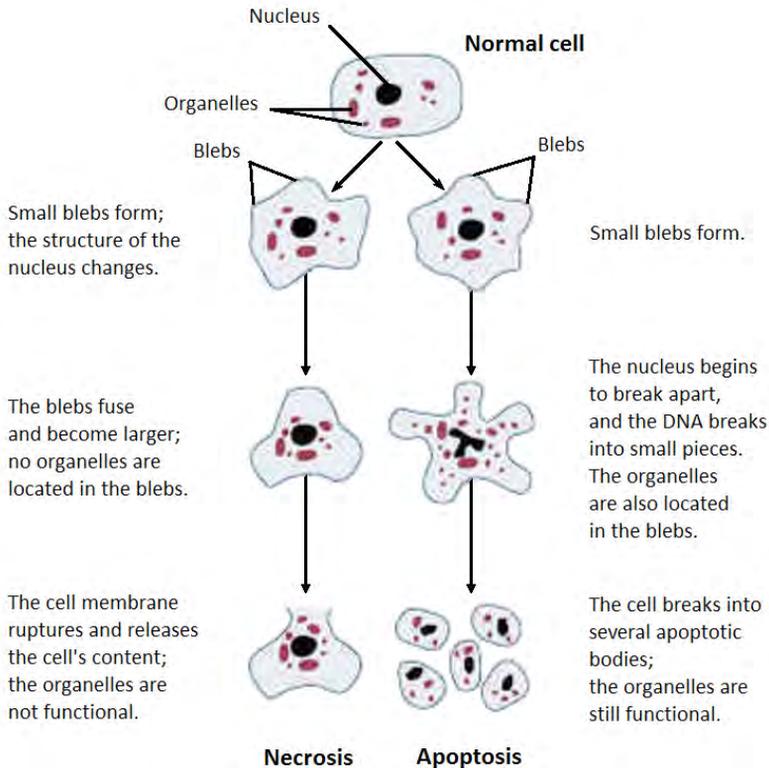


Figure 9. Major differences between apoptosis and necrosis (*figure used with permission under Creative Commons license*)

The intrinsic pathway, also referred to as the mitochondrial pathway, can be triggered by a signal from the inside of the cell, such as cytotoxic DNA damage or elevated intracellular ROS.

The mitochondrial pathway is firmly controlled by the opposite actions of the members of the Bcl-2 family. These proteins are divided into functionally distinct groups: inhibitors of apoptosis (including Bcl-2, Bcl-XL), which inhibit their pro-apoptotic counterparts that promote apoptosis. The pro-apoptotic

proteins include BAX, BAK, BID, PUMA and many others. Upon detection of cytotoxic internal stimuli, two pro-apoptotic proteins, BAX and BAK, undergo structural changes that lead to their activation. Both BAX and BAK migrate to the mitochondrion, where they undergo homodimerization and introduce pores in the mitochondrial outer membrane. This results in Mitochondrial Outer Membrane Permeabilization (MOMP), and disrupts the mitochondrial function. The release of cytochrome c, which interacts with Apoptotic Protease Activating Factor 1 (APAF-1) and procaspase-9, leads to apoptosome formation – a complex structure in which procaspase-9 dimerizes and undergoes auto-activation. The principal function of the apoptosome is to trigger a cascade of caspase activation beginning with the effector caspases (3 and 7) being responsible for cell death.

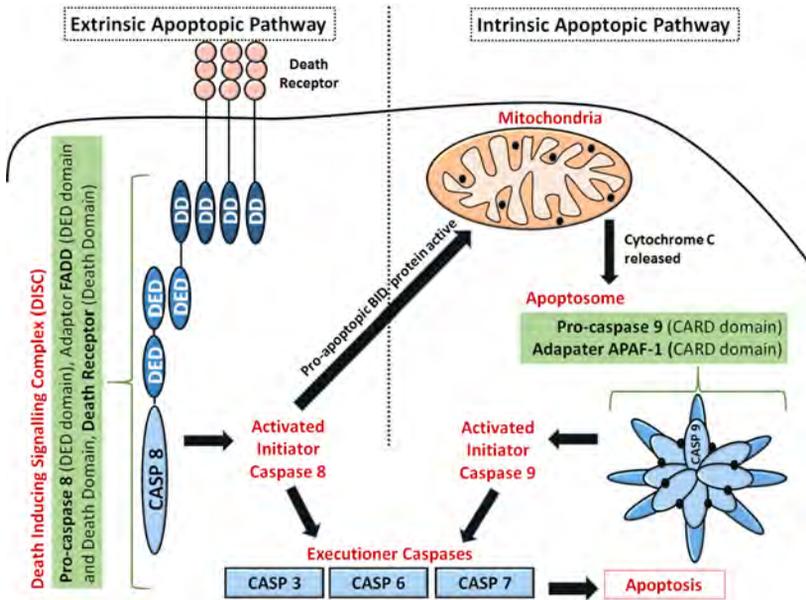


Figure 10. The extrinsic and intrinsic apoptotic signalling pathways (figure used with permission under Creative Commons license)

The extrinsic apoptotic pathway is also involved in IR-induced apoptosis. This pathway is known as the receptor-mediated pathway because it requires a ligand-dependent activation of the specific transmembrane proteins, the so-called death receptors. Death receptors comprise a subset of the tumour necrosis factor (TNF) receptor superfamily (which includes Fas and TNF α), characterized by distinct protein motifs – namely Death Domains (DD) and Death Effector Domains (DED). The death receptor (for example, Fas) is stimulated by the Fas ligand (FasL) which attracts the DD-containing molecule, the adapter protein – Fas-Associated protein with Death Domain (FADD). Recruitment of FADD attracts other DD/DED-

containing proteins, such as pro-caspase-8 (and -10), to promote the formation of the so-called Death Inducing Signalling Complex (DISC) in the cytoplasmic compartment. DISC formation is necessary for the cleavage and activation of procaspase 8 to form caspase 8, which goes on to cleave and activate other caspases such as procaspase 3 initiating the caspase cascade, which leads to cellular death.

IR-induced necrosis is regarded as a passive, pathological process, characterized by cellular swelling, rupture of the plasma membrane and uncontrolled release of cytoplasmic content into the intercellular space. It is associated with increased inflammation of the surrounding tissues (Fig. 9). In necrotic cells, the level of ATP dramatically decreases as a result of depolarization of the mitochondrial membrane, followed by impairment of the electron transport. Not only mitochondria, but also other cytoplasmic organelles undergo destruction (nucleus, lysosomes, endoplasmic reticulum, ER). The influx of calcium ions to the cytosol from the ER is a typical sign of necrosis. Elevation of the calcium concentration activates nucleases that cleave the DNA. Necrosis in general results from more severe stress compared to apoptosis and senescence. For example, high IR exposure (≥ 30 –50 Gy), was shown to induce necrosis in neurons. Although necrosis has long been considered an accidental cell death, recent studies suggest that there are several genetically regulated forms of necrosis, including necroptosis.

Mitotic catastrophe is also one of the major forms of IR-induced cell death, which denotes a mechanism of delayed mitotic-linked cellular death. Mitotic catastrophe (also called mitotic death), involves a sequence of events that are caused by premature or inappropriate entry of cells into mitosis and aberrant chromosome segregation due to severe DNA damage. Mitotic catastrophe leads to the formation of giant cells with aberrant nuclear morphology or multiple nuclei, and/or several micronuclei. It is noteworthy that this type of death is believed to be the p53-independent pathway, and is executed by rapidly dividing cells (stem cells, epithelial cells), and by most non-haematopoietic tumour (fast proliferating) cells in response to IR. Aberrant mitosis in response to DNA damage (also caused by IR) and mitotic death can also be associated with the aberrant duplication of centrosomes, the structures responsible for spindle microtubule formation. The characteristic feature of the cells that follow mitotic catastrophe is that the death is delayed, occurring 2–6 days post-irradiation. Mitotic catastrophe in apoptosis-competent cells is frequently followed by a delayed type of apoptotic death, or in other cells, by delayed necrosis.

Autophagy is an important catabolic process in which the cell digests itself *via* degradation of intracellular components such as proteins and organelles, to gain energy and nutrients (metabolic precursors). Autophagy mainly contributes to cell survival under adverse conditions, but when a stress factor persists for longer, autophagy can lead to so-called Type II programmed cell death.

Additionally, IR can induce a permanent cell cycle arrest, called senescence, a state in which the cell remains viable but with altered functions, and is no longer

competent for proliferation. Two major types of senescence have been identified: replicative senescence, which is a consequence of telomere shortening, and accelerated cellular senescence, in which the cell functions in the state of persistent, chronic DDR signalling. Senescent cells display characteristic phenotypic traits – they become enlarged and flattened with increased granularity. Senescent cells can be identified by their positive staining for the senescence-associated- β -galactosidase. In some cases, it has been demonstrated that increasing radiation doses shift the cellular response from senescence to apoptosis and/or autophagy, with higher doses leading to necrosis. However, there is no absolute response of all cells to a given dose of radiation exposure. Some cell types rapidly undergo apoptosis in response to the same level of IR that induces senescence in other cell types.

5.4.3. Cell survival curves

Cell survival curves describe the relationship between the surviving fraction of cells and the absorbed dose. Cell survival as a function of radiation dose can be graphically presented by plotting the surviving fraction (using a logarithmic scale) on the ordinate against dose (a linear scale) on the abscissa (Fig. 11).

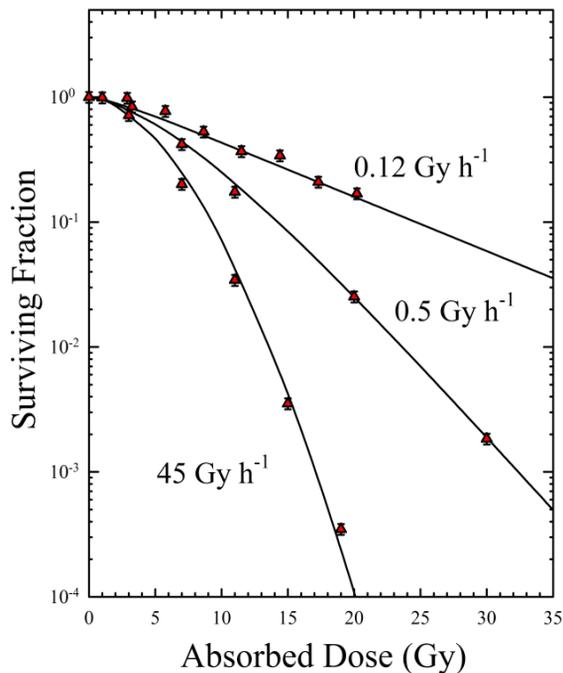


Figure 11. An example of a cell survival curve (figure used with permission under Creative Commons license)

The shape of the cell survival curve is influenced by the type of radiation. High LET (densely) IR exhibits cell survival curves that are almost exponential functions of the dose, shown by an nearly straight line on the log-linear plot. For low LET (sparsely) IR, however, the curves show an initial slope followed by a shoulder region that then turns nearly straight at higher doses.

5.5. The effects of ionizing radiation on the human organism

Throughout their lifetime, humans are exposed to low doses of IR from natural sources. The man-made sources which also contribute to IR exposure include industrial (nuclear power plant workers), and medical (radiotherapy, diagnostic X-rays) sources. Radiation accidents and incidents (nuclear weapon explosions, terrorist acts with radioactive materials) have led to much higher exposure doses. Despite the many practical applications of IR, exposure to high radiation doses has fatal consequences. The major sources of data on the health risks of IR exposure are the epidemiological studies of the Japanese survivors of atomic bombing, the workers who cleaned up after the Chernobyl and Fukushima nuclear plant accidents, and other populations accidentally exposed to high (even lethal) radiation doses. Secondary, also important sources of information on the effects of IR exposure on humans, are studies of oncological patients undergoing radiotherapy. Radiation harm can occur from external irradiation (outside the body), external or internal contamination with radioactive materials (the latter include inhalation, digestion or absorption through the skin), as well as from combinations of all of these exposure types. Injury from a nuclear detonation depends on the location of the victim relative to the hypocentre and the resulting exposure to heat, bomb blast, and radiation. Heat and light cause thermal injury, such as skin and/or retinal burns and blindness (due to the temporary depletion of photopigment from retinal receptors). The blast wave can result in fractures, wounds, rupture of internal organs and pulmonary haemorrhage and oedema. Acute overexposure to IR results in Acute Radiation Syndrome (ARS). Radiation also causes cutaneous injury and scarring, chorioretinal damage (from the exposure to infrared energy), and depending on the dose and dose rate, a variety of long-term effects (late toxicity). Cutaneous injury is characterized by loss of epidermis, sometimes dermis. Skin damage can cover small areas but it may extend deeply into the soft tissues, reaching the underlying muscle and bone.

The effects of IR on the human population are commonly divided into somatic and genetic. Somatic effects include harm that individuals exposed to IR suffer during their lifetime, such as the increased risk of IR-induced cancers (carcinogenesis), opacification of the eye lens, infertility, and shortened lifespan. Genetic effects (also called hereditary effects) include IR-induced changes in genomic DNA of the exposed individual's (chromosome aberrations, mutations),

which can contribute to the birth of defective descendants. Radiation is a known teratogenic agent; it disturbs the development of the embryo or foetus. Depending on the dose and the stage of development at the time of exposure, the main effects of radiation on a foetus include: foetal or neonatal death, malformations, growth retardation, congenital defects and cancer induction.

The biological effects of ionizing radiation on the human body depend on the nature and energy of the radiation, the time and mode of interaction and radiosensitivity of the exposed cells.

5.5.1. Radiosensitivity

Radiosensitivity is a broad term which can be applied to cells, tissues, organs and individuals. Cellular radiosensitivity defines the degree of response of the cells to IR. It refers to a wide range of events (endpoints) measured at the cellular level, such as cell viability, DNA damage and repair, markers of the cell cycle, apoptosis (or other cell death), chromosomal damage, etc. The response to IR can vary by cell type. The vulnerability of tissue to radiation injury depends on the degree of differentiation of the cells in the tissue and their proliferative activity. In 1906, French radiologists Bergeron and Tribondeau noted that radiosensitivity of a mammalian cell is proportional to the cell division rate and inversely proportional to the degree of their differentiation. This observation is called the law (or principles) of Bergon and Tribondeau. Tissues of the human body are well-organized structures composed of cells that have an epithelial and connective tissue origin. The epithelial cells (e.g. in the intestinal epithelium) and haematopoietic bone marrow cells continuously self-regenerate, they are cells renewing within a few days. Conversely, the endothelial vascular cells and fibroblasts that compose an underlying stroma are slowly renewing (proliferating). Therefore, the most radiosensitive are bone marrow and lymphoid tissue, germ cells and intestinal epithelial cells. Less sensitive are muscle cells, parenchymal organs (such as the liver), the nervous tissue, and the connective tissue. There is also a concept of 'relative radiosensitivity', which takes into account the importance of tissue to the body and the effects of its radiation damage. Taking this approach, the concept of the critical organ has been introduced, defined as the most important organ to the body that is the most damaged by radiation. For example, to X-rays and γ -rays, the critical organs are the bone marrow, the gonads and the eye lens. Generally, the radiation's effects can be modified by different factors, such as the type of radiation, dose, dose rate, dose fractionation (in radiotherapy), the mass of the irradiated tissue, tissue oxygenation, the organ irradiated, and the addition of radical scavengers. Rich vascularization, and thus a good supply of oxygen to tissues, increases their radiosensitivity.

At the organ level, IR-induced injury results from the direct destruction of highly radiosensitive cells, such as the stem cells in the bone marrow

(a rapidly renewable system). Damage may also result from the constriction of microcirculation, from oedema and inflammation of the basement membrane, and this injury can progress to fibrosis. The IR may have little effect on the parenchymal cells (slowly renewable/non-renewable system), but ultimate parenchymal atrophy and death over several months can result from fibrosis and occlusion of the microcirculation.

The individual radiosensitivity of various representatives of the population is, of course, relatively diverse. It can also vary within the same individual, for example, with age and with health conditions. For the description of radiosensitivity of the given population the concept of lethal dose (LD) has been introduced. All variants of this dose assume a single irradiation in a short time (up to several hours) of the whole body and no medical assistance after irradiation. The most useful for comparison is the mean lethal dose; it can be measured by assays such as $LD_{50/30}$ ($LD_{50/60}$) which defines the radiation dose required to kill 50% of a given population within 30 days (60 days) of exposure. Minimal lethal dose (LD_{min}) refers to the smallest radiation dose at which deaths can occur due to irradiation of a given population, while the maximal lethal dose (LD_{max}) defines the minimal radiation dose that causes death of all individuals of the irradiated population.

As an example, detonation of the nuclear device over Hiroshima, in 1945, resulted in approximately 150,000 casualties and 75,000 fatalities, in all survivors the estimated exposure was below 3 Gy. It has been suggested that the mean lethal dose of radiation (of the whole-body radiation) that kills 50% of human population within 60 days ($LD_{50/60}$) is at the range of 3.25–4 Gy in persons that have no supportive care, and 6–7 Gy in those having transfusion and antibiotics support.

5.5.2. Acute and late radiation toxicity, stochastic and deterministic effects

The timescale involved between the breakage of the chemical bonds in the vital macromolecules and the biological effect may be hours to years, depending on the type of damage. When a cell death is the result, it may happen in hours to days. If the damage is cancer induction, then its expression may be delayed for years. Based on the timescale as well as functional and histopathological endpoints, habitually, the effects of IR on tissues (or organs) has been divided into acute (early) responses and chronic (late) responses. Acute radiation toxicity are manifested soon after exposure to radiation (mostly within a few weeks) and are characterized by death of the critical cell populations. Especially damage is more evident in the haematopoietic and epithelial cells. Radiation at doses higher than those applied radiotherapy may completely destroy these cells. Early responses also involve gene activation resulting in tissue dysfunction followed by

the increased vascular permeability, tissue oedema, production of cytokines and growth factors, chemoattraction of macrophages and other white cells, leading to inflammation. These early responses to the stromal cells can persist of weeks or even months until have been settled. Late effects are delayed (may occur after 12 months) and may be consequential to acute damage in the overlying tissues, such as mucosa or the epidermis. Late effects can include, for example fibrosis, atrophy, ulceration, stenosis or obstruction of the intestine. Late effects may also be genetic and caused by absorption of radiation directly in the target tissue (mutations, chromosomal aberrations, carcinogenesis).

The acute/subacute effects of IR are known as so called deterministic effects (non-stochastic effects). They have a specific threshold dose. A deterministic effect (tissue reaction) is defined as a one that increases in severity with increasing dose, usually above a threshold dose, in affected individuals. Skin erythema, organ dysfunction, fibrosis, cataract, blood changes, decrease in sperm count are all examples of the deterministic effects. For example, the total body irradiation at the dose >5 Gy results in the bone marrow suppression, but this suppression is not observed for the dose <5 Gy. A stochastic effect is defined as a one in which the probability of occurrence increases with an increasing dose but the severity in affected individuals does not depend on the dose. These are statistically measurable effects. There is no threshold dose for the effects that are truly stochastic, because these effects arise in single cells and it is assumed that there is always some small probability of the event occurring even at very small doses. Stochastic effects (usually chronic effects) are for example IR-induced genetic mutations, chromosome aberrations, carcinogenesis.

5.5.3. Whole body irradiation

The response of animals, used for *in vivo* studies, to a single dose of whole body irradiation (WBI) can be characterized by four overlapping syndromes, including prodromal, haematological, gastrointestinal, and neurovascular syndrome, which are manifested following different doses, at different post-radiation time. The similar syndromes also apply to human victims of radiation accidents; the dose ranges after which the particular syndrome is seen vary between rodents and human. The neurovascular syndrome occurs following large IR doses (more than 20 Gy) and usually causes a rapid death (within hours to days) due to dysfunction of cardiovascular and nervous system. The gastrointestinal syndrome occurs following exposure to doses above 8–12 Gy, and in rodents the upper doses of this range generally result in death (within a week) which is mainly caused by a severe damage of the gastrointestinal tract mucosa. A subsequent loss of the protective barrier results in infection, loss of electrolytes and the fluid volume imbalance. In human victims, intensive treatment with antibiotics, replacement of fluids and electrolytes can prevent early death from this syndrome. However, these

patients can die due to the injury of other organs. The haematopoietic syndrome in rodents occurs at doses in the range of 3–10 Gy (2–8 Gy in human), which is caused by a severe depletion of blood morphotic elements, such as red blood cells (RBCs), white blood cells (WBCs) and blood platelets (PL), due to killing of the precursor cells in the bone marrow. As a result, the animals die usually between 12–30 days after irradiation, and in case of human a death happens fairly later. Treatment of human victims may include bone marrow transplantation to prevent death, provided that the radiation dose was not too high. There are considerable differences in radiation doses required to induce death due to the haematopoietic syndrome (for example, in LD_{50} values) between different animal species, even between strains of the same species. For human the mean lethal dose (LD_{50}) has been estimated at 4 Gy (4–7 Gy depending on the supportive care).

In relation to human, high instantaneous doses (>10 Gy) can occur accidentally (explosions of nuclear weapons, nuclear power plant accidents, handling unshielded radiation sources or radioactive waste). Data on some post-radiation symptoms were also collected based on the medical human exposure to TBI (a routine procedure before the bone marrow transplantation). Health effects after an individual is exposed to low or high doses of gamma irradiation have been described in several publications. In general, doses of IR below 0.15 Gy (15 rad) produce no noticeable symptoms or signs. This range includes, for example, lifetime radiation exposure from natural background radiation, the majority of nuclear diagnostic tests or nuclear power plant functioning. Increased radiation doses (0.15 to less than 0.5 Gy; 15 to <50 rad) result in subclinical responses, characterized by very few, if any, clinical or haematological symptoms. This level of IR exposure produce no visible manifestations, with the probability of chromosomal breaks occurring. At radiation doses from 0.5 to 30 Gy (50 to 3,000 rad) or more, clinical responses do occur. Acute radiation syndrome (ARS) is seen in individuals following acute whole body irradiation with doses of 1 or more Gy (≥ 100 rad).

Acute Radiation Syndrome (radiation sickness)

Acute Radiation Syndrome (ARS), is defined as ‘an acute illness caused by irradiation of the entire body (or most of the body) by a high dose of penetrating ionizing radiation in a very short period of time (usually a matter of minutes)’. ARS is also known as radiation sickness, and can be seen after exposures to doses >1 Gy. The degree of ARS may be classified by the absorbed dose and the time over which the energy from the radiation has been deposited in the tissues. As mentioned earlier, clinical components of the ARS include haematopoietic, gastrointestinal and cerebrovascular syndromes. However, the clinical phase of ARS can also be divided into four overlapping stages:

- 1) a mild phase (0.5–1 Gy, 50–100 rad);
- 2) the haematopoietic (or the bone marrow) syndrome (1–8 Gy, 100–800 rad);

- 3) the gastrointestinal syndrome (8–30 Gy, 800–3,000 rad);
- 4) the central nervous system (or cerebrovascular) syndrome (>30 Gy, >3,000 rad).

Each syndrome can be divided into four stages: the initial (prodrome), latent, and manifesting illness stages. The last of these is recovery or death. Depending on the dose absorbed, symptoms can appear at different times. The initial phase of ARS usually occurs in the first two days, but it can develop up to six days after exposure. The latent phase is a short period, lasting from several days to a month, when the symptoms transiently improve. Then, the manifest illness symptoms appear, characterized by strong immunosuppression. This period can last for weeks and is the most difficult to treat. If the individual survives this third stage, recovery is possible. Victims exposed to supralethal doses of IR can experience all these stages in just a period of several hours, leading to early death. If the energy is deposited over more than a few days (i.e. at a lower dose rate), the severity of the effects can be greatly reduced and the time of onset delayed. The phases of ARS as a function of the exposed dose, at high exposure rates, are summarized in Table 3. Clinical responses to radiation at doses in a range of 0.5 to above 30 Gy will be discussed below.

The **mild phase** of ARS (0.5–1 Gy) is characterized by mild, non-specific signs of toxicity. At a dose of 1 Gy, the majority of persons express temporary haematopoietic manifestations. The first clinical symptoms, nausea and vomiting, usually appear within 4–8 hours. Within 7–15 days after exposure, a moderate leukopenia (low WBC count) appears. However, blood cell counts finally go back to normal within 4–6 weeks after exposure.

The **haematopoietic syndrome** (1–8 Gy) can be seen after WBI exposures that exceed 1 Gy; it is hardly ever clinically relevant below this radiation dose. The dividing capability of the haematopoietic progenitor cells significantly decreases after a whole-body radiation dose of 2–3 Gy, leading to haematological crisis. This crisis is characterized by hypoplasia or aplasia, and bone marrow failure syndromes that result in development of pancytopenia – a condition in which the number of all blood cells dramatically falls. However, small subpopulations of stem and/or progenitor cells are selectively more radio-resistant, and these are responsible for recovery from haematopoiesis after exposure to doses even as high as 6 Gy. Pancytopenia, which includes leukopenia (low counts of lymphocytes and granulocytes; WBC counts), thrombocytopenia (low PL count) and anaemia (low RBC count), is a condition that predisposes to infection, bleeding and poor wound healing. Lymphopenia is the most common, occurring before manifestation of other cytopenia. Since the rate of the lymphocyte depletion is predictable (a 50% decline in total lymphocyte count occurs within the first 24 h after exposure, and becomes more severe after 48 h), the lymphocyte depletion kinetics can be used as an important component of biodosimetry (Waselenko et al. 2004). Individuals with additional injuries, such as burns and trauma – very

common after detonation of a nuclear device (60–70% of victims) – can develop lymphopenia as a result of these injuries alone.

Table 3. Dose dependent-injury of humans exposed to high doses of IR

Radiation dose (Gy)	severity	symptoms	Prognosis (without treatment)	Radiation syndrome
0.5–1	mild	minor decline of blood cell counts	survival –almost certain	-
1–2	mild/ moderate	early signs of BM damage	>90% victims survive	BMs
2–3.5	moderate	moderate/severe BM damage	survival – probable	BMs
3.5–5.5	severe	severe BM and minor GI damage	death within approx. 6 weeks (50% victims)	BMs
5.5–7.5	severe	pancytopenia, moderate GI damage	death – probable within 2–3 weeks	BMs/GIs
7.5–10	severe	hypotension, noticeable GI and BM damage	death – probable within 1-2.5 weeks	GIs
10–20	severe	Severe GI damage, pneumonitis, mental status, cognitive dysfunction	death-certain within 5–12 days	GIs/CNSs
20–30; >30	severe	fever, cerebrovascular collapse, shock	certain death within 2–5 days	CNSs

BM – bone marrow; BMs – bone marrow syndrome; GI – gastrointestinal; GIs – gastrointestinal syndrome; CNS – central nervous system; CNSs – central nervous system syndrome

The onset of other cytopenia differs and is much more dose- and dose rate-dependent. Although red blood cell precursors are affected at these doses, because of the lifespan of a peripheral red blood cell (90–120 days), anaemia may not become clinically evident for several days or weeks after exposure. Cells that proliferate more slowly (e.g. the cells of the central nervous system, connective tissues, etc) are largely unaffected.

Different clinical manifestations of the haematopoietic syndrome of ARS, depending on the phase, have been described. The prodromal phase, which

typically lasts up to 2–3 days, is characterized by fatigue, sleeplessness and lethargy that progress to headache, anorexia, nausea, and vomiting within – depending on the dose – approximately 8 hours after initial exposure. Varying alterations in the peripheral blood can be seen, with the earliest changes demonstrated as a marked lymphopenia. The latent phase lasts from 3–4 days to 3 weeks. The marked signs of this phase include a progressive decrease in total blood leukocyte counts and hair follicle death. The third phase, the symptomatic or bone marrow depression phase (18–21 days after exposure), is characterized by a plethora of symptoms, such as chills, fever, depression, a swollen throat, inflammation of the gums (gingivitis), bleeding gums, small blood blisters, and bruises. The leukopenia and thrombocytopenia weaken the body's natural defences against disease and haemorrhage causing anaemia and acute infectious diseases. Coagulopathies begin to appear due to blood platelet anomalies (purpura, haemorrhage), as well as hair loss (hair epilation). Depending on the dose and intensity of the treatment protocols, the clinical image can vary from serious to fatal. The recovery phase is marked by a general improvement of the patient over a 3–6 month period. There is a good prognosis for recovery if the doses received do not exceed 6 Gy (1–6 Gy). For doses of 6–8 Gy the prognosis is rather poor. Nevertheless, some victims are expected to survive if they receive intense medical treatment. The $LD_{50/30}$ for whole-body irradiation is estimated to be between 3.5–4.5 Gy for those who receive minimal or no medical treatment.

Gastrointestinal syndrome (8–30 Gy) is most often a result of the loss of intestinal crypts and the breakdown of the mucosal barrier. The prodromal phase of this syndrome is very rapid in onset and characterized by nausea and diarrhoea, which typically decreases after several days, followed by a short latent period. The symptoms then return, and these include WBC depletion as seen in haematopoietic ARS syndrome, nausea, vomiting, diarrhoea (sometimes bloody), abdominal pain, fever, and massive electrolyte imbalances, which ultimately then result in death. Other systemic effects can include malabsorption, malnutrition, small-bowel obstruction, dehydration and hypotension. Damage of the intestine mucosa and microcirculation leads to gastrointestinal bleeding, that increases anaemia and the risk of sepsis and/or acute renal failure. At these doses the mortality rate of the gastrointestinal syndrome exceeds that of the haematopoietic syndrome. Treatments are palliative. People exposed to absorbed doses of ≥ 10 Gy are expected to die, although aggressive medical intervention can improve the survival rate. It's worth noting that there is an exception – if the dose is fractionated, as with the bone marrow transplantation patients who receive a standard whole-body dose of 15.75 Gy and are well managed, with fluids, antibiotics, and a sterile environment, the individual has a reasonable chance of survival.

The central nervous system syndrome (>30 Gy). Symptoms of this syndrome typically have an immediate onset, and include violent nausea and vomiting, diarrhoea, headache, irrational behaviour, circulatory system collapse,

and lack of neuromuscular coordination, occurring within a few minutes after irradiation. This syndrome is less well defined than other syndromes. Note that the signs and symptoms of different organ systems significantly overlap at each radiation dose; cerebrovascular symptoms do not appear until exposure to a high whole-body dose. Individuals exposed to supralethal exposures (>20–30 Gy) can present fever, hypotension and major impairment of cognitive function. The prodromal stage of the cerebrovascular syndrome is characterized by disorientation, confusion and prostration, and can also be accompanied by a loss of balance and seizures. A physical examination can show papilledema (optic nerve swelling) due to increased intracranial pressure, ataxia (a neurological sign consisting of lack of voluntary coordination of muscle movements), reduced or absent deep tendon and corneal reflexes. Diarrhoea, respiratory suffering, fever, and cardiovascular shock can occur within 5–6 hours post-exposure. This phase mimics the clinical symptoms of acute sepsis and septic shock, both of which should be considered. Convulsions, coma, and death follow within 48 hours after irradiation.

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6. METHODS OF DETECTING BIOLOGICAL MATERIALS

The rapid detection of Biological agents is one of the most important steps in the process for first responders to bioterrorist attacks. Confirmation of a bio-agent's presence and where it most likely occurred, as well as precise identification of the agent, are key elements of action in addressing the effects of human exposure using appropriate medical procedures, and limiting the agent's spread. Biological weapons can be deployed in two ways: as hidden attacks, or as part of public displays of terrorism. In the case of a hidden attack, a diagnosis of the use of biological agents will always be delayed because the first suspicions will occur only after analysis of the epidemiological data, and when it indicates that non-endemic variables are present. In this case, analysis of the agent used will be based on typical microbiological methods (bacterial culturing), and advanced molecular biology techniques (genotyping of the pathogen). Detection and characterisation in this case will be carried out at specialised biological laboratories. However, in the second type of attack, involving the explicit use of a biological agent, initial detection of the material used should be one of the most important elements in any organised response system. A quick analysis allows for possible acknowledgment of the threat, and thus exclusion of false alarms. Information about the initial determination of whether a dangerous agent has been used and spread, as well as other potential factors in the attack, provides the basis for the on-site incident manager to take further action – evacuation, isolation, and medically preventive actions (such as rapid implementation of antibiotic treatment). It should be noted, then, that only true, accurate knowledge of the exact characteristics of the biological agent used allows for proper preventive and therapeutic action.

The methods used for detecting biological pathogens should consist of the following criteria:

- Quantitative abilities;
- Multiplexing capabilities;
- High specificity;
- High sensitivity.

Traditional detection methods, such as microscopic identification of characteristic morphological differences in bacterial, enzymatic and staining

test methods can be less specific than needed, and can be time-consuming and inefficient. Analysis should include a cascade of actions using increasingly more sophisticated research methods that narrow down the exact agents involved. As such, the system described in the following pages has been developed as a result of the analysis of various existing methodologies. Proposition of this scheme is presented below:

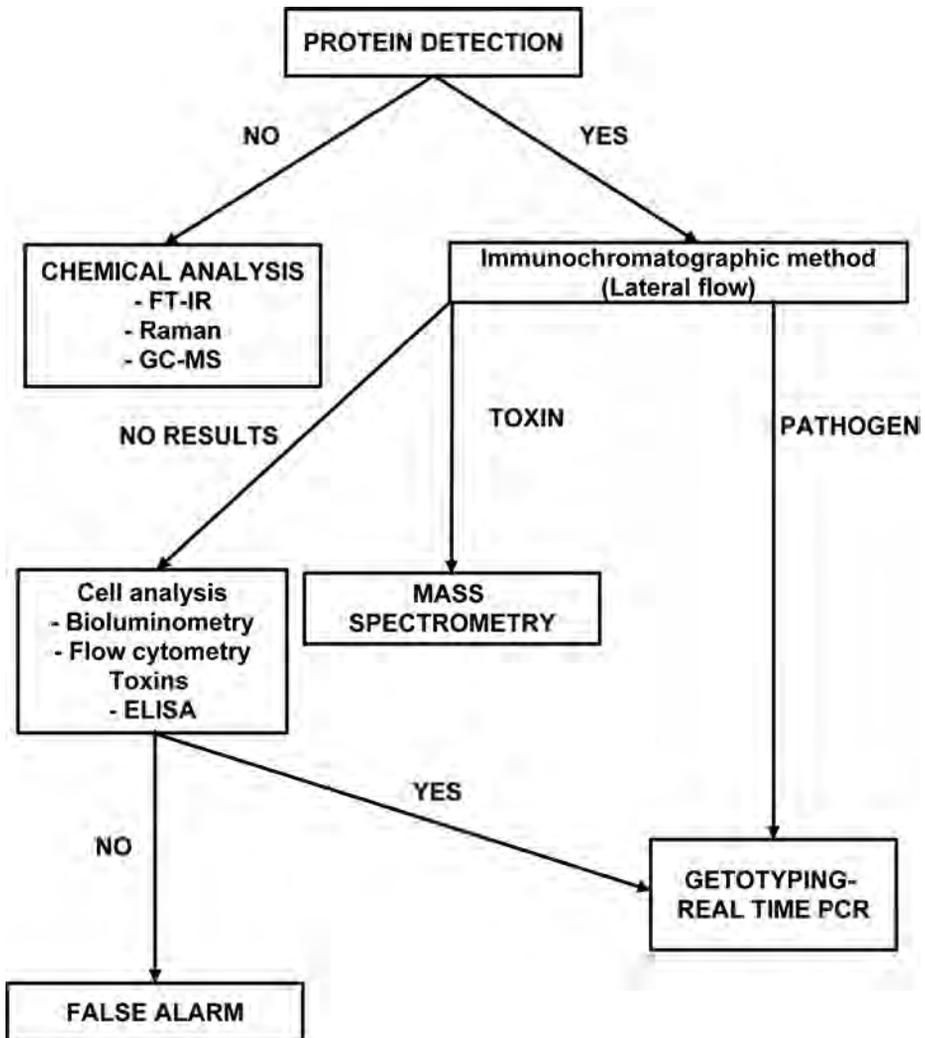


Figure 1. Proposition of cascade biodetection scheme

6.1. Proteins

Proteins are the basic building element of each living cell, as well as the main component of the outer shell of viruses, which according to the current concept of biology, is not considered part of a living organism. In addition, protein structure also contains a great amount of biological toxins (although there are some exceptions, for example T-2 mycotoxins). Thus, the confirmation or exclusion of the presence of protein in a sample will allow for an initial assessment of the presence of a hazardous biological agent. For this reason, determination of the presence of protein should be the first step in analysing biological materials recovered from the action scene. Such analysis should be very simple to implement and the time needed to complete it short. In addition, a very important element of the selected diagnostic method should be its sensitivity (i.e. the detection threshold), because the amount of bio-agent in the sample may be relatively small compared to the filler material, which is very often a non-protein substance/substances.

Proteins are large biomolecules, or macromolecules, consisting of one or more long chains of amino acid residue. All amino acids possess common structural features, including an α -carbon to which an amino group, a carboxyl group, and a variable side chain are bonded. Only proline differs from this basic structure as it contains an unusual ring to the N-end amine group, which forces the CO-NH amide moiety into a fixed conformation. A linear chain of amino acid residues is called a polypeptide. The amino acids in a polypeptide chain are linked by peptide bonds. The peptide bond is formed when the carboxyl group (COOH) of one amino acid molecule reacts with the amino group (NH₂) of the other amino acid molecule, causing the release of a molecule of water (H₂O). Hence the process is a dehydration synthesis reaction (also known as a condensation reaction). Proteins in their structure can contain the residue of from a few dozen to several thousand amino acids.

In biochemical analysis, many methods of determining the protein content of a sample have been used over the years. The most popular of these are based on the UV absorbance method, as well as on colorimetric methods such as the Biuret method, the Lowry Protein Assay, the Bradford method, and the bis-cinchonine acid method (BCA).

Spectrophotometric methods (including colorimetric analyses) of protein concentration in different samples are based on measurements of sample absorbance at different light wavelengths. Measurements can be made using different types of cuvettes or microtiter plates. Most popular cuvettes have a volume of 1.5–3 ml and are made of plastic, optic glass or quartz glass. The choice of cuvette type should be appropriate to the volume of the material being examined, and in particular to the spectroscopic method used (e.g. ultraviolet/UV

assay, colorimetric assay or fluorimetric methods). When the use of spectroscopic equipment is not an option, a simple colorimetric assay can be made by eye.

Spectrophotometric analysis under ultraviolet (UV) light is one of the simplest methods of protein detection, or quantification. Polypeptide chains contain amino acids with a characteristic cyclic (aromatic) structure. For example, tyrosine, phenylalanine and tryptophan, which are able to absorb UV radiation at 280 nm. The presence of these amino acids may be a basis for protein detection or simple quantification of protein content by A_{280} measurement. Other methods for protein quantification include measurements made at 205 nm, corresponding to the UV absorption of peptide bonds. However, there are numerous chemical and practical aspects that limit the possibility of quantitative evaluation of protein concentration using these methods. The most significant of these is that an extinction coefficient of the examined protein must be established. Measurements in the UV range (wavelengths up to 400 nm) need to be made using quartz glass or plastics, without UV absorption, especially made for use in this spectrum.

The vast majority of current biochemical methods of protein quantification are based on various types of colorimetric assays. Colorimetry is an analytical technique that is widely used to determine the concentration of substances based on the intensity of their colour (measured at appropriate wavelengths). Chemical structures of many compounds, including proteins and other biomolecules, contain some structural fragments that are able to absorb visible spectrum (VIS) radiation, and these are called 'chromophores'. The visible spectrum covers wavelengths of between 400 and 800 nm, which may be detected using quartz glass, optic glass and plastic laboratory equipment. The absorptive capability of VIS radiation at different wavelengths by various substances results in the substances taking on distinctive colours. For instance, compounds able to absorb radiation at wavelengths of 400–420 nm turn yellow, while substances strongly absorbing radiation of about 600–750 nm are mainly purples, or intense blues. Thus, a chromophore may be defined as the part of a molecule responsible for its colour. The C=C- and N=N bonds, C=O and N=O groups as well as aromatic rings all belong to the most common chromophores. However, only part of the molecules (inc. proteins such as haemoglobin) possesses a natural colour that can be assayed directly. Therefore, the concentration of most substances can be determined indirectly, using chemicals that react with the examined compound (or a product of its chemical activity), in order to generate a coloured substance.

A panel of colorimetric assays commonly applied in protein quantitation includes different protocols based on the Lowry assay, Bradford assay, Pierce 660 assay and BCA assay. Mostly, colorimetric assays are based on the detection of coloured products/complexes resulting from reactions between cuprous ions (Cu^+) and proteins and/or reagents. These methods are classified as copper-based assays. The reagent mixtures contain cupric ions (Cu^{2+}), which undergo

a reduction to Cu^+ under alkaline conditions. The Cu^+ ions are able to react with peptide bonds (the 'biuret reaction'), the aromatic ring of tyrosine, the indole part of tryptophan, the thiol groups of cysteine residue in proteins, and with chemical reagents, leading to the formation of detectable products. In most proteins the content of tryptophan or cysteine is significantly lower than the amount of tyrosine residue, and so these assays are primarily dependent on the presence of tyrosine.

The Lowry assay is one of the most popular methods for determining the total protein content of a biological sample, including biological material, originated from human, animal and bacterial sources. The combination of the Biuret method and two types of reaction provides a very sensitive reaction, for example oxidation of peptide bonds generating Cu^+ ions (1), and the reaction of the ions with Folin-Ciocalteu reagent (sodium 1,2-naphthoquinone-4-sulfonate) (2). This allows for detection of proteins even at concentrations of $10 \mu\text{g}/\text{ml}$ (the sensitivity range is $10\text{-}1000 \mu\text{g}/\text{ml}$). The formation of an intensively blue product can be recorded at 750 nm .

Another popular method, based on a combination of the biuret reaction and complexation of the generated Cu^+ ions, is the bicinchoninic acid (BCA) assay. This method is based on a combination of two chemical reactions. The first is the biuret reaction, in which amino acids in the protein structure reduce the cupric ions (Cu^{2+}) to cuprous ions (Cu^+) in an alkaline solution. The second step is colorimetric detection of the product of cuprous ions and BCA complexation/chelation. The chelation of one cuprous ion by two molecules of BCA yields a purple-coloured product, with a maximum absorption of 562 nm . Since the formation of these complexes has a linear and dose-dependent character, and is reproducible in a relatively broad range (approx. $20\text{-}2000 \mu\text{g}/\text{ml}$), the BCA assay is considered to be very useful for testing different samples containing proteins. It is easy to execute and requires only small amounts of protein samples ($20\text{-}25 \mu\text{l}$). The procedure can also be modified according to the type or concentration of the protein-containing samples. However, there are some very important limitations and disadvantages, chief of which is that the reactions develop over time. Additionally, certain substances (such as detergents, reducing agents, biogenic amines or chelating compounds), are known to interfere with this method. But modified BCA reagents that can be used in the environment of detergents and reducing agents are available on the commercial market.

The next most popular method of fast protein analysis is the Bradford assay, which represents a different type of analytical method. The chemical basis of this reaction is the direct reaction of Coomassie Brilliant Blue G-250 dye with amino acids in the protein structure. This reaction is primarily mediated by reactions with arginine, lysine and histidine. During the assay, a brown/red coloured dye reacts with proteins under acidic conditions, yielding complexes with a blue colour that can be detected by eye. The sensitivity of this method is dependent on

the molecular weight of the assayed proteins and is proportional to the number of positive charges in the proteins. The assay is compatible with most buffers, as well as reducing and chelating compounds. The main limitations and disadvantages of the Bradford assay is that it is not suitable for polypeptides of low molecular mass – the molecular weight of the assayed proteins must attain at least 3,000 Daltons, but most bio-threats will have proteins with a higher mass.



Figure 2. Changes of Bradford dye in different protein concentrations

The Pierce™ 660 nm Protein Assay is a very interesting, commercially available reagent kit. It is based on the use of a dye-metal complex (polyhydroxybenzenesulfonephthalein-type dye and a transition metal), which as in the Bradford reacts with amino acid residue. The dye-metal complex begins as a reddish-brown colour and changes to green upon protein binding. The colour change is produced by deprotonation of the dye at low pH, facilitated by interactions with positively charged amino acid groups in proteins. Therefore, the dye mainly interacts with basic residues in proteins, such as histidine, arginine and lysine, and to a lesser extent tyrosine, tryptophan and phenylalanine. The colour produced in the assay is stable and increases in proportion to a broad range of increasing protein concentrations. In comparison to the Bradford, this assay is more linear and compatible with higher concentrations of most detergents, reducing agents and other commonly used reagents. The Pierce 660 nm is recommended as a quick method of quantifying proteins in a range of 25–2000 µg/ml. Although the Pierce 660 nm Protein Assay produces a higher level of protein-to-protein variation (37%) than other assays, such as the BCA Protein Assay, the simpler single-reagent format and broader substance compatibility make the Pierce 660 nm Assay more convenient for many routine applications.

However all of protein detection methods have one big limitation. To proper detection of proteins to smart detection kit must be added some bacterial cell lysing agent such as DNAzol or Lyse it®.

At present, there are a number of test-tube-based protein test kits dedicated to fast detection of substances on-site at bioterrorist events. One of the most popular is sold under the trade name BioCheck by 20/20 GeneSystems Inc. The BioCheck Powder Screening Test Kit is designed for first-responders to investigate suspicious powders for bioterrorism agents. The kit is characterised by remarkable ease of use. It consists of two tubes of reagents that identify the proteins and determine

the pH of the test sample. According to the manufacturer's instructions (<http://biocheckinfo.com/schematic/>), it takes up to 5 minutes to verify the presence of proteins. A positive test result is indicated by purple staining. The sensitivity of the BioCheck test is quite high, at about 10 $\mu\text{g}/\text{test}$. The price of the kits is also not excessive, at EUR 1,000 for 25 kits (plus postage from the United States). The use of such a simple diagnostic test allows for the exclusion of the presence of biological hazards, or if confirmed, subsequent, more detailed testing can be carried out. This kit has mixture of protein detection reagent and strong lysing agent in tested tube which provide very variable results of analysis.

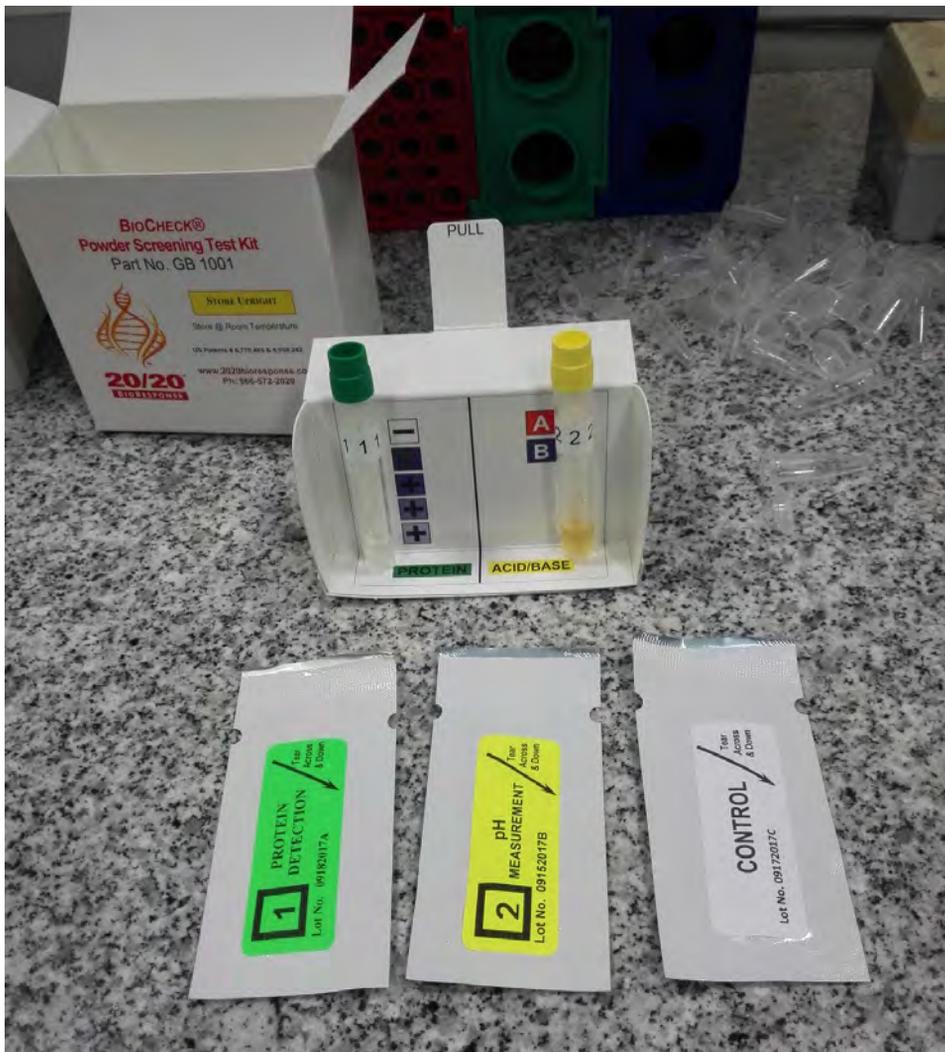


Figure 3. Composition of BIO-CHECK kit



Figure 4. Positive and negative results of BIO-CHECK

6.2. Immunoenzymatic tests

The word ‘immunoenzymatic’ can be used to describe the types of analytical and diagnostic methods discussed in this text. They can be defined as the use of specifically designed antibodies (from immunoglobulins G, IgG) to enable detection of unique target proteins (antigens), after which the results are visualized *via* enzymatic reactions that can be classified as absorbance or fluorescence. Briefly, the biochemical background of immunoenzymatic reactions involves the use of antibodies dedicated to the protein of interest. The interaction of an antibody and its antigen is made possible due to the presence of specific epitopes (antigen determinants) in the target molecules or cells. Two types of antibodies are used in these assays: polyclonal and monoclonal antibodies. Polyclonal antibodies detect multiple epitopes in the antigen structure, while monoclonal antibodies are able to recognize only one epitope in the antigen.

In the case of proteins, antibodies are able to recognize the detected antigen on the basis of the amino acid sequence or three-dimensional structure of the polypeptide chain. The reaction is very selective and allows detection of even nanograms of the investigated protein (antigen). Antibody-antigen complexes are

detected with the use of enzymatic reactions. The enzyme can be directly attached to the antibody structure, or added separately, in subsequent steps of the assay. The most frequently used enzymes are horseradish peroxidase (HRP) and alkaline phosphatase (AP). Substrates for these enzymes are commercially available as separate reagents, or are part of the assay kits. For the HRP enzyme, the most popular substrates are 3,3',5,5'-tetramethylbenzidine (TMB), (O-phenylenediamine dihydrochloride (OPD), and 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS), while p-nitrophenyl phosphate (PNPP) is mainly used as a substrate for alkaline phosphatase.

Different types and variations of immunoenzymatic assays are widely applied in diagnostics, scientific research and the food industry. The most common variations include the Enzyme-Linked Immunosorbent Assay (ELISA), Western blotting, radioimmunoassays (RIA), lateral flow tests and protein microarrays.

6.2.1. ELISA tests

The Enzyme-Linked Immunosorbent Assay (ELISA) requires the use of a solid phase (usually a 96-well polystyrene microtiter plate). The plate is a base for antibody or antigen absorption (depending on the ELISA subtype used). In general, ELISAs may be classified into three main types: direct ELISAs, indirect ELISAs, and the 'sandwich' ELISA (an extended indirect ELISA). It should be emphasized, however, that ELISAs are often modified at different stages of their protocol, including in both the antigen immobilization stage and the detection/probing steps. For example, antigens may be directly coated on microplate wells, or bound to antigen-specific antibodies that were previously adsorbed onto the microplate. In order to enhance the sensitivity of the tests, some modifications of the further stages can also be made. The secondary antibodies can be associated with biotin (vitamin B₇). The biotin label enables further interactions with other components of the assay, including reactions with avidin (a protein of animal origin), and streptavidin (a protein isolated from *Streptomyces avidinii* bacteria), display a high ability to biotinylate itself or biotinylated molecules, and this connection is based on a strong, non-covalent reaction. This is very useful during construction of certain kinds of ELISA protocols. Enzymes used in immunoenzymatic assays can be conjugated with avidin or streptavidin. Biotinylated antibodies effectively bind the avidin- or streptavidin-labelled enzymes (HRP or AP). Although most of the available ELISA assays involve the use of enzymatic reactions to produce coloured products, ELISAs based on the fluorescent signal of antibodies labelled with fluorophores (immunoassays without enzymes) are also available. This type of immunoassay is particularly practical in conducting multiplex arrays that require simultaneous detection of more than one antigen (with the use of antibodies conjugated to different fluorophores).

Direct ELISA. The antigen is detected in direct reaction with primary antibodies connected with the enzyme. Typically, the procedure contains four basal steps:

- Immobilization (coating) of antigens to the surface of microplate wells;
- Blocking of the remaining unsaturated surface-binding sites on the microplate wells by incubation with a solution of other proteins that are not-reactive in this assay;
- Incubation with enzyme-coupled antibodies (probing);
- Detection – determination of enzyme activity products (visualisation of the results).

Indirect ELISA. The fundamental difference between direct and indirect ELISA is the use of secondary antibodies. The procedure for indirect assay involves the use of primary antibodies (recognising the antigen, but lacking the enzyme), and secondary antibodies (recognising the primary antibodies and bearing the enzyme). Thus, the procedure is longer when compared to the direct ELISA. However, the use of secondary antibodies significantly improves the sensitivity of the assay and decreases the extent of undesired non-specific effects. The simplest version of the indirect ELISA protocol can be presented in the following five steps:

- Immobilization (coating) of antigens to the surface of microplate wells;
- Blocking of the remaining unsaturated surface-binding sites on the microplate wells by incubation with a solution of other, not-reactive in this assay protein;
- Incubation with the primary antibodies (without the enzyme);
- Incubation with enzyme-coupled secondary antibodies (probing);
- Detection – determination of enzyme activity products (visualisation of the results).

Sandwich ELISA. This subtype of the ELISA was developed to potentiate the selectivity and sensitivity of immunodetection, especially when certain complex mixtures are assayed. The sandwich method is based on the use of two different antibodies that are able to bind specifically to the antigen, but recognizing different epitopes. The first antibodies (the 'capture' or 'coating' antibodies) are immobilized on the microplate well. The second antibodies are added to the antigen as a solution – this is the detection antibody.

A simple sandwich ELISA requires the following steps:

- Immobilization the first antibodies (capture/coating antibody) to the surface of microplate wells;
- Incubation with the antigen;
- Blocking of the remaining unsaturated surface-binding sites on the microplate wells by incubation with a solution of other, not-reactive in this assay protein;
- Incubation with the secondary antibodies (the detection antibody);
- Detection – determination of enzyme activity products (visualisation of the results).

Furthermore, analogous to the indirect ELISA protocol, the sensitivity of the sandwich assay can also be enhanced with use of biotinylated secondary antibodies and avidin- or streptavidin-labelled enzymes.

6.2.2. Western blot

The western blot (also called western blotting or immunoblotting) is an analytical technique commonly used in biochemistry and molecular biology to identify particular proteins in a sample after its transfer to a membrane – the proteins are transferred by electric current into a thin membrane of nitrocellulose or Polyvinylidene Fluoride (PVDF), and are usually detected by specific antibodies.

To increase detection sensitivity, a sandwich of two layers of antibodies is usually used. The first antibodies are specific to the target protein, and the second antibodies are able to bind the first antibodies and start an enzymatic reaction, as they are conjugated with horseradish peroxidase (HRP). The enzyme catalyses reactions in which colourful products, or chemiluminescence, appear. Images can be registered by cameras or scanners (the first case), or by digital dark chambers, sensitive sophisticated scanners and contact with light-sensitive photo paper (second case).

The membranes can be dyed with Amido Black or Ponceau S dyes to visualize all transferred proteins. Only Ponceau S. staining is reversible, by rinsing with water, and can be used before immunodetection. The Amido Black dye is irreversible and disturbs the binding of antibodies, so it can be implemented only after immunodetection.

6.2.3. Lateral Flow Assays

Lateral flow assays (LFA) are the technology behind low-cost, simple, rapid and portable detection devices popular in biomedicine, agriculture, food and environmental sciences. Lateral flow immunoassays, also known as immunochromatographic assays, or strip tests, are unidirectional assays which are used to quickly and easily establish whether a target analyte is present in a test sample. Lateral flow immunoassays are essentially immunoassays adapted to operate along a single axis, to suit the test strip format. The lateral flow assay is a membrane-based platform for the detection and quantification of analytes in complex mixtures, in which the sample is placed on a test device. The results are displayed within 5–30 min.

The basic of this technology was first described in the 1960s, while the first real commercial application was Unipath's Clearview home pregnancy test, in 1988. Since then, the technology has been employed to develop a wide and ever-growing range of assays for clinical, veterinary, agricultural, food and bio-defence purposes.

The advantages of the lateral flow immunoassay system are well-known, and include:

- High sensitivity and specificity;
- Only a low sample volume;
- Applicable in a wide range of settings;
- One-step assay, no wash steps necessary, short time to result;
- Possibility of multiplexing;
- Low cost;
- Simple, user-friendly operation;
- High potential for commercialization.

A typical lateral flow immunoassay consists of four key components: a sample application pad, a conjugate release pad, a nitrocellulose membrane and a wicking pad.

1. Sample pad – an adsorbent pad onto which the test sample is applied.
2. Conjugate or reagent pad – contains antibodies specific to the target analyte and conjugated to coloured particles (usually colloidal gold nanoparticles, or latex microspheres).
3. Reaction membrane – typically a nitrocellulose or cellulose acetate membrane onto which anti-target analyte antibodies are immobilized in a line that crosses the membrane to act as a capture zone or test line (a control zone will also be present, containing antibodies specific to the conjugate antibodies).
4. Wick or waste reservoir – a further absorbent pad designed to draw the sample across the reaction membrane by capillary action, and collect it.

The components of the strip are usually fixed to an inert backing material and may be presented as a simple dipstick, or within a plastic casing with a sample port and reaction window showing the capture and control zones.

The mechanism of analysis in lateral flow testing is very simple: a liquid sample (or its extract) containing the analyte of interest moves without the assistance of external forces (capillary action), through various zones of polymeric strips, on which molecules that can interact with the analyte are attached. The sample is applied at one end of the strip, on the adsorbent sample pad, which is impregnated with buffer salts and surfactants that make the sample suitable for interaction with the detection system. The sample pad ensures that the analyte present in the sample is capable of binding to the capture reagents of conjugates and on the membrane. Once soaked, the fluid migrates to the second element (conjugate pad), in which the manufacturer has stored the so-called conjugate, a dried format of bio-active particles in a salt-sugar matrix that contains everything to guarantee an optimized chemical reaction between the target molecule – an antigen and its chemical partner – antibody that has been immobilized on the particle's surface. The antibodies that are specific to the target analyte are conjugated to coloured or fluorescent particles, most commonly colloidal gold or latex microspheres.

While the sample fluid dissolves the salt-sugar matrix, it also dissolves the particles, in one combined transport action. The sample, together with the conjugated antibody bound to the target analyte, migrates along the strip through the porous structure into the detection zone. This is a porous membrane with specific biological components immobilized in one or more lines (often called stripes). Their role is to react with the analyte bound to the antigen-conjugated antibody.

By the time the sample-conjugate mix reaches these stripes, the analyte has been bound on the particle and the third 'capture' molecule binds the complex. Recognition of the sample analyte results in an appropriate response on the test line, while a response on the control line indicates the proper liquid flow through the strip. Typically, there are at least two stripes: one (the control) that captures any particle and thereby shows that the reaction conditions and technology worked properly; the second contains a specific capture molecule and only captures those particles onto which an analyte molecule has been immobilized. After passing these reaction zones the fluid enters the final porous material, the wick, which simply acts as a waste container. The read-out, represented by lines appearing with different intensities, can be assessed by eye or using a dedicated reader.

To test multiple analytes simultaneously under the same conditions, additional test lines of antibodies specific to different analytes can be immobilized in an array format.

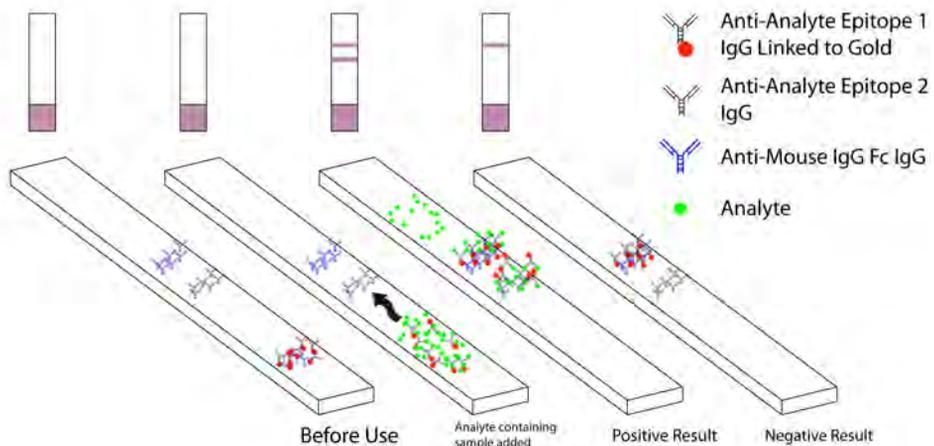


Figure 5. Scheme of lateral flow test analysis (figure used with permission under Creative Commons license)

Different commercially-available lateral flow tests dedicated to multiple bio-threat detection are available. The most popular are produced by Drager (BIO AGENT); Alexeter Technologies LLC (RAID (Rapid Assessment Initial

Detection)), and BBI Detection (IMASS). BIO AGENT and RAID are typical lateral flow multiplex kits that contain a plate with equipment necessary for sampling, while the IMASS is quite different. IMASS stands for Integrated Multiplex Assay and Sampling System, and is an integrated system of sampling and fast detection in one kit. It provides the ability to rapidly sample powders, liquids or surfaces using an integrated sampling sponge. Once the sampling has been completed and the device is run, the sample is transferred simultaneously to all of the integrated assay strips.



Figure 6. Positive results of lateral flow tests

6.3. Flow cytometry

Flow cytometry is a method that allows a sample of cells or particles in suspension to be separated through a narrow, rapidly flowing stream of liquid. Flow cytometry provides rapid analysis of multiple characteristics of single cells. In essence, a flow cytometer is a fluorescence microscope that collects light signals emitted from particles flowing across an objective lens. The information obtained is both qualitative and quantitative. The sample is passed through a laser, which allows for detection of size, granularity, and the fluorescent properties of

individual cells/particles in the sample. Flow cytometry measurements are based on two types of markers: light scattering on the cell, and the fluorescence of labelled antibodies linked to antigens on the surface or inside the cell. This method consists in passing a liquid stream containing the test cells through a focused laser beam. In the flow cytometer, the cell suspension is pumped under vacuum in an envelope of screening liquid, in the form of a thin, flowing laminar stream containing a row of sequentially arranged cells (a so-called hydrodynamic focus). A fluorescently-labelled sample containing the particles of interest is connected to the flow cytometer, where the particles are oriented by hydrodynamic focusing into a thin stream surrounded by fluid of higher speed (sheet fluid). As a result of the interaction with the cell, the intensity of the scattered radiation is recorded by carefully selected and set up photomultiplier and photodetectors. For a few years, with the use of new generation fluorescent dyes, this method is significantly more sensitive, allowing for a much higher number of parameters and a lot of information from a single experiment. A beam of laser light is directed onto the stream of fluid and a number of detectors are aimed at the same point.



Figure 7. FSC vs SSC dot plot showing different cell populations

In light scattering analysis, two parameters are used. The first is Forward Scattering (FSC), for which the detector is mounted behind the measuring chamber. This first parameter carries information about cell size. It is assumed that the intensity of light is proportional to the size of the dispersion particle. The second parameter defines the light scatter perpendicular to the Side Scattering (SSC). The strength of this radiation is a defined function of the granularity of the illuminated cell – the more internal structures that diffuse the light beam, the greater the angle of diffused incident radiation. This in turn leads to an increase in

the radiation intensity recorded at an angle of 90° . FSC and SSC detectors record signals for each specific object, so that the populations of the analysed cells can be distinguished or concluded on their own individual characteristics. Typically, particles in a range from 0.2 to 150 micrometres passing through the beam scatter the light at a detectable level based on SSC or FSC.

Flow cytometry measures the optical and fluorescence characteristics of single cells. Physical properties, such as size and internal complexity, can resolve certain cell populations. Fluorescent dyes may bind or intercalate with different cellular components, such as DNA and RNA. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells. When labelled cells are passed by a light source, the fluorescent molecules are excited to a higher energy state. Upon returning to their resting states, the fluorochromes emit light energy at higher wavelengths. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths (or 'colours'), allows for several cell properties to be measured simultaneously.

Modern instruments usually have multiple lasers and fluorescence detectors allowing for a more complex experimental setup. The process of collecting data from samples using the flow cytometer is termed 'acquisition'. Acquisition is mediated by a computer connected to the flow cytometer and the software that handles the digital interface with the cytometer. Typically, a flow cytometer has five main components:

- A flow cell where the liquid stream (sheath fluid) that carries the cells/particles is aligned, so that the cells/particles pass single-file through the light beam for sensing;
- A measuring system, commonly including diode lasers of different wavelengths (blue, green, red, violet), resulting in light signals;
- A detector, which is most often a Photo Multiplier Tube (PMT), and Analogue-to-Digital Conversion (ADC) system that turns FSC and SSC as well as fluorescence signals from light into electrical signals that can be processed by a computer;
- An amplification system (linear or logarithmic);
- A computer, for analysis of the signals.

Inside a flow cytometer, cells in suspension are drawn into a stream created by a surrounding sheath of isotonic fluid that creates a laminar flow, allowing the cells to pass individually through an interrogation point. At the interrogation point, a beam of monochromatic light, usually from a laser, intersects the cells. Emitted light is given off in all directions and is collected *via* optics that direct the light to a series of filters and dichroic mirrors that isolate particular wavelength bands. The light signals are detected by photomultiplier tubes and digitized for computer analysis. The resulting information is usually displayed in histogram or two dimensional dot-plot formats. In the case of histograms, the x-axis will represent either fluorescence or scatter intensity collected from a single photo-

detector on either a linear or logarithmic scale, and the y-axis represents the number of particles with the corresponding light intensity. For instance, if a cell is tagged with a fluorescently labelled antibody directed towards a surface protein, the fluorescence intensity will be directly proportional to the expression level of this antigen. By using multiple antibodies one can then assess the expression levels of several membrane-bound and/or intracellular proteins of a single cell. Any two of these parameters can be displayed simultaneously on two dimensional dot-plots.

Flow cytometry is a powerful technique for correlating multiple characteristics on single cells. This qualitative and quantitative technique has made the transition from a research tool to standard clinical testing.

Flow cytometry is routinely used in a number of research and diagnostic areas. In health care, flow cytometry is used to diagnose different diseases, including blood cancers, as well as for help with other clinical decisions in the fields of transplantation, haematology, tumour immunology and chemotherapy, genetics etc.

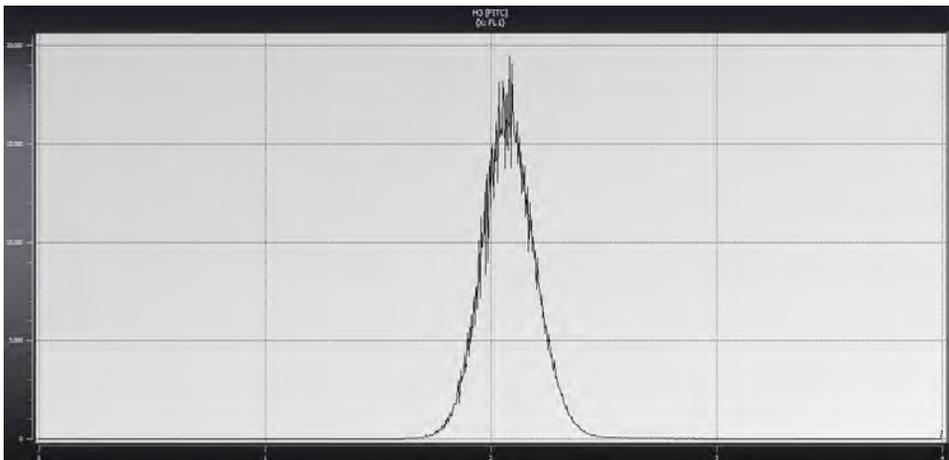


Figure 8. Fluorescence signal for cells connected with fluorescent antibodies

Using a combination of fluorescent dyes sensitive to cellular states, polyclonal antibodies targeting specific pathogens, specific assay additives, and a carefully defined cytometer gating logic, we can have a system for detecting target microbes in tested probes with a lot of advantages:

- Requires no or minimal enrichment;
- Achieves single cell sensitivity;
- Delivers a quantitative response;
- Offers analytical specificity (i.e. target pathogens without false negatives from non-target particles);

- Provides consistent and robust instrumental operation;
- Utilizes reasonably stable and relatively inexpensive reagents;
- Does not require extensive operator training.

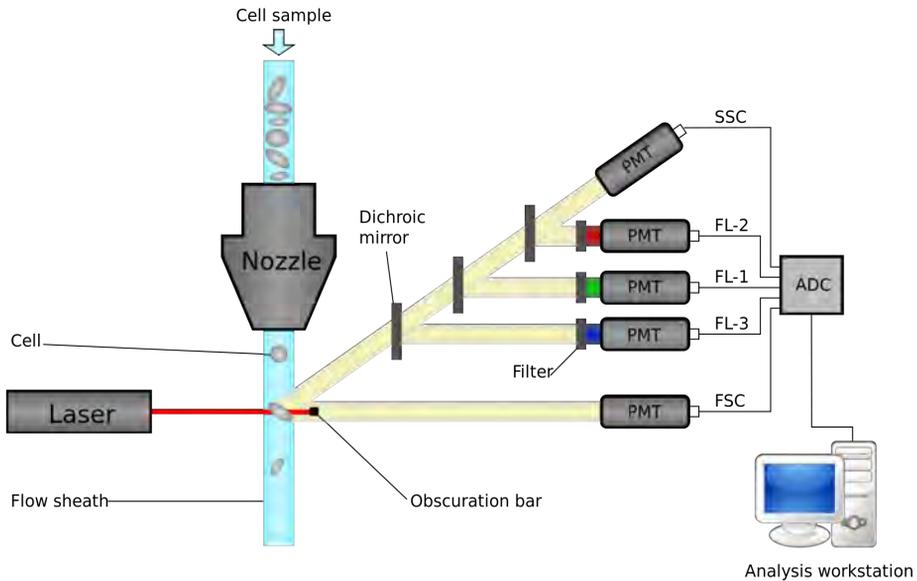


Figure 9. Scheme of flow cytometer mechanism (*figure used with permission under Creative Commons license*)

From the point of view of microbiologists, a key advantage of flow cytometry is the ability to simultaneously collect multiple data outputs from an individual cell. As bacteria are much smaller than mammalian cells, the detection and analysis of pathogenic microorganisms poses particular challenges for flow cytometry. For this reason very sensitive equipment is required. Additionally, beads are available for calibration and counting and are used as standards to provide a reference point allowing the operator to optimize instrument settings for detection of particular pathogens. These beads allow detection at a size level required for analysis of small particles, such as bacteria, and are available in a range of 0.1 μm upwards. Fluorescence and side-scatter or forward scatter ratio information allow the operator to anticipate where, for example, labelled pathogens are located on a two-parameter plot. The concept of using flow cytometry to analyse bacterial presence in a sample is not new. However, the successful commercial development or modification of flow cytometers devoted entirely to the detection of a specific bacterial pathogen is still relatively uncommon.

As with most research and clinical equipment, flow cytometers are typically laboratory devices, but the development of the technique has led to their

miniaturization, and nowadays, commercially-available mobile devices are available for field applications. One example of such a device used for field infection diagnosis in African countries is the Cyflow® miniPOC from Partec, which has a rechargeable battery dock (for 4–5 hours operation per battery charge). This equipment is dedicated to establishing populations of CD4/CD4% cells, but other antibodies (for example, bacillus spores), can be used for pathogenic field detection.

6.4. Real-time PCR

Real-time polymerase chain reactions (real-time PCR), also known as real-time quantitative polymerase chain reactions (real-time-qPCR), allow for specific and highly sensitive quantification of nucleic acids. RT-PCR is based on a revolutionary method in molecular biology – PCR – which was developed by Kary Mullis in the 1980s. PCR allows for more than a billion-times amplification of specific fragments of DNA using sequence specific oligonucleotides, heat stable DNA polymerase, dNTPs and thermal cycling. A pair of primers are complementary to the sequence of interest, and during PCR reactions are extended by the DNA polymerase. The copies produced after the extension, so-called amplicons, are re-amplified with the same primers, thus leading to exponential amplification of the DNA molecules.

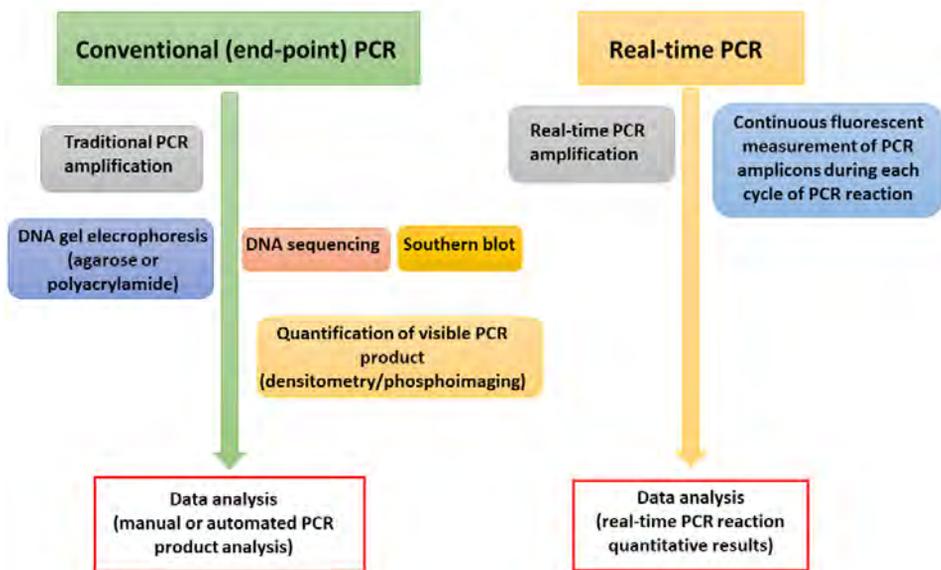


Figure 10. Conventional PCR *versus* Real-time PCR

In conventional (traditional) PCR, detection and quantification of the amplified sequence (DNA product) are performed at the end of the reaction after the last PCR cycle – this is a so-called end-point analysis. After amplification, additional post-PCR analysis, such as gel electrophoresis (agarose or polyacrylamide) and image analysis are required, and this makes conventional PCR time consuming. Real-time-qPCR overcomes this problem – the amplified DNA product is measured in each cycle, as the reaction progresses.

The advantages of real-time PCR include:

- Ability to precisely measure the amount of amplicon in each cycle, which allows for highly accurate quantification of the amount of DNA starting material in each sample;
- Ability to monitor the PCR reaction progress in real time;
- Amplification and detection of PCR product occurs in a single tube, eliminating post-PCR manipulations (high throughput, low contamination risk);
- Elimination of non-specific amplification;
- Confirmation of specific amplification by melting curve analysis;
- Most specific, sensitive and reproducible;
- Not much more expensive than conventional PCR (except for the cost of the Thermal Cycler);
- Increased dynamic detection range (up to 10^{10}).

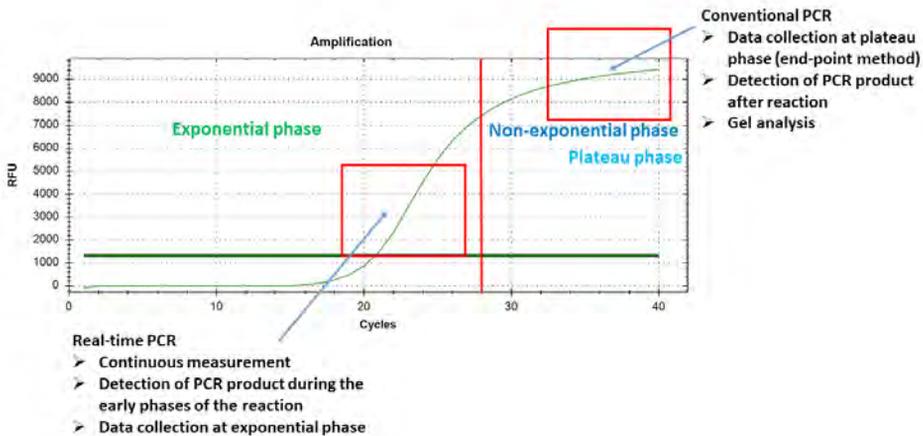


Figure 11. Reaction kinetics in real-time PCR. Comparison conventional PCR with real-time PCR

To better understand the differences between conventional PCR and Real-time PCR and how real-time PCR works, in Figure 2 we show the kinetics of the PCR reaction using a typical amplification plot. On the x-axis the number of PCR cycles is shown, while on the y-axis is the fluorescence from the PCR

amplification reaction, proportional to the amount of amplified product in each tube. Generally, an amplification curve presents three different phases:

1. Exponential;
2. Non-exponential;
3. Plateau or end-point phase.

During the exponential phase, all components are present in a sufficiently high quantity to guarantee good amplification, and the amount of PCR product approximately doubles in each cycle. As the cycles progress and reagent components of the reaction begin to be depleted, the reaction will begin to slow down, the PCR product will no longer be doubled in every cycle, and non-exponential amplification will occur where the samples begin to diverge in their quantities. After several rounds of PCR amplification, the PCR reaction will no longer generate a template, due to the lack of critical reagents in the reaction tube. This is commonly referred to as the plateau phase, or end-point.

All samples that start with the same quantity of all PCR components and DNA concentration at the beginning of the reaction will end up at different points in the plateau phase, due to the different kinetics each sample had during thermal cycling. More precise measurements are made during the exponential phase, in which the replicate samples are amplified exponentially. Therefore, only in the exponential phase in a real-time reaction is quantification possible. In conventional PCR, the amplified products are analysed by gel electrophoresis (size-fractionation), and visualized by ethidium bromide. This process will therefore measure the final DNA obtained during the PCR reaction, which can be compared to measuring the amount of DNA in the plateau phase. At this point, quantification is exceedingly difficult, since the PCR gives essentially the same amount of PCR product independently of the initial amount of DNA template molecules that were present at the beginning.

6.4.1. Basic terms used in real-time PCR

Before the levels of a nucleic acid (DNA or cDNA) target can be quantified and analysed by real-time PCR assay, the raw data must be processed. Basic terms used in data analysis are described below, and in Figure 12.

Baseline

The baseline of the real-time PCR reaction is the signal level during the initial cycles of PCR, usually cycles 3 to 18, in which increases in fluorescence are not detectable. The baseline in real-time PCR can be determined empirically by user analysis for each reaction, or automated analysis of the real-time amplification plot. The baseline should be carefully set to allow accurate determination of the threshold cycle (Ct; defined below). The baseline determination should take into

account enough cycles to eliminate the background found in the early cycles of amplification, but should not include the cycles in which the amplification signal begins to rise above the background.

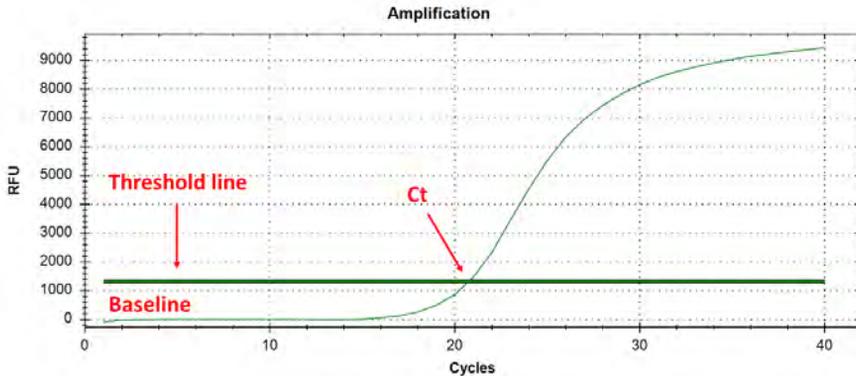


Figure 12. Example of a single amplification plot illustrating the nomenclature commonly used in real-time PCR

Threshold

The threshold of the real-time PCR reaction is the level at which the signal shows a statistically significant increase over the calculated baseline signal. Threshold is set to distinguish relevant amplification signal from background, and must be set within the linear region of the amplification curve, which represents the detectable log-linear range of the PCR. Generally, real-time PCR instrument software automatically sets the threshold at 10 times the standard deviation of the fluorescence value of the baseline.

Ct (threshold cycle)

The threshold cycle (Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold. The Ct allows calculation of the starting template amount present in the reaction, because the Ct value is measured in the exponential phase when the reagents are not limited. The Ct of a real-time PCR reaction is determined mainly by the amount of template DNA/cDNA present at the start of the amplification reaction. If a large amount of template is present at the start of the reaction, relatively few amplification cycles will be required to accumulate enough product to give a fluorescence signal above background. Thus, the reaction will have a low Ct. In contrast, if a small amount of template

is present at the start of the reaction, more amplification cycles will be required for the fluorescence signal to increase above background. Thus, the reaction will have a high Ct. This relationship forms the basis for the quantitative aspect of real-time PCR.

ΔCT value

The ΔCT value describes the difference between the CT value of the target gene and the CT value of the corresponding endogenous reference gene (so-called housekeeping gene) and is used to normalize for the amount of template by following formula:

$$\Delta CT = CT (\text{target gene}) - CT (\text{endogenous reference gene})$$

Efficiency and slope

The slope of the log-linear phase of the amplification reaction provides an indication of the efficiency of the real-time PCR. To obtain accurate and reproducible results, reactions should have an efficiency as close to 100% as possible, equivalent to a slope of -3.32. The efficiency of a real-time PCR assay can be calculated by analysing a template dilution series, plotting the CT values against the log template amount, and determining the slope of the resulting standard curve. From the slope (S), efficiency can be calculated using the following formula:

$$\text{PCR efficiency (\%)} = (10^{-1/S} - 1) \times 100$$

Generally, most amplification reactions do not reach 100% efficiency due to experimental limitations.

6.4.2. Real-time PCR reaction components

As in traditional PCR, real-time PCR reaction requires the addition of deoxynucleotides (dNTPs) solution mix, reaction buffer, sequence-specific primers, *Taq* polymerase, magnesium and water. Moreover, real-time PCR requires one additional component, either a fluorescent dye that binds double stranded DNA or a fluorescent labelled probe.

Thermostable DNA polymerase

DNA polymerases play the most important role in replicating the target DNA/cDNA. *Taq* DNA polymerase, isolated from the eubacterium *Thermusaquaticus*, is the most commonly-used enzyme for standard end-point PCR, as well as real-

time PCR reactions. *Taq* DNA polymerase has relatively high thermostability, with a half-life of approximately 40 minutes at 95°C. It incorporates nucleotides at a rate of about 60 bases per second at 70°C and can amplify lengths of about 5 kb. One of the key factors which affecting PCR specificity is the fact that *Taq* DNA polymerase has residual activity at low temperatures. Primers can anneal non-specifically to DNA, allowing the polymerase to synthesize nonspecific product. This problem can be minimized by using a 'hot-start' enzyme. Using a hot-start enzyme ensures that no DNA polymerase is active during the reaction setup or the initial DNA denaturation step. Usually, the optimal concentration of *Taq* DNA Polymerase ranges from 5–50 units/ml (0.25–2.5 units/50 µl reaction), are sufficient for amplification of target DNA/cDNA.

Deoxynucleotides (dNTPs) solution mix

A dNTPs solution mix is used in real-time PCR reactions, is an equimolar solution of ultrapure dATP, dCTP, dGTP and dTTP, and is used as the building blocks of new DNA/cDNA strands. These four nucleotides are typically added to the PCR reaction in equimolar amounts for optimal base incorporation. In common real-time PCR applications, the recommended final concentration of each dNTP is generally 0.2 mM. Note, however, that dNTPs exceeding optimal concentrations can inhibit real-time PCR reaction.

Template

Both traditional PCR as well as real-time PCR templates for replication can have any DNA source, such as genomic DNA (gDNA), complementary DNA (cDNA), or plasmid DNA. Recommended amounts of DNA template for a 50 µl reaction are as follows: 10 to 1,000 copies of template nucleic acid for each real-time PCR reaction. This is equivalent to approximately 100 pg to 1 µg of genomic DNA, or cDNA generated from 1 pg to 100 ng of total RNA. Note, however, that excess template nucleic acid can bring with it higher contaminant levels that can greatly reduce PCR efficiency. Depending on the specificity of the PCR primers for cDNA rather than genomic DNA, it may be important to treat RNA templates to reduce the chance that they contain genomic DNA contamination. One of the better options is to treat the template with DNase I.

Primers (sequence-specific oligonucleotides)

Oligonucleotides, also known as 'oligos' or 'primers', are short (approx. 15–30 bases), single-stranded polymers of nucleic acid. PCR primers are designed to bind by sequence complementarity to sequences that flank the region of interest in the template DNA/cDNA. During PCR amplification,

DNA polymerase extends the primers from their 3' ends. As such, the primers' binding sites must be unique to the vicinity of the target with minimal homology to other sequences of the input DNA to ensure specific amplification of the intended target. Generally, primers should be 18–24 nucleotides in length. This provides for practical annealing temperatures. In addition, primers should be designed according to standard PCR guidelines. They should be specific to the target sequence and be free of any internal secondary structure. Primers should avoid stretches of homopolymer sequences (e.g. poly (dG)) or repeating motifs, as these can hybridize inappropriately. Primer pairs should have compatible melting temperatures (within 5°C) and contain approximately 50% GC content. Primers with high GC content can form stable imperfect hybrids. Conversely, high AT content depresses the T_m of perfectly matched hybrids. If possible, the 3' end of the primer should be GC-rich (GC clamp) to enhance annealing of the end that will be extended. Primer pair sequences should be analysed to avoid complementarity and hybridization between primers (primer-dimers).

Primers for qRT-PCR reactions should ideally be designed to span an exon-exon junction, with one of the amplification primers potentially spanning the actual exon-intron boundary of the mRNA, to allow differentiation between amplification of cDNA and potential contaminating genomic DNA by melting curve analysis. To confirm the specificity of designed primers, a BLAST search of public databases should be performed, to ensure that primers only recognize the target of interest. It has been shown that the amplicon length should be approximately 50–150 bp, as longer products do not amplify as efficiently and because the final concentration of 200 nM for each primer is effective for most reactions.

Optimal primer sequences and appropriate primer concentrations are essential for maximal specificity and efficiency in both traditional PCR and real-time PCR. Good primer design is one of the most important parameters in real-time PCR, and is especially critical when using DNA-binding dyes for amplicon detection. There are multiple free primer design tools available online that produce high quality primers based on the criteria listed above. One popular web-based program for primer design is *Primer3.0* ([http://bioinfo. ut. ee/primer3-0. 4. 0/.](http://bioinfo.ut.ee/primer3-0.4.0/))

Magnesium

Magnesium ion (Mg^{2+}) functions as a cofactor for activity of DNA polymerases by enabling incorporation of dNTPs during DNA/cDNA polymerization. The magnesium ions at the enzyme's active site catalyse phosphodiester bond formation between the 3'-OH of a primer and the phosphate group of a dNTP. Furthermore, Mg^{2+} facilitates formation of the complex between the primers and DNA templates by stabilizing negative charges on their phosphate backbones. In real-time PCR, magnesium chloride or magnesium sulfate is typically used at a final concentration of 3 mM. It has been shown that this concentration works

very well for most targets, however the optimal magnesium concentration can vary between 3 and 6 mM. Note, however, that low Mg^{2+} concentrations result in little or no PCR product, because of the polymerase's reduced activity. On the other hand, high Mg^{2+} concentrations often produce nonspecific PCR products from enhanced stability of primer-template complexes, as well as increases in replication errors from mis-incorporation of dNTPs.

6.4.3. Real-time PCR protocol

There are three main steps that make up each cycle in a real-time PCR reaction: denaturation, annealing and extension, which are described in detail below.

1. Denaturation step: High temperature incubation is used to 'melt' double-stranded DNA into single strands and loosen the secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 95°C). The denaturation time can be increased if the GC template content is high.

2. Annealing step: During this phase, complementary sequences have an opportunity to hybridize, so an appropriate temperature is used, based on the calculated melting temperature (T_m) of the primers (5°C below the T_m of the primer).

3. Extension step: At 70–72°C, the activity of the DNA polymerase is optimal, and primer extension occurs at rates of up to 100 bases per second. When an amplicon in real-time PCR is small, this step is often combined with the annealing step using 60°C as the temperature.

6.4.4. Reverse Transcription Quantitative PCR (RT-qPCR) – RNA as the starting material

RT-qPCR terms is used when RNA is a starting material. It is used in a variety of applications, such as gene expression analysis, RNA validation, microarray assays, pathogen detection and also various genetic tests. In general, two methods are available for quantification of gene expression by RT-qPCR: two-step RT-qPCR and one-step RT-qPCR. In both cases, RNA is reverse-transcribed into cDNA, and the cDNA is then used as the template for qPCR amplification. 'One-step' and 'two-step' refer to whether the RT and real-time PCR amplification are performed in the same, or separate tubes. In the two-step method, RNA is first transcribed into cDNA in a reaction using reverse transcriptase. Reverse transcriptase is an enzyme that makes a molecule of DNA from an RNA template. Some enzymes have RNase activity that allows for degradation of the RNA strand in the RNA-DNA hybrid after transcription. If an enzyme does not possess RNase activity, an RNaseH may be added for

better qPCR efficiency. Commonly used enzymes include Moloney murine leukaemia virus reverse transcriptase, and Avian myeloblastosis virus reverse transcriptase. RNA is reverse transcribed into cDNA using oligo-dT primers, random oligomers, or gene-specific primers. Next, an aliquot of the resulting cDNA is then used as a template for multiple qPCR reactions. In the one-step method, RT and qPCR are performed in the same tube, and so this method is also referred to as 'one-tube RT-PCR'.

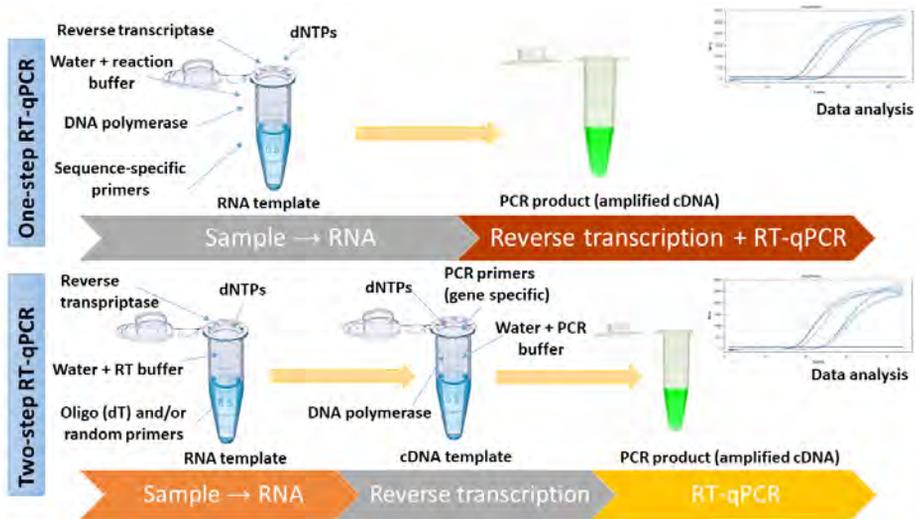


Figure 13. One-step vs two-step RT-PCR (*figure used with permission under Creative Commons license*)

6.4.5. PCR product detection in real-time PCR

Real-time PCR and RT-PCR allow for accurate quantification of starting amounts of DNA, cDNA, and RNA targets. Fluorescence is measured during each cycle, which greatly increases the dynamic range of the reaction, since the amount of fluorescence is proportional to the amount of PCR product. PCR products can be detected using either fluorescent dyes that bind to double-stranded DNA (e.g. SYBR® Green I and EvaGreen), or fluorescently labelled sequence-specific probes, such as TaqMan and Molecular beacons. The change in fluorescence over the course of the reaction is measured by an instrument that combines thermal cycling with fluorescent dye scanning capability. By plotting fluorescence against the cycle number, the real-time PCR instrument generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction.

6.4.6. Molecular detection of Bacteria by real-time qPCR assays

Bacterial genome

Deoxyribonucleic acid (DNA) is present in all living organisms. This amazing macromolecule encodes all of the information needed to program a cell's functions, such as reproduction, metabolism and other specialized functions. DNA is comprised of two strands of deoxynucleotides. Each deoxynucleotide contains a phosphate, a 5-carbon sugar (2-deoxyribose), and one of four nitrogenous bases: adenine, cytosine, thymine or guanine. The phosphate and sugar make up the backbone of each strand of DNA, while the bases are responsible for holding the two strands together *via* hydrogen bonds in a structure called the double helix. The order of the bases in a DNA strand contains the coded genetic information. All of the DNA found in an organism is collectively referred to as the genome.

Bacterial genomes are generally smaller and less variant in size among species when compared with genomes of animals or single cell eukaryotes. Generally, a bacterial genome is composed of a single, circular chromosome. In addition, bacteria may have one or more smaller circular DNA molecules, called plasmids, that contain (usually) non-essential genes.

Bacterial identification methods based on biochemical tests, such as API system and conventional culture methods, have been traditionally used for the detection and identification of pathogens. In recent years, new molecular methods based on DNA amplification and quantitative polymerase chain reaction (qPCR) have been proposed for the rapid and high-sensitive detection of pathogens.

DNA and RNA extraction from bacteria

To extract a functional macromolecular component from bacterial cells, three things need to happen:

1. Efficient disruption of the bacterial cell wall and cell-membrane system, to facilitate extraction of the desired components.
2. Everything must be done under conditions that either inhibit or destroy the many degradative enzymes released during cell disruption, including the nucleases and proteases.
3. You must employ a fractionation procedure that separates the desired macromolecule from other cellular components with high-efficiency and purity.

The typical procedure for genomic DNA preparation from a culture of bacterial cells can then be divided into four steps:

1. Probing the sample.
2. Preparation of a cell extract (lysozyme, EDTA or combination of both; SDS).

3. Purification of DNA from a cell extract (organic extraction; phenol or phenol chloroform, or enzyme digestion – protease, ribonuclease).

4. Concentration of DNA samples (ethanol precipitation).

RNA isolation from a bacterial cells is based on the following principle: RNA is separated from DNA after extraction with an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol and chloroform, followed by centrifugation. Under acidic conditions, total RNA remains in the upper aqueous phase, while most of the DNA and proteins remain either in the interphase or the lower organic phase. Next, total RNA is then recovered by precipitation with isopropanol.

Either total RNA can be used for most real-time RT-qPCR applications. One critical consideration in working with RNA is to eliminate RNases in your solutions, consumables and labware. RNase-free solutions can be purchased, or your solutions can be treated with diethyl pyrocarbonate (DEPC), and then autoclaved. RNases on labware can also be inactivated with DEPC treatment or by baking at 250°C for 3 h. Extracted RNA samples may also need DNase treatment to prevent potential amplification of any contaminating genomic DNA, which could lead to overestimation of the copy number of an mRNA.

6.4.7. Detection and quantification of *Bacillus* sp. by real-time qPCR methods

A large number of real-time PCR procedures are available for the specific detection and quantification of these bacteria, which are described below.

SYBR Green-based real-time qPCR assays

SYBR Green assay uses a pair of PCR primers that amplifies a specific region within the target sequence of interest and includes a double-stranded DNA (dsDNA)-binding dye for detecting the amplified product. A SYBR Green qPCR reaction contains the following components:

- PCR master mix with SYBR Green dye;
- DNA template;
- Specific primers.

It can be used for the following assay types:

- One-step RT-PCR for RNA quantitation;
- Two-step RT-PCR for RNA quantitation;
- DNA/cDNA quantitation.

SYBR Green binds to the minor groove of dsDNA – when it is free in a solution it displays relatively low fluorescence levels, but when dsDNA is added, the fluorescence increases over 1,000 times. Therefore, the greater the amount of dsDNA present in the reaction tube, the greater the amount of DNA

binding and fluorescent signal from the SYBR Green. As the accumulation of new amplicons increases with the PCR reaction cycles, the intensity of fluorescence also increases, because more SYBR Green molecules bind to the dsDNA. Thus, the accumulation of product can be measured in real time. However, nonspecific PCR products and primer-dimers also contribute to the fluorescent signal. Therefore, qPCR reactions using SYBR Green should be sensitive and specific, and should exhibit good amplification efficiency over a broad dynamic range. In addition, melt-curve analysis of reaction product from a SYBR Green assay should be used to distinguish specific products from non-specific products.

TaqMan probe-based real-time PCR assays

A TaqMan probe assay uses a pair of PCR primers and a dual-labelled, target-specific fluorescent probe. A TaqMan probe-based qPCR reaction contains the following components:

- PCR master mix;
- DNA/cDNA template;
- Primers;
- Probe(s).

TaqMan probes are also known as 5'-nuclease probes, because the 5'-exonuclease activity of DNA polymerase cleaves the probe. In terms of structure, hydrolysis probes are sequence-specific dually fluorophore-labelled DNA oligonucleotides. One fluorophore is the 'quencher', and the other is the 'reporter'. When the quencher and the reporter are in close proximity – that is, they are both attached to the same short oligonucleotide – the quencher absorbs the signal from the reporter. This is an example of fluorescence resonance energy transfer (also referred to as a 'Forster transfer'), in which energy is transferred from a 'donor' (the reporter) to an 'acceptor' (the quencher) fluorophore. During DNA amplification, the oligonucleotide is broken apart by the action of DNA polymerase (5'-nuclease activity), and the reporter and quencher separate, allowing the reporter's energy and fluorescent signal to be liberated. Thus, destruction or hydrolysis of the oligonucleotide results in an increased reporter signal, and corresponds with the specific amplification of DNA. As with SYBR Green assays, an optimized TaqMan assay should be sensitive and specific, and should exhibit good amplification efficiency over a broad dynamic range.

The TaqMan probe can be used for the following assays:

- One-step RT-PCR for RNA quantitation;
- Two-step RT-PCR for RNA quantitation;
- DNA/cDNA quantitation.

Over numerous studies, this method has been developed and validated for quantitative and specific detection of *Bacillus* spp.

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MODULE III

**PRACTICAL FIELDWORK
TRAINING IN THE
DETECTION AND
SAMPLING OF
BIOLOGICAL MATERIALS**

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1. ORGANISATION OF THE BIOLOGICAL SAMPLING PROCESS

1.1. Introduction

A well-planned and coordinated biological sampling process during the CBRN incident is extremely important for further operations, both military and civil. For this reason, the collect of different types of samples must be carried out in accordance with a detailed plan, to provide as much information as possible. Further procedures in the detailed diagnosis and analysis of the collected material is carried out by specialized laboratories (mobile field labs and stationary reference laboratories).

The process of planning and conducting sampling activities involves thorough coordination and careful execution. Teams performing sampling activities must be well trained and have the specialized equipment to perform the sampling process correctly. CBRN command officers must plan and coordinate the entire sampling process to ensure the safety and high quality of the samples. Task include command and control, sampling, packing and transport, analysis and interpretation of the data within the chain of custody.

1.2. Composition of survey, sampling and decontamination tasks

A CBRN investigation is the directed effort to determine the nature and degree of CBRN hazards in the area of contamination, and to outline the boundaries of the hazard area. Environmental sampling is the first important and critical step in determining the nature and scope of the threat from a biological agents. Since sampling is a key issue for biological detection, the way a sample is taken and how it is handled will important affect the result of the analysis. In this reason well-developed and detailed planning is essential element in the sampling process.

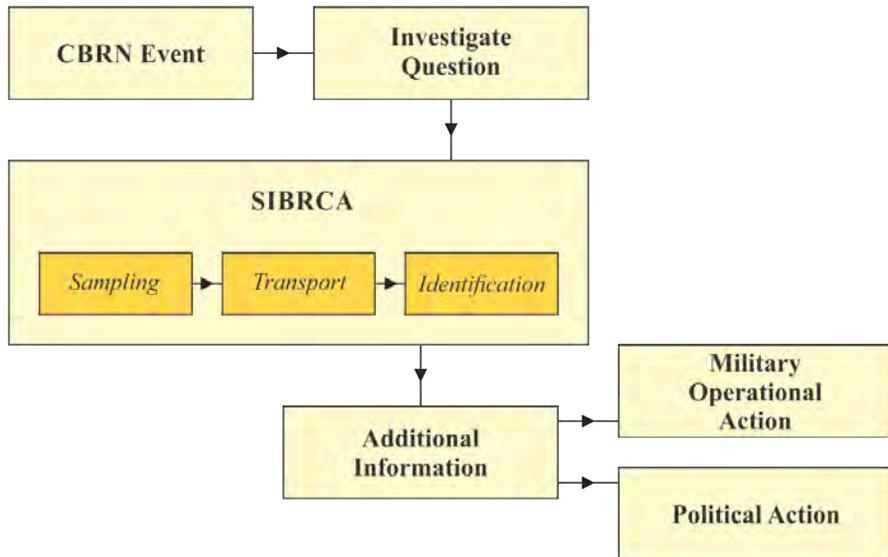


Figure 1. The place of SIBCRA missions in the CBRN event-response chain

All substantial procedures concerning sample collection and processing should be based on documented Standing Operating Procedures (SOP). The SOPs should describe necessary equipment/instrumentation, proper equipment use, and step-by-step procedures in each operational phase (immediate, urgent, late).

An extremely important step during the sampling process is the decontamination of personnel, equipment and vehicles involved in the SIBCRA mission.

The aim of decontamination is to neutralize or eliminate contamination completely or at least to the extent that the operation can be continued without individual protection. The levels of decontamination include: **immediate decontamination** (minimize casualties and limit spread of contamination), **operational decontamination** (done by the sampling personnel or specific decontamination teams), **clearance decontamination** (conducted by specialized CBRN units). All elements/units, including sampling issue, should be capable of conducting their own operational decontamination.

1.3. Essential equipment for survey, sampling and decontamination personnel

Sampling Team (SIBCRA team) are responsible for the collecting samples, preliminary identification (in situ analysis *via* portable instruments and immunochemical hand-held kits), packaging and transport samples to the

laboratory (field deployable lab or reference lab). The collection of specimens from humans and small animals as a result of any suspected contamination, requires the augmentation of these teams by additional technician specialists.

The Sampling Team must operate according to established and verified operational procedures. Uniformity, safety, and accountability in sampling procedures must be ensured. The procedures for transport and handling of CBR samples and specimens are covered by national directives. Proper chain of custody has to be ensured and documented to establish forensic evidence, so that unambiguous identification by certified laboratories is achievable. SOPs should be exercised regularly as part of the basic training.

The size of a typical sampling team (Specialist Sampling Team) is four to five personnel. If it required the SIBCRA team size will have to be increased to take into account the additional duties. The tasks assigned to the people on the Specialist Sampling Team are:

- Team Leader (directs the team);
- Sampling Technician(s) (responsible for collect/transport samples);
- Communications Technician (responsible for recording all important information concerning the sample and sample collection process, use communication systems, operate a video camera);
- Transportation Technician (responsible for movement of sampling personnel, equipment, and samples).

The Specialist Sampling Team must be well-trained and be involved in systematic basic and specialized training (**basic training** needed: Specialist CBRN Unit training; Chemical and Biological Occupational Protection, Emergency Procedures and Personal Protection; **specific training** needed: Environmental Sampling Techniques; Sample management, Shipping Procedures).

Training of personnel responsible for sample collection, handling, and analysis is important to providing the reliability of results from sample analysis. Consequently, proper training in sampling methods, handling of biological samples, and understanding of the effects of biological and environmental factors on analytical results should be a permanent and repeatable element of specialist training.

Additional Specialists will be needed to carry out specific SIBCRA missions. The standard team may be supplemented with different specialists, for example: **forensic specialist** (trained in proper crime scene investigations and particularly for forensic SIBCRA missions), **medical specialist** (for all issues involving biomedical sampling), **CBRN technology specialists** (for specific knowledge of agent production facilities), **EOD specialists** (Explosive Ordnance Disposal). A specialist may be required to distinguish agent production from other 'dual use' applications. Such extended teams are called to as **Scientific Advisor Sampling Team/Scientific Advisor Forensic Sampling Team** for a team increased with a forensic specialist.

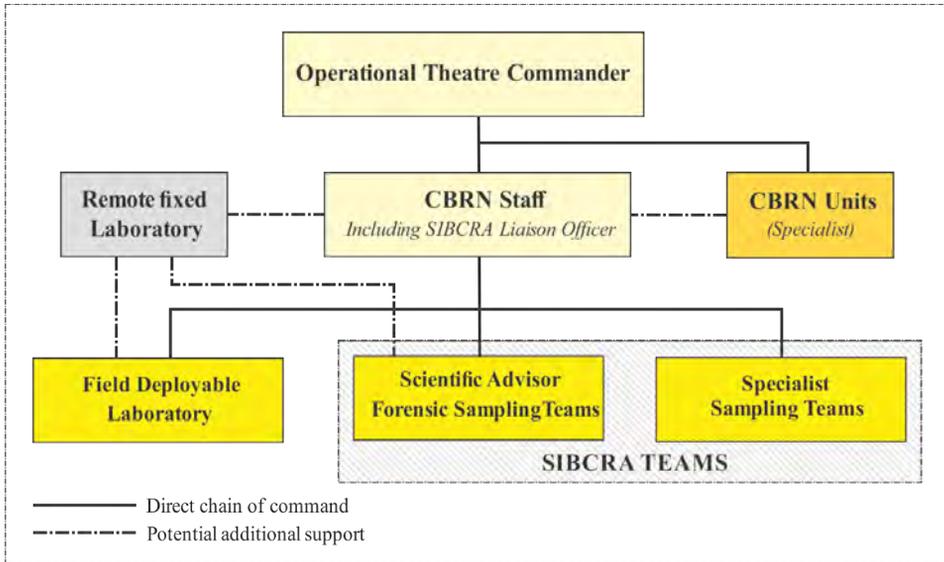


Figure 2. Suggested operational structure for SIBCRA missions

The Forensic Sampling Teams must be qualified to collect different types of samples required for an investigation. Scientific advisors can be both civilian and military personnel and they should have considerable expertise. It is expected that the Scientific Advisor staff will have in-depth knowledge of SIBCRA procedures and national or international forensic procedures. Scientific advisors can be employed in sampling teams or can be recruited as an external expert to manage SIBCRA missions. Scientific Advisor expertise should be involved in the risk analysis and selection appropriate protection equipment.

A very important step in the SIBCRA mission is decontamination. It is carried out by the sampling team (at the sampling site to prevent contamination of the material being collected, to avoid cross-reactions) and after leaving the sampling zone by a specialized **Decontamination Team**. Decontamination is carried out to reduce contamination on personnel, equipment, materiel and working areas. The primary aim of decontamination is restoring normal operational tempo during the SIBCRA mission. It is required that sample containers must be decontaminated before they leave the contaminated zone. Decontamination Team is responsible for decontamination of sampling personnel, material and equipment of all components the whole NBC contamination. In order to properly perform their tasks, the 'decon' team should be provided with appropriate decontamination equipment, for example: specialized decontamination chamber.

Sampling equipment necessary to conduct SIBCRA missions includes different supplies and instruments and depends on the nature of the threat and

type of sampling methods (air sampling, liquid sampling, solid sampling, surface sampling, biomedical sampling). The SOPs should list the equipment necessary for sampling and instruct personnel on proper use of the instruments.

Equipment used during sampling operation:

- Equipment common to all sampling/surveying: supplies, personal protective equipment, communication/location equipment, supporting documentation, samples transportation;

- General sampling equipment and supplies: general sampling tools, specific sampling equipment and supplies, container related supplies;

- Specific biological sampling equipment.

All samples should be collected and handled using clean sampling instruments (recommended single-used materials). For biological agents sterilised equipment is necessary to avoid sources of contamination and samples containers should be inert to avoid absorption or reaction with collected samples. All material should be inspected periodically to assure it is in good condition and will function as expected. Sufficient numbers of spare sampling tools should be maintained in the event that some equipment breaks or becomes contaminated during sampling. Larger equipment or electronic instruments should be protected from contamination by plastic or other protective container.

Commanders must be aware of the risks their forces take in performance of sampling mission. Similarly, sampling personnel must be responsible for their own protection in a contaminated environment. In this reason is required that the SIBCRA Team was equipped with Individual Protective Equipment (required, recommended, optional equipment). The basic protective equipment for most scenarios consists of respirators, disposable garments, hand and foot protection and other items which comprise the individual protective ensemble.

1.4. Sampling strategies for CBRN incidents

Sampling process is the first key element of a CBRN incident investigation (Fig. 3). Correct sampling of representative samples is very important to further operational and political action.

The aim of the sampling process is gaining of the representatives samples of material to the laboratory analysis to detect of the used biological agent and to defined of the scale of the contaminated area. To warrant the correct sampling process (considered choice of representative samples, no cross-contamination, viability, safety etc.) it should be conducted by trained personnel with specific equipment. In CBRN incident investigation are two types of sampling process: field and forensic sampling.

Field sampling is designed to allow the commander to making early operational decisions about further operational activities of various services

(emergency, medical, decontamination) in the field, as well as the selection of appropriate protective equipment (protective clothing, respiratory protection etc.). The samples are usually collected by the sampling team in the primary composition.

Forensic sampling is performed for strategical and/ or political/ military purposes. The samples have to be collected by the sampling team extended by additional specialists (investigator, forensic expert, medical expert, epidemiologist, CBRN specialist), and the samples are used to confirm the unambiguous use of the CBRN agents by the opponent. Sample analysis is performed using the advanced diagnostic techniques in the reference laboratory to ensure that the result is undeniable. The design of sampling strategies for CBRN incidents needs to provide to sampling team the key information (sample types, size and numbers, places where the samples should be collected, needs equipment) to correct perform the sampling event. The design should be objective in nature, technically defensible, and practical to implement. A well designed strategy is needed to obtain the maximum amount of information from the number of samples.

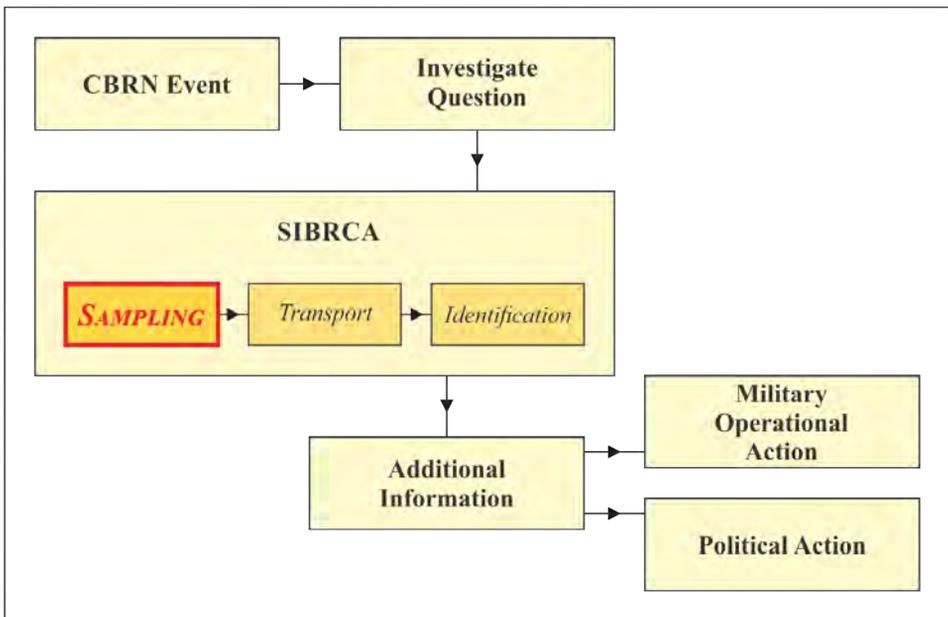


Figure 3. Position of the sampling process in the SIBRCA missions in the CBRN event-response chain

Designation of the sampling process can be delineated for three separate goals: identification, determination of intensity, or confirmation.

Accomplishing each of these goals is possible through the using of different sampling strategies:

- Judgmental for identification purposes;
- Systematic for determining the intensity;
- Random for confirmation purposes.

For some types of operations (CBRN or criminal investigation, environmental repair) it is possible to use a combination of these strategies.

Judgmental Sampling. In judgmental sampling, only a small numbers of samples are collected and sampling is directed toward a specific incident where the probability of finding the contaminated material is very high. The advantages of the judgmental strategy of sampling that it operational cost is less, than other strategies. However in interpretation of the results of analysis the high care level have to be taken, because the valid of the data is dependent on intelligence activities and knowledge about the site.

Judgemental sampling can be useful to:

- Provide information on which agent or agents were used in a particular CBRN incident;
- Confirm the presence or level of contamination in a specific location (e.g. 'the worst' location);
- Provide the information needed to identify the next operational phases.

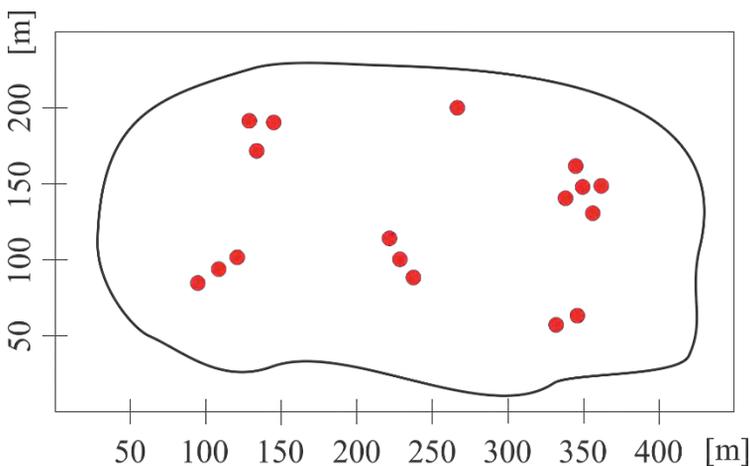


Figure 4. Judgmental Sampling Strategy

Systematic Sampling. Systematic sampling is much less biased than previous sampling strategy. To ensure complete coverage of the area, a grid model is used where the samples are collected in the intersection of the grid lines. This type of strategy helps in defining the level of contamination in different places and boundary of contamination but needed larger numbers of samples.

Systematic sampling can be useful to:

- Shows gradient of contamination of area (help created the border between hot and warm zone in CBRN incident);
- Criminal/ CBRN investigation, decontamination preparations.

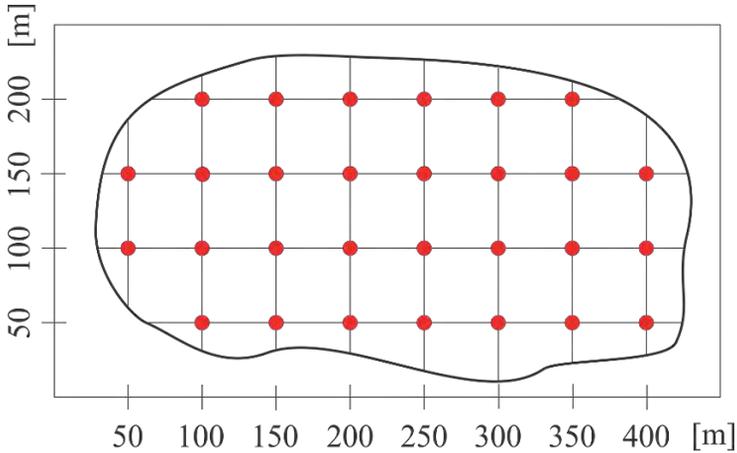


Figure 5. Systematic Sampling Strategy

Random Sampling. Random or unsystematic strategy of sampling may be very useful when the specific contaminated area is no known or when there is concern, but a lack of definitive information to selection a specific sampling zone. It requires a very large number of samples. This strategy of sampling is also known as a surety operation, assists in determining whether a threat remains in the location, e.g. after decontamination process.

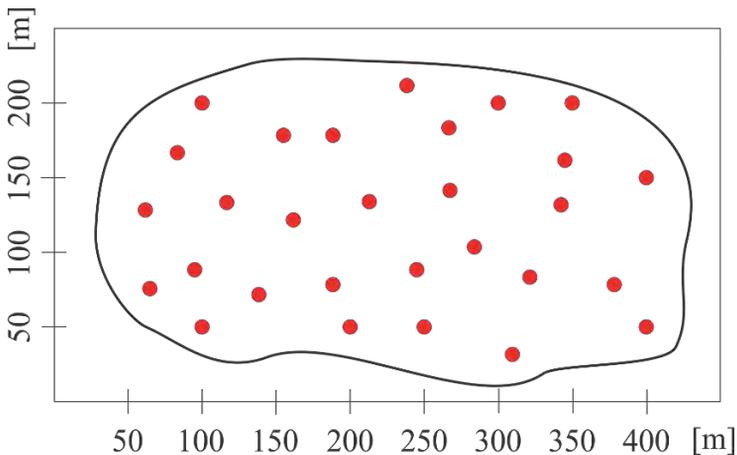


Figure 6. Random Sampling Strategy

During development of the strategy and selection of the sampling methods to be used, the following elements must be considered: legal regulations; available intelligence (risk analysis, type of CBRN incident, activity of military and/or paramilitary organisations, the epidemiological situation etc.); existing equipment; required size and number of samples; the diagnostics capacity of field and/or reference laboratories (available procedures and analytical equipment), and costs.

1.5. Phases in the process of biological sampling

Biological sampling process (as well as other CBRN factors) have the following phases:

- Immediate Phase Operation;
- Urgent Phase Operation;
- Late Phase Operation.

The task of the first phase (*Immediate Phase Operation*) should be made as soon as possible (within the first minutes after the incident to several hours maximum). Commanders must prepare a model decision tree CBRN operations which include force protection, the management of casualties and confirmation of the hazard. The success of the operation depends on the use of airborne and surface detectors (including CBRNs), as well as having regard the following vectors:

- Mission (establish level and type of threat);
- Enemy (adequate protection of the personnel);
- Terrain and weather (impact on the spread of hazardous material);
- Military (support of specialized military forces);
- Time (to be effective, protective actions must be implemented as soon as possible post incident. This requires an intensive and sustained effort of surveying and sampling, particularly during the first minutes to hours post event).

The listed factors are included in the first steps of model post-incident decision tree (Fig. 7) and should be prepared by the SIBCRA Team (Flow Chart Actions).

Where the information provided by the intelligence reports the high risk of CBRN agents, it is necessary to notify, consult or deploy specialized groups. Deployment of these groups is also indispensable if the threat is detected by the reconnaissance and monitoring forces. When a threat is detected, you should adopt a protective attitude and employ detection by using personal security and detection measures. It is also important to generate and send a report of the incident to the higher headquarters. If the incident is the result of criminal, terrorist or enemy activity, the higher headquarters may request the deployment of additional forensic sampling teams.

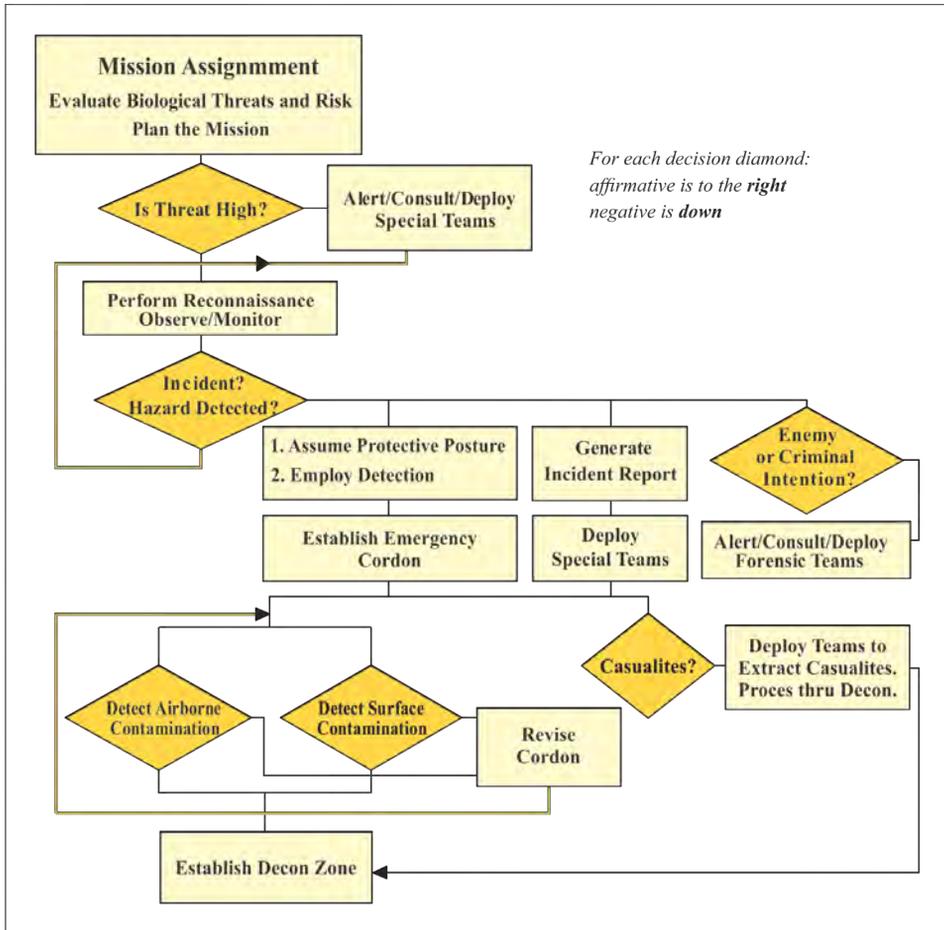


Figure 7. Immediate Phase Operation

Next to, and with the information from the monitoring and protection activities the Emergency Cordon is created (minimum 100 meters up to several kilometres depending on the incident size and expected hazard area). The revise of Emergency Cordon is the result of continuous monitoring hazard area (SIBCRA Team task) and have simultaneously affects to the location of the decontamination zone. Contamination control area (CCA) should be established in the upwind warm zone border with the cordon area. If there are also casualties, they should be secured by appropriately trained medical personnel.

The purpose of Urgent Phase Operations is to allow military and political authorities to initiate effective protective actions and countermeasures in order

to prevent further direct exposure, ingestion, inhalation, and dispersion of contamination, and to begin the process of site recovery (Fig. 8).

SIBCRA Team in this phase should:

- Air sampling (assessing airborne contamination);
- Soil or road dust sampling (assess environmental contamination level);
- Field identification (in situ analysis *via* portable instruments, *via* deployed laboratory, preliminary data from immunochemical hand-held test kits);

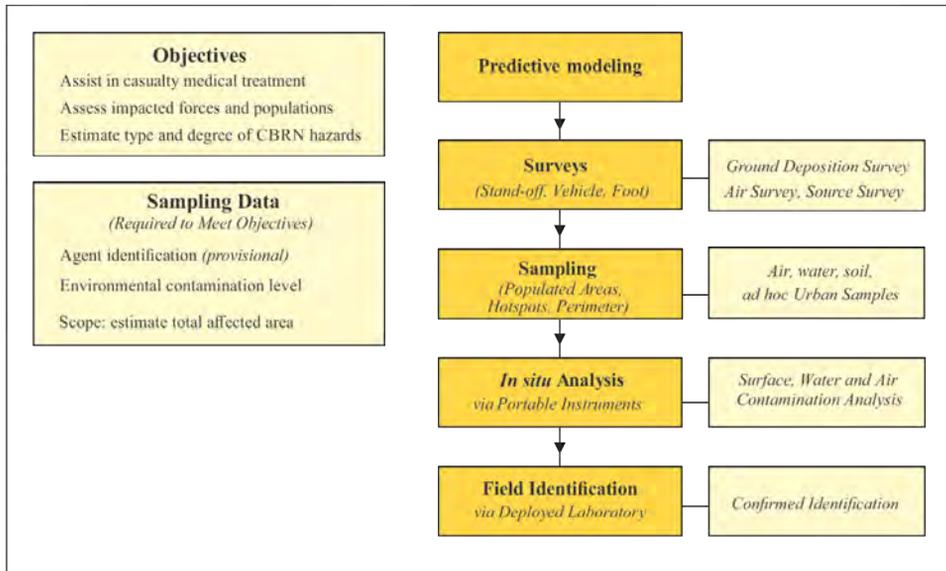


Figure 8. Urgent Phase Operation

The third Late Phase Operations (Fig. 9) require monitoring contamination levels for the protection of local populations and deployed forces from direct exposures, ingestion of contaminated materials, and inhalation of re-suspended material. Current research include determining the location and degree of the environmental contamination and comparing the result with the exposure limits.

Types of samples:

- Soil/ urban sampling;
- Water, milk, and other foodstuffs originating from contaminated areas;
- Bioassay samples (to support post-exposure assessment);
- Quality control samples to support accuracy and precision of laboratory measurements;

This activity is likely to occur days to potentially months following the incident.

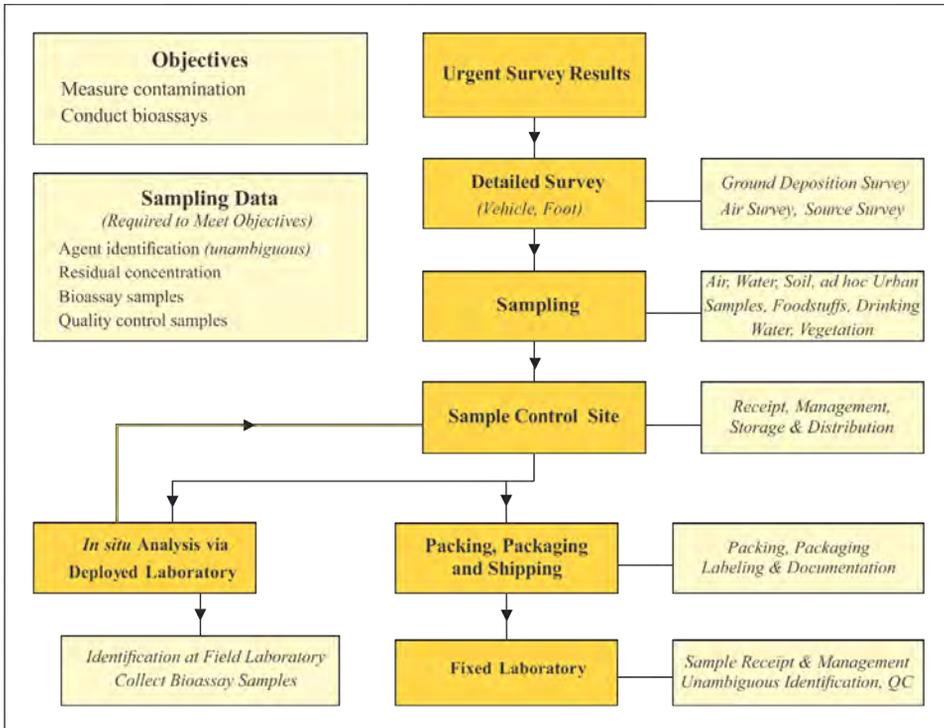


Figure 9. Late Phase Operations

1.6. Documenting the sampling process

Each sample can be identified if it is written on or affixed to the sample container. The safest and simplest way to label a sample is writing on the sample container. The other method of labelling samples is to use tags or adhesive labels which should be affixed immediately after placing the sample in container. In addition to resistance to external factors (e.g. degradation, fading, temperature) labelling should not contaminate the sample. The sample number or ID should be clearly described on the sample and additional sample data sheet. Further the label should contain as much information as possible. Individual sample identifier can be a combination of site location and time. The following method is recommended: **ALATLOGDDT'TT'ZMMMYYYYXX**.

These tags are described in the table below:

Table 1. Sample ID tags

Designation	Specification	Examples
A	Media description	A – Air S – Soil H – Hydrological samples (Water and Snow) V – Vegetation D – Dairy (Milk) G – Grain M – Meat O – Other Foodstuffs U – Urine F – Faeces W – Wipes (Smears and Swipes)
LATLOG	6 digit latitude-longitude GPS or other geographic coordinate system (np. UTM)	514155021974 – 51° 24' 56" N 21° 58' 29" E 34N 567782 5696489 – UTM
DD	Day of the month	05
TTTT	Time (24 hours system)	1654
Z	Time zone	A – alpha Z – Zulu time
MMM	Alphabetically abbreviated month	AUG – August JUN – June
YYYY	Year	2017
XX	Sequential number (identify field duplicate or split samples, or sampling method)	02 – second field sample

Each sample for analysis must have accompany sample data sheet containing the following critical information:

- Data sheet general code number;
- Specific identification number of sample;
- Sample distinction, field blank and duplicate;
- Operation (or incident) identification;
- Date and time of operation (incident);
- Grid and GPS sampling location;
- Sample site description (may refer to photographs or video);
- Casualties symptoms;

- Sampled Medium;
- Method of sampling and used equipment;
- Preparation and preservation of sample;
- Name (or ID) of person (sampling team) collecting the sample;
- Physical and meteorological condition (at sampling time);
- Special handling, temporary storage or safety precautions;
- Result of field expedient assays using hand-held instruments;
- Signatures.

Examples of all data sheets for CBRN agents are included in Appendix C of AEP-66. The example sheets used for biological agents are shown below.

BIOLOGICAL ENVIRONMENTAL SAMPLE			
SAMPLE NUMBER:	<div style="border: 1px solid black; height: 20px; width: 100%; background-color: #e0e0e0;"></div>		
Team Leader:	-----		
Location ID:	GPS Coordinates: <table border="1" style="display: inline-table; border-collapse: collapse; text-align: center; width: 150px; height: 20px;"> <tr> <td style="width: 50%;">Latitude</td> <td style="width: 50%;">Longitude</td> </tr> </table>	Latitude	Longitude
Latitude	Longitude		
	Time and date (global): <table border="1" style="display: inline-table; border-collapse: collapse; width: 150px; height: 20px;"></table>		
Location Description: <i>(Address if applicable)</i>	-----		
Weather Conditions:	-----		
Field Observations:	----- -----		
Sample Type	<input type="checkbox"/> Normal sample		
QC:	<input type="checkbox"/> Duplicate <input type="checkbox"/> Co-located <input type="checkbox"/> Control sample		
	QC: <input type="checkbox"/> Background <input type="checkbox"/> Field blank		
Sample Media	<input type="checkbox"/> Swipe <input type="checkbox"/> Biota <i>(specify)</i> -----		
	<input type="checkbox"/> Soil <input type="checkbox"/> Gutter dirt		
	<input type="checkbox"/> Sediment <input type="checkbox"/> Surface water <i>(specify)</i> -----		
	<input type="checkbox"/> Vegetation <input type="checkbox"/> Drinking water		
	<input type="checkbox"/> Precipitation <input type="checkbox"/> Road / surface dust		
	<input type="checkbox"/> Roof tiles <input type="checkbox"/> Ad hoc		
Sample Description <i>e.g. type of soil, quantity (volume or mass), sampled area and depth</i>	----- -----		
Sample Tool - Area:	----- cm ²		
Sampling method	-----		

Figure 10. Biological Environmental Sample Form

BIOLOGICAL FOODSTUFF SAMPLE

SAMPLE NUMBER:

Team Leader: _____

Location ID: _____

GPS Coordinates:	Latitude	Longitude
Time and date (global):		

Location Description:
(Address if applicable) _____

General Foodstuffs

Food Type: _____

Location of acquired sample in production line _____

Source location of raw product _____

Distribution points for foodstuffs _____

Reason for Sampling: _____

Milk Specific Cow Goat Other: _____

Location of acquired sample in production line _____

Source location of raw product _____

Distribution points of milk: _____

Farm where cows originated: _____

Feed Type: Pasture Stored Feed Other: _____

Grazing Location: _____

Sample Description
e.g. type of soil, quantity (volume or mass), sampled area and depth _____

Figure 11. Biological Foodstuff Samples Form

CHAIN OF CUSTODY				
Sample ID	Date Time	Relinquished by <i>(Print and Sign Name)</i>	Received by <i>(Print and Sign Name)</i>	Purpose for Custody Change

FOR LAB USE ONLY Yes No NA

Package Received with Custody Seals Intact: Yes No

Sample Forms, Tags, and Chain of Custody Agree: Yes No

Figure 12. Chain of custody Form

BIOLOGICAL AIR SAMPLE					
SAMPLE NUMBER:	<div style="border: 1px solid black; height: 20px; width: 100%; background-color: #e0e0e0;"></div>				
Team Leader:				
Location ID:	GPS Coordinates: <table border="1" style="display: inline-table; border-collapse: collapse; text-align: center;"> <tr> <td style="width: 50%; padding: 2px;">Latitude</td> <td style="width: 50%; padding: 2px;">Longitude</td> </tr> <tr> <td colspan="2" style="padding: 2px;">Time and date (global):</td> </tr> </table>	Latitude	Longitude	Time and date (global):	
Latitude	Longitude				
Time and date (global):					
Location Description: <i>(Address if applicable)</i>				
Weather Conditions:				
Field Observations:				
Sampler	Model:				
	Type:				
	Filter Holder:				
Filter Media	Serial #:				
	Type:				
	Size:				
	Media:				
	Pre-sampling Weight:grams				
	Post-sampling Weightgrams				
Sample	Time From To Totalmin				
FLOW RATE	Begin l/min End l/min				
Total volume m ³				

Figure 13. Biological Air Sample Form

BIOLOGICAL BIOASSAY SAMPLE					
SAMPLE NUMBER:	<div style="border: 1px solid black; height: 20px; width: 100%; background-color: #e0e0e0;"></div>				
Team Leader:	-----				
Location ID:	GPS Coordinates: <table border="1" style="display: inline-table; border-collapse: collapse; text-align: center;"> <tr> <td style="width: 50%; padding: 2px 5px;">Latitude</td> <td style="width: 50%; padding: 2px 5px;">Longitude</td> </tr> <tr> <td colspan="2" style="padding: 2px 5px;">Time and date (global):</td> </tr> </table>	Latitude	Longitude	Time and date (global):	
Latitude	Longitude				
Time and date (global):					
Location Description: <i>(Address if applicable)</i>	-----				
Weather Conditions:	-----				
Field Observations:	----- ----- -----				
PATIENT					
Last Name:	-----				
First name:	-----				
ID Number	-----				
UNIT	-----				
Address	-----				
SEX	<input type="checkbox"/> MALE <input type="checkbox"/> FEMALE <input type="checkbox"/> Pregnant				
Sample					
Type	-----				
Quantity	-----				

Figure 14. Biological Bioassay Sample Form C-9

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2. PERSONAL PROTECTIVE MEASURES

2.1. Introduction

Collecting samples in an area potentially contaminated with CBRN agents is a challenging task. There are not only the hazardous materials to consider, but also other threats related to hostile activity or environmental hazards, such as IEDs or wild animals. These problems can also pose a significant risk to first responders and CBRN survey teams. Physically securing areas with armoured guards and antecedent activity of EOD patrol may reduce risk and allow personnel to focus on sampling mission. Nevertheless, there are unlimited scenarios for sampling which may happen during CBRN incident or terroristic attack, the common sense and general procedures constitute the base for successful missions. Depending on organizational approach or specific scenario, CBRN survey and sampling may rely on general NBC teams or specialized teams (chemical, radiological and biological). The biological agents with their intrinsic pathogenic and/or toxic properties, which include incubation period, communicability or unspecific symptoms and delayed diagnosis, are one of the greatest potential threats. Thus appropriate methods, practices and countermeasures have to be applied to reduce biological risk for first responders or sampling personnel. These include personal protective equipment, medical pre- and post-exposure prophylaxis as well as antiseptic and aseptic procedures during mission. Moreover training in special practices related with decontamination of PPE and equipment which leaving the zone, including enhanced general hygiene or even food consumption can't be neglected.

All measures which have positive influence on personal safety of potentially exposed duty officers, need to be undertaken. Considering specific nature of biological warfare agents they also protect non-exposed personnel, as well as their relatives and general public health. This chapter will focus on these aspects.

2.2. Biorisk assessment

Biological risk assessment and management is prerequisite action for successful biological sampling mission preparation. Even though there are unlimited numbers of CBRN scenarios, there are also defined lists of pathogens which can be used as

biological warfare agents, which have been discussed in previous chapters. Considering their properties as well as disease characteristic and availability of prophylaxis and treatment medications, the general or specific biological countermeasures may be planned and applied. Following aspects of biological warfare agents need to be considered during the biorisk assessment process:

- Survivability/stability in environment – bacteria are substantially more stable in the environment (bacterial spores can survive for many years in soils), while viruses are less stable, but non-enveloped viruses are more resistant than enveloped ones;
- Sensitivity to disinfection substances – the vegetative forms of bacteria and enveloped viruses are sensitive to majority of disinfectants. However spores are resistant to many disinfectants. Additionally, non-enveloped viruses, mycobacteria and fungi may be resistant to some types of chemicals;
- Probable dissemination method – the most likely pathway for the spread of many pathogens is an aerosol form, however there are some agents which can be used to contaminate water or food chain, or with using vectors;
- Infectious dose – the number of microorganisms required for development of an infection. This also depends on pathogen species or port of entry. For example, approximately one virus particle or 5–10 cells of *F. tularensis* (respiratory route) are sufficient for infection, whereas in case of *B. anthracis* from 8,000 to 50,000 endospores are required. In many cases the infectious dose is unknown or only rough estimated.

The other significant input for biorisk assessment is characteristic of diseases and available adequate prophylaxis and treatment medicines. These are:

- Incubation period – for different microorganisms is varied. It can last for several hours (*Escherichia coli*), a few days (*Influenza Virus*, *Yersinia pestis*, *Dengue virus*), and even a few months. This information may be valuable to predict quarantine and observation time;
- Mortality (number of fatal casualties related to number of cases) and prevalence. These factors determine a disease severity and directly allow to assess hazard related with agent, and potential consequences of exposure. It is also important to distinguish these factors among treated patients and non-treated patients as well as vaccinated and not vaccinated ones;
- Communicability of disease – there are direct and indirect ways of spreading of the infection. The direct way can be divided into: direct contact with sick person (e.g. contact with body fluids (*Ebola virus*, *Marburg virus*), droplets spreading during coughing and sneezing (*Influenza viruses*), sexual contact (*Zika virus*), vertical infection, from mother to child (through the placenta, during labour or breastfeeding). While the indirect contact includes: skin contact with contaminated material (*Bacillus anthracis*), faecal-oral route, consumption of contaminated water and food (*Escherichia coli*, *Vibrio cholerae*), aerosol route (pulmonary plague and tuberculosis) and infection by vectors e.g. direct contact with sick animal (e.g. bites or stings by infected animals (*Hantaviruses*) or insect

bites (*West Nile virus*, *Dengue virus*, TBE) or contact with waste containing microorganisms particles (*Hantaviruses*);

– Availability of vaccines and/or antibiotics – the presence of preventive measures and effective therapy to a particular pathogen significantly minimizes related biorisk. Basically, antimicrobial therapy is widely available, but when it comes to viruses, the specific treatment opportunities are very limited, rather supportive treatment is available. Moreover there might be some discrepancies of treatment measures in various countries depending on condition of medical service and wealth.

This information above will contribute to appropriate selection of disinfection substances, respiratory and personnel protection measures, needed vaccination or chemoprophylaxis measures. The characteristics of resulted disease will influence on medical procedures in case of exposed personnel or rescued casualties. The most challenging situation will occur in case of unknown agent. The worst case scenario has to be always foreseen in biorisk management. However, it might be presumed that in real situations (such as Anthrax letter incidents from year 2001), additional information about anticipated biological agents may be available. These may allow for more narrowed and specific biorisk assessment and management.

2.3. Medical measures

Medical prophylaxis and treatment medicines are very effective countermeasures in the long-lasting war of humanity against infectious diseases. Antibiotics and vaccines can also be very useful to protect personnel against biological warfare agents (Table 1). Thus, knowledge about available prophylaxis and medical measures is an essential point in mission preparation. Basing on results of biorisk assessment concerning particular agents and activities, vulnerable personnel has to be advised or obligated to undertake necessary medical measures to minimize the risk of infection. A special stockpile of these pharmaceuticals need to be prepared and stored in advance of any event. There might be requirements of prophylactic vaccination for duty officers in case of potential occupational exposure. There are for example legal requirements of vaccination in Poland for national defence and interior affairs ministries units. Vaccines are the main and the most effective medical prophylactic measures from the medical point of view. However, biological warfare agents and related diseases currently are not the top listed threats for public health. Thus, vaccines for them may be not easily available or there exist no vaccine on the market for some biological agents. On the other hand, widely available antibiotics, may be used for post-exposure prophylaxis or treatment of bacterial diseases. For viral biological warfare agents, special antiviral drugs may be applied, however often their efficacy are not fully tested or confirmed. Moreover, for some biological warfare agents specific immune antibodies (as well as antitoxins and antisera) can be applied against selected viruses, bacteria or toxins.

In case of directed or suspected exposure on biological warfare agent, especially in case of unvaccinated personnel – there is a need to implement special post-exposure prophylaxis procedures. The detailed action is heavily related to the characteristic of agent and disease (communicability, mode of transmission, infectivity, course of disease and availability of pharmaceuticals).

Table 1. List of diseases caused by potential biological warfare agents, with available vaccines and drugs

Disease	Causative agent	Vaccine	Drugs
Anthrax	<i>Bacillus anthracis</i>	available (purified antigen)	penicillin (except for inhalation anthrax in which the mortality remains high), ciprofloxacin, doxycycline, tetracyclines, erythromycin, chloramphenicol
Pneumonic plague	<i>Yersinia pestis</i>	available	streptomycin, tetracycline, chloramphenicol (for cases of plague meningitis), kanamycin, ROM therapy
Tularemia	<i>Francisella tularensis</i>	during the tests	streptomycin, ciprofloxacin, gentamycin, tobramycin, kanamycin, tetracyclines, chloramphenicol, aminoglycosides
Brucellosis	<i>Brucella suis</i>	not available for humans	tetracyclines, doxycycline with rifampicin, streptomycin, trimethoprim/ sulfamethoxazole (TMP/SMX)
Q-Fever	<i>Coxiella burnetii</i>	available	cotrimoxazole, rifampin, doxycycline, minocycline, tetracycline, clarithromycin, sparfloxacin, quinolones
Glanders	<i>Burkholderia mallei</i> , <i>Burkholderia pseudomallei</i>	not available	trimethoprim/sulfamethoxazole (TMP/SMX), ceftazidime, imipenem, doxycycline, minocycline, ciprofloxacin, gentamicin, ciprofloxacin sulphas, chloramphenicol, tetracycline
Smallpox	<i>Variola virus</i>	available	no specific treatment is available but cidofovir may be effective
Venezuelan Equine Encephalitis	Venezuelan Equine Encephalitis virus	available	no specific treatment is available

Disease	Causative agent	Vaccine	Drugs
Marburg Fever	<i>Marburg Fever virus</i>	not available	no specific treatment is available
Botulism	toxin of <i>Clostridium botulinum</i>	not available	antitoxin
Ricin-intoxication	Ricin	available	no specific treatment is available
SEB-intoxication	<i>Staphylococcal enterotoxin B</i>	not available	no specific treatment is available

Post-exposure measures may include: observation of casualty's health status, quarantine, application of antibiotics or antivirals or antibodies (antitoxin or antisera) as well as vaccination or revaccination (booster-doses) or combining these measures. Once symptoms of diseases developed, patients may be treated in isolation wards to prevent spread of disease in case of highly contagious and dangerous disease.

2.4. General protective equipment

Personal protective equipment (PPE) consists of specialized clothing or equipment used to prevent contact of personnel with hazardous CBRN materials. The PPE provides physical barriers that protect the potential entry ports for biological agents (hands, skin, eyes, nose, respiratory system and mouth) against contact with infectious agents, and potential infection or intoxication. This includes garments (suit, gowns, uniform), respiratory protection equipment, gloves, safety glasses, helmets, hoods, face shields, rubber boots and others. The PPE must be worn at all times during work with dangerous substances or sampling mission. Final selection of PPE, as a result of the risk assessment, has to base on mission-work environment description, analysis of potential hazardous agent, type of activity as well as individual capacities to fulfil the task in PPE. It is mandatory in European Union for employer to select and provide PPE, train personnel how to use it, as well as clean/decontaminate or dispose it. Legal requirements for PPE production and testing, including CE marking of PPE fall under European directive, which will be suppressed in near future with the directly applicable European regulation.

The main type of garment PPE for biological sampling mission are disposable or reusable coveralls (suits), which are more preferably used than other type of garment PPE, due to comfort of movement and readiness for decontamination

procedure. There are currently three categories of work-protective garment. Categories II and III are considered PPE, but for CBRN sampling missions the garment must belong to Category III (specialized cloths), which is applied to the life and health risks situations.

Current European standards for PPE, which can be used in biological sampling, relate to general demands for protective clothing but there are more specific document which cover biological safety. Since unknown event areas can also be burdened with other threats, other hazards like chemicals or radiation mustn't be neglected. Moreover decontamination procedure very often envisages disinfecting shower with harmful chemical substances. Various types of protective garments can be applied for situations, where chemical, biological, radiological or nuclear warfare hazards may be present (Table 2).

The European Standards specify the tests methods for materials intended to be used in the manufacturing of each type of protective clothing. General division into six types (depending on form of the threat: gas, particles liquid, vapours), relates to chemical safety but each type may be tested against biological agents according to EN 14126 which specify requirements and tests methods for protective clothing against infective agents (letter 'B' in suit code name).

Additional elements of protective suits may also be necessary to fully equip the personnel. These are for example gloves, which have to be both chemically and biologically resistant. On the other hand, they have to be thin enough to allow for precise work. Appropriate gloves must be worn for all procedures that may involve direct or accidental contact with blood, body fluids and other potentially infectious materials or infected animals. A special attention has to be paid for potential allergenic properties of material which has direct contact with skin. Many CBRN sampling teams work in double gloves regime (gloves are changed after changing location of sampling or collection of another sample), it is a reasonable approach compared to alternative option basing on frequent disinfection of gloved hand. Depending on suit type and presumed activity, many other items can also be useful: aprons, sleeves, rubber boots, safety glasses, face shields (visors) or other protective devices which must be worn when it is necessary to protect eyes and face from splashes or impacting objects.

Depending on the type and form of biological agent, exposure levels, type of work and risk of infection, consideration should be given to selection of the most adequate type of category III garment. General rule is that personnel which enters the unknown hot zone have the most advanced PPE (for example overpressure suits) whereas personnel engaged in warm zone (e.g. buddy decontamination, undoing or sample processing) wear lighter suits (e.g. with negative pressure respirators).

Table 2. Personal protective equipment – category III cloths types

Type	Pictogram		European standards
Type 1a		Full body protection against gaseous and airborne agents (respiratory device inside the suit).	EN 943-1 and tested by EN 464 :1994 if used by the emergency team, also EN 943-2 (code name: 1a-ET)
Type 1b		Full body protection against gaseous and airborne agents. (respiratory device outside the suit).	EN 943-1 and tested by EN 464 :1994 if used by the emergency team, also EN 943-2 (code name: 1b-ET)
Type 1c		Full body protection against gaseous and airborne agents. (breathing air provided by hose from outer stationary source).	EN 943-1 and tested by EN 464 :1994
Type 2		Full body protection against gaseous and airborne agents. (by positive pressure inside the suit).	EN 943-1, not tested against EN 464:1994
Type 3		Protect against jets of liquid.	EN 17491-3 EN 14605+A1 :2009
Type 4		Protect against sprayed liquid.	EN 17491-4 EN 14605+A1 :2009
Type 5		Protect against dust and airborne solid particles .	EN 13982-1:2004+A12010
Type 6		Limited protection against splashing of liquid.	EN 13034:2005+A1:2009
Type 1-6- B		Protect against biological agents.	EN 14126:2003

The other consideration related with thermal comfort of the personnel has to be included in planning. The majority of current biological suits are made from multilayer fabrics, which intend to provide both adequate resistance, barrier and improved vapour-permeability for comfortable usage. These fabrics

or synthetic materials are sophisticated laminates – multilayer materials made from polyurethane, polypropylene, polyethylene, polyester, cellulose, porous materials and breathable membranes. More resistant and thick fabric may result in decreased permeability and consequently decreased comfort of work in suit in hot or humid environments. This circumstance need to be overcome by shortening of the mission or application of cooling vests which allow to decrease temperature under the suit. The cooling effect may be obtained by thermoelectric effect (it needs electricity from portable battery), a phase change effect or ice packs. On the other hand in colder climate, the appropriate warm underwear have to be used to keep warmness under the suits.

Below four examples of the disposable Category III protective suits, which can be used in various type of missions and activities, are presented. The first suit (PROTEC® Plus) is rather light and may be used in presence of limited threats or warm zone activities (Fig. 1). It is made from breathable layer/microporous film (PE/PP). This suit is type 4B, 5B, 6 (protection against dust, particles, fibres and diffused substances) with protection against radioactive contaminating particles and biological hazardous substances. It is intended for work with lighter activities involving work with liquid and solid moderately hazardous substances (non-pressurized). It may be combined with any respiratory protection measure and other add-ons.



Figure 1. Example of a light Category III suit (type 4B, 5B, 6)

The second example suit (ProChem® II C/F – category III, type 3B, 4, 5 and 6 – Fig. 2) may be easily and tightly combined with negative pressure respirator (full-face facepiece) *via* butyl face shield seals as well as gloves (various types: butyl, nitrile, chloroprene) and rubber boots (or shoe covers with non-slip and antistatic sole). Entrance is from the back (a safer ‘solid front’ approach) and secured with two tabs closed with double-sided adhesive tape. It is much heavier (material: Tychem® F or C) compared to previous one (version C is lighter, though). It is intended for more intensive works including entering hot zone and direct sampling. It provides protection against inorganic and organic chemicals (only version F) in high concentration, dangerous biological hazards and contamination with particles, fibres and dust, radiation and protection against toxic warfare agents. It is resistant against even intensive personnel decontamination shower applied before leaving the hot zone.



Figure 2. Example of a heavier Category III suit (type 3B, 4, 5, 6)

The following example (ProChem® III F or C – category III type 3 and 5; Fig. 3) is interesting due to combining it with powered air purifying respirator (PAPR) as a respiratory protection measure. It is made from the same material (Tychem® F or C) and provides similar properties capacities as previous ones. It can also be supplemented with similar additional add-ons. The main

advantage of this type is application of the battery operated filtering device which eliminates the use of full-face facepiece respirator. Hence, it allows for prolonged (few hours) mission in more comfortable conditions – breathing is not restricted by negative pressure respirator. Impressive panoramic visor and size of suit's head part will improve observation field and it may help users who has problem with claustrophobia. Placing the PAPR device under the coverall (only the filtering units are outside) prevents it from being contaminated. The air from outside is filtered (at least P3 filters which may be combined filter against chemical/radiological agent) and clean air is blown over the head. This gives additional advantage for thermal / humidity comfort. Even though it was not tested against requirements of type 2 garment, it provides additional protection due to relative overpressure inside the garment, which prevents penetration of hazardous gases and particles. There are also similar types of suits, but combined with SCBA (self-contained breathing apparatus) instead of PAPR, which have their own advantages and disadvantages.



Figure 3. Example of a heavier Category III suit (type 3B, 4, 5, 6) with PAPR

The last example (ProChem® VI TK – category III, type 1a – Fig. 4) is the most advanced solution, providing maximum gaseous barrier for CBRN

hazards, even in higher concentration and under the pressure. The suit is made from heavy material Tychem® TK (triple dense as previous ones). It is equipped with breathing apparatus with compressed air and full-face facepiece as respiratory protection measure, which provide additional overpressure inside the suit. As a whole the suit and breathing apparatus provide protection against highly concentrated substances as well as in presence of toxic industrial materials, for which regular military or civilian chemical filter based negative respirators may be not sufficient. As the type 1a, the self-contained breathing apparatus is under the suit, which protects this expensive device against potential contamination. Panoramic visor of facepiece respirator and suit provide adequate observation field. It may be supplemented with sealed 'socks' or air-tight sealed rubber boots. The suit is combined with double glove work system, thinner gloves inside and thicker ones outside. It is tested additionally against standard EN 943-2 to meet special requirements for emergency rescue teams equipment.



Figure 4. Example of a heavy Category III (type 1a -ET), air-tight, SCBA-equipped suit for emergency teams

2.5. Respiratory safety

The main probable dissemination route for majority of biological warfare agents is aerosol. The primary threat: aerosolized bacteria, viruses or toxins are odourless and rather invisible. Once settled down, they contaminate affected area and equipment. The resulting biological fallout is source re-aerosolization and secondary threat. Thus protection respiratory tract is the indispensable part of biological PPE. There are two approaches for respiratory protection – isolation and filtration. The first method bases on supply of clean air (or oxygen) from an independent source. There are two types: stationary (air is supplied by air-hose) and autonomous (such as self-contained breathing apparatus SCBA). The second one, which bases on bottle of compressed air is preferably used in CBRN protection and by first responders. It provides the superior safety, which also includes other hazards (chemical gases, vapours, decreased level of oxygen) over filtration based methods. Moreover, they are positive pressure respirators (the pressure of clean air inside the facepiece is greater than outside) which is another safety advantage. However limited air amount in an autonomous high-pressure tank allows for missions which take less than one hour for the open circuit systems or four hours for the closed circuit system. The closed circuit system is more sophisticated and it is based on recirculation and recycling of exhaled gas. Thus the closed circuit SCBAs are much more expensive and complex in terms of routine maintenance, hence they are less widely used in CBRN protection.

The filtration based approach is more widely implemented in CBRN protection. It is simpler in use, cheaper in production and maintenance as well as suitable for long term storage. It consists of reusable facepiece (full-face facepiece or half-mask) and filtration unit (sometimes also adsorbing) or disposable filtering facepiece (FFP), where the whole facepiece functions as filter. The majority of filtration based masks are negative respirators (the pressure of clean air inside the facepiece during the inhalation is lower than outside). Full-face facepieces (e.g. regular military mask) are more favourable than half-masks, because they also offer face and eyes protection, however observation field and comfort of work is limited. Thus shape of facepiece has to be precisely fitted to the face, to reduce the risk of inhalation of unfiltered air (max. 0.05%). The single-use filtering facepieces (half masks) are being more popular nowadays. They are cheaper, lightweight and disposable, thus more comfortable (they do not need to be decontaminated after action). The disadvantages of this solution is related with their shape (half masks, which generate higher inward leakage) and difficulties to obtain adequate face seal.

The filtration based respiratory protection PPE may be enhanced with airflow supported by e.g. battery operated electric blower. This enhancement

leads to improved safety with applied mask, half mask as facepiece (*power assisted filtering devices*) and an implementation of positive pressured but loosely fitted facepieces like hoods or helmets (*powered filtering devices*). For example powered air purifying respirator (PAPR) connected with hood or overall suit (Fig. 4), offers better thermal comfort, observation field, longer work and comparable safety to other options (SCBA or negative pressure protective mask). This approach is being more popular in case of the absence of co-occurring radiological or heavy chemical contaminations.

Table 3. Filter classes under standards EN 143 and EN 149

Filter class EN 143:2000	Min. efficacy (%)	Other marks:	FFP class EN 149:2001	Minimum efficacy (%)	Inward leakage (%)	Occupational Exposure Limit
P1	80	NR – disposable R – reusable	FFP1	80	<22	4 x OEL
P2	94		FFP2	94	<8	9x OEL
P3	99.5	Colour: white	FFP3	99.5	<2	20 x OEL

Filtration efficacy of filtration units or filtration facepiece against solid and liquid aerosols falls into three classes in European Union (Table 3). Similar classifications exist in, for example, the USA (Table 4).

Table 4. Filtration efficacy based on 42 CFR Part 84 (US)

Filter class	Minimum efficacy (%)	Type of test
95	95	N – non-oil aerosols (e.g. N95, N99, N100) R – include oil aerosols with restrictions (e.g. R95, R99, R100) P – include oils aerosols (e.g. P95, P99, P100)
99	99	
100	99.97	

Filtering units may be combined with adsorbing materials and also provide protection against chemical agents. The respiratory protection equipment may be sensitive to inappropriate storage condition (temperature, humidity). Filter units are normally packed in hermetic bags, once they were opened, there may be some restrictions to reuse them. Respiratory protection PPE has maximum time of storage, which is marked on filtering unit or facepieces. It must never be used after the expiration date. For some *power assisted* or *powered* devices, a special attention has to be paid on batteries (in blower part) – they may need periodical recharging to sustain their lifetime and efficacy.

The prolonged usage of respiratory protection equipment may be related with serious sensorial stress (limited hearing, smell and observation field). That is why decision about enrolment of personnel to use the respiratory protection equipment and its careful selection has to include: type of activity, individual capacities and environmental conditions. Moreover individual capacities are related with age, sex, hair and shape of face (e.g. beard), psychological and neurological conditions (e.g. claustrophobia), health status (pregnancy, respiratory insufficiencies or chronic conditions). Since respiratory protection equipment usage need some fluency – the enrolled personnel has to undergo initial and refreshing trainings to sustain practical skills and readiness to unexpected events during the mission.

2.6. Personnel decontamination

Leaving the hot zone comprises significant risk related with transferring biological warfare agent out of contaminated zone. Thus all necessary actions should be undertaken to prevent uncontrolled release of agents into the 'clean' environment and to protect staff against potential infection or contamination by dangerous substances. Thus a comprehensive and credible decontamination is indispensable both after finishing work with dangerous substances and after work in the field where unidentified agents would have occurred. A whole process of decontamination should concern both equipment (including reusable elements and devices) and clothes (including boots, garment, gloves) of every person who may have been exposed to potential hazardous substances or agents.

Wastes remaining after sampling mission (such as used disposable garments, plasticware), and wastewater after decon procedure are hazardous wastes due to potential presence of harmful CBRN warfare agents or chemical disinfecting agents. Hence they are also subject to appropriate utilization processes like other hazardous wastes. The hazardous wastes management are regulated by series of general policies and specific regulations. Due to chemical and biological threats the waste from biological sampling mission may be included in group *H9 Infectious*, *H4 Irritant*, *H5 Harmful*, *H6 Toxic*. There are different methods and schemas which may be applied for infectious wastes utilization: autoclaving, chemical or microwave treatment, alkaline hydrolysis. Despite final burning in incinerator is not the cheapest method, it is the most effective one for reduction of waste volume and hazard.

On-site decontamination process often is undertaken in specially assigned warm (yellow) zone between hot zone (red) zone and cold (green) one. It can relate to the first responders' equipment, as well as to potentially exposed casualties (mass-decontamination corridors). The simplest but also the most effective approach is physical removal of dangerous agent by water solutions under field showers. Warmer water and some additives of detergents increase comfort and efficacy of process. This method is also environmentally friendly and has not

harmful side-effects to human health. On the other hand the increased risk may impose application of additional inactivating chemical agents. The combination of both methods (physical removal and chemicals) is more effective. There are several biocidal chemicals which can be used in solutions for decontamination procedures in the field condition. These are for example solutions based on: chlorhexidine, active chlorine (e.g. sodium hypochlorite), hydrogen peroxide, potassium monoperoxysulfate, peracetic acid or quaternary ammonium compounds (QAC). There are also several factors which have to be taken into account in planning effective decontamination and selection of appropriate biocide chemicals. The first one is its spectrum of activity, some disinfectants may not be equally active against all type of biological agents. There are some discrepancies especially in activity against bacterial spores, un-enveloped viruses, mycobacteria and fungi. Sometimes activity of disinfectant may be impaired by presence of organic substances. Hence the type and concentration of disinfectant or deactivator should be adjusted to the type of contaminant. Recently, solutions based on potassium monoperoxysulfate (and other ingredients) have been considered as disinfectants with the most broad spectrum. The side effects of disinfectants to human health (e.g. skin irritation, potential inhalation of vapours) and environment are also important factors in the selection process. It should be underlined that there is need to ensure appropriate contact between disinfection solution and contamination in proper time and temperature. Considering different properties of selected disinfectants and scenarios, there may be a need for additional action e.g. a pre-treatment shower (removing the organic substance, soaking which enhances contact), additional shower with neutral solution (removing the disinfectants) or application of PPE for protection against harmful activity of disinfecting substance. Thus, the knowledge about the components of the reagents used during work in the field and anticipated biological warfare agents may be helpful in selecting the adequate decontaminant which is simultaneously safe in use.

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3. TRANSPORT OF DANGEROUS BIOLOGICAL MATERIALS

3.1. Introduction

Transport of biological samples is an important stage for successful laboratory identification and confirmation of bio-threats in field or reachback facilities. Transport of biological samples should be carried out properly to assure security and safety for humans, environment and specimen. Moreover package with infectious substances must be delivered safely, efficiently and timely to the place of destination to reduce content degradation. Hence, shippers must prepare package containing sample in way that minimize the risk of damage during transport and uncontrolled release of dangerous material into the environment. Samples must also be protected from contamination by the appropriate vessels or jars. Type of sample may determine the need to use refrigerants such as dry ice or coolers. Depending on potential and real content of infectious biological materials in specimen, it may be considered as dangerous goods or dual-use material, which falls under legal restrictions. Due to that, it is very important for shippers to be familiar with the current requirements concerning transport of infectious materials. They should be trained frequently to make sure that they will handle with the package confidently and will classify the material properly.

3.2. Dangerous goods and dual-use materials regulations

The biological infectious materials belong to dangerous goods, which may present hazards for human and environment during the transport. Transport of dangerous good is an area of recommendations of United Nations. These recommendations are a base for international, national and internal regulations for example for roads, maritime, railways or air. These regulations describe dangerous goods in 9 classes, including biological ones (subclass 6.2 – biohazard). For purposes of transport, Infectious substances are defined as substances which are known or are reasonably expected to contain pathogens. Pathogens are defined as microorganisms (including bacteria, viruses, rickettsiae, parasites,

fungi) and other agents such as prions, which can cause disease in humans or animals. There are several types of goods in this subclass: biological substances Cat. A (UN 2814) and Cat. B (UN 3373), bio-medical and clinical wastes (UN 3291), infectious substances affecting animals only (UN 2900). Moreover, the conditions of transport of hazardous substances or technical requirements concerning packages and their testing are described. WHO periodically issues comprehensive guideline solely on transport of infectious substances, which is very helpful tool for shippers of biological materials. This chapter bases mainly on this publication.

Furthermore, some biological substances are considered as dual-use materials due to biological weapons proliferation threat. A certain species of viruses, bacteria or toxins which may be pathogenic for human, animal or cultivation – they additionally represent a threat for national or international security. That is why, there might be some national or international restrictions in possession, transfer, export or import of these materials. There is a regulation in European Union, which covers dual-use biological substances. It establishes control measures for their export and transits, thus knowledge about it and other national regulations is important for successful transport of selected biological materials. The informal international forum of states – the Australian Group – is aimed at harmonising export controls in the field of biological and chemical dual-use materials and enhance implementation of the Biological and Toxin Weapons Convention and the Chemical Weapons Convention. Its unofficial publication control list handbook is a helpful tool for shippers of biological materials (Australia Group Common Control List Handbook Volume II: Biological Weapons-Related Common Control Lists, Revision 2, January 2016).

3.3. Transport of infectious samples

Laboratory analysis cannot be done right if the material has been collected or transported incorrectly or stopped during transport. Hence, the organization of shipment plays important role in timely, safe and secured transport. Shippers are mostly responsible for planning, activity during the preparatory phase, sample delivery and all the associated aspects. Shippers should ensure that packages are prepared in a proper way and that they won't be damaged during transport or pose any threat to surrounding (people or animals). The shipper discusses and arranges with the courier the type of packaging, conditions and means of transport (most direct and shortest is preferable), and also obtains potential export permission. Their role is also to prepare the documentation. Shipment has to be supplemented with the appropriate consignment note. Depending on internal procedures and current legislation, it typically contains: information of a person who prepared the package, sender, recipient responsible person for shipment

(with telephone number), packing list (a short description of the sample such as the kind of material (liquid, solid, gas substance), quantity, volume, weight of the material, number of vials, real or potential name of biological agents), assignment to appropriate hazard class connected with proper labelling and marking on the outer package. Packages from sampling mission often contain more information such as: place of sampling (its description, grid coordinates, images), date and time of sampling, sample identification number, results of rapid tests which were done on site as well as sample collection method, type of preservation and analysis requested by shipper. Depending on the content and way of transport package may contain the shipper's *Declaration of Dangerous Goods*, import/export permits, an air waybill for air transport or equivalent documents for road, rail and sea shipments or other document required by courier. In case of international transport, for customs purposes a pro-forma invoice is also mandatory, which contains the sender and recipient's address, the number of packages, details of content and purpose, weight, commercial value (for diagnostic specimens *No commercial value* should be written). Before shipment sender has to ask recipient about the readiness to accept the shipment in anticipated time.

Furthermore, the shipper has to choose the right means of transport adjusted to a specific sample and to secure the shipment against access by unauthorized persons. In military, alleged use of biological warfare and criminal investigation contexts additionally, a chain of custody is observed. It is the process of tracing a sample from the place of collection to the laboratory until the analysis. Each stage of transfer and receipt of the sample must be appropriately documented, to assure and maintain the sample integrity. This is done using the appropriate *Chain of Custody (COC)* documentation. The form must be filled out by all persons who take part in transport. A chain of custody is initiated by the sampler whose task is to properly collect and mark samples. During the transport, each person receiving and transmitting the sample is required to complete the form. The basic information contained in the form may include: short description of shipment (codes number), information about shipper and recipient – addresses and phone numbers, special handling requirements, printed name and signature of person relinquishing custody (date and time when custody was relinquished), printed name and signature of person receiving custody (date and time receipt of the sample). An example COC is shown below.

On the other hand courier role is to advise the shipper about current regulations and packages selection, archiving documentation and taking a part in chain-of-custody if applicable. The role of recipient is to confirm readiness to receive shipment, organize timely package collection and if applicable, obtain import permission and organize custom procedures. Finally recipient confirms sender about successful shipment arrival, including information about quantity and quality of content.

Sender and contact name			Recipient and contact name	
Ad- dress			Address	
Phone			Phone	
Sample ID	Data and time	Sample description	Type of sample	Special instructions/ comments:
Chain of custody				
Received from		Released by:	Received from:	Received by:
Signature:		Signature:	Signature:	Signature:
Date/time:		Date/time:	Date/time:	Date/time:
Received from:		Received from:	Received from:	Received by:
Signature:		Signature:	Signature:	Signature:
Date/time:		Date/time:	Date/time:	Date/time:

Figure 1. Example chain-of-custody form (COC)

3.4. Types of infectious material categories and packages

The dangerous goods shipment, has to be marked and identifiable by UN numbers and shipping names, according to their classification and composition. The main division of infectious shipments (Subclass 6.2) falls into two categories: Category A (UN 2814 or UN 2900), and Category B (UN 3373).

Category A infectious substances are defined as any material that contains or is reasonably expected to contain a pathogen transported in a form that, when exposure to it occurs, is capable of causing permanent disability, life-threatening or fatal disease in otherwise healthy humans or animals. An exposure occurs when substance released from its protective packaging contact with humans or animals. If so, it has to be marked as UN 2814 (INFECTIOUS SUBSTANCE, AFFECTING HUMANS). Infectious substances cat. A which cause disease in animals only should be assigned to UN 2900 (INFECTIOUS SUBSTANCE, AFFECTING ANIMALS). An substance which does not meet the criteria for category A (such as effects of exposures related with transport, severity and prognosis of potential disease) should be assigned to UN 3373 (BIOLOGICAL SUBSTANCE, CATEGORY B).

Assignment to any of this category should be based on the known medical history and symptoms, form of transport (culture or clinical or environmental samples), endemic conditions and professional judgment. The WHO Guidance

includes an indicative (not exhaustive) list which helps in final categorization of biological material. Examples of infectious substances classified into category A are listed in Tab. 1.

Table 1. Indicative list of examples of infectious substances category A (adapted from the 18th edition of the United Nations Model Regulations).

UN number and shipping name		Microorganism
UN 2814 Infectious Substances, affecting humans	Viruses shipped in any form	Variola virus; Monkey pox virus; Ebola virus, Marburg virus; Crimean- Congo haemorrhagic fever virus; Lassa virus; Nipah virus; Omsk haemorrhagic fever virus; Junin virus; Kyasanur Forest disease virus; Machupo virus; Sabia virus; Guanarito virus; Hantaan virus; Hantaviruses causing haemorrhagic fever with renal syndrome; Hendra virus; Flexal virus
	Viruses shipped in form of culture	Yellow fever virus; West Nile virus; Dengue virus; Japanese encephalitis virus; Venezuelan equine encephalitis virus; Eastern equine encephalitis virus; Russian spring-summer encephalitis virus; Tick-borne encephalitis virus; Hepatitis B virus; Herpes B virus; Human immunodeficiency virus; Highly pathogenic avian influenza virus; Poliovirus; Rabies virus;
	Bacteria shipped in form of culture	<i>Yersinia pestis</i> , <i>Bacillus anthracis</i> ; <i>Francisella tularensis</i> ; <i>Clostridium botulinum</i> ; <i>Brucella abortus</i> ; <i>Brucella suis</i> ; <i>Brucella melitensis</i> ; <i>Coxiella burnetii</i> ; <i>Burkholderia mallei</i> - <i>Pseudomonas mallei</i> ; <i>Burkholderia pseudomallei</i> - <i>Pseudomonas pseudomallei</i> <i>Escherichia coli</i> (verotoxigenic); <i>Chlamydia psittaci</i> (avian strains); <i>Mycobacterium tuberculosis</i> ; <i>Coccidioides immitis</i> ; <i>Rickettsia prowazekii</i> ; <i>Rickettsia rickettsia</i> ; <i>Shigella dysenteriae</i> type 1
UN 2900 Infectious substances, affecting animals only	Viruses shipped in form of culture	African swine fever virus; Classical swine fever virus; Foot and mouth disease virus; Avian paramyxovirus Type 1-Velogenic Newcastle disease virus; Lumpy skin disease virus; Peste des petits ruminants virus; Rinderpest virus; Sheep-pox virus; Goatpox virus; Swine vesicular disease virus; Vesicular stomatitis virus

There are some biological materials which are *NOT* subject of dangerous goods regulations:

- Substances containing microorganisms that are non-pathogenic to humans or animals;
- Substances with neutralized or inactivated pathogens;
- Environmental samples (including food and water samples) not posing a significant risk of infection;
- Some clinical materials, such as: dried blood spots (collected by applying a drop of blood onto absorbent material), faecal occult blood screening tests, blood or blood components (for the purposes of transfusion or for the preparation of blood products), tissues or organs (for transplantation).

Human or animal specimens (patient specimens) for which there is very little likelihood of pathogens presence (e.g. transported for routine testing not related to the diagnosis of an infectious disease), are also exempted from Subclass 6.2 under the following conditions: triple leak-proof packaging marked as Exempt human or animal specimen. The likelihood assessment has to be based on professional and comprehensive judgement.

Medical or clinical wastes containing infectious substances category A should be shipped under the name UN 2814 or UN 2900, as appropriate. Wastes containing substances category B should be shipped under the name UN 3291 - CLINICAL WASTE, UNSPECIFIED, N.O.S. or (BIO) MEDICAL WASTE, N.O.S. or REGULATED MEDICAL WASTE, N.O.S.

The general triple packaging system consists of three layers as follows:

- Primary receptacle – a watertight, leak-proof or stiff-proof receptacle containing the specimen;
- Secondary packaging – resistant, watertight, leak-proof or stiff-proof packaging to enclose and protect the primary receptacle(s);
- Outer packaging – shipping packaging with suitable cushioning material protecting their contents from physical damage during transport.

Overall external dimension should be at least 10 x 10 cm. In case of liquids, there should be an absorbent material placed between the primary receptacle(s) and the secondary packaging to absorb the entire contents released during transport. Several cushioned primary receptacles may be placed in one secondary packaging, but with the use of sufficient additional absorbent material; if they are fragile, they should be either individually wrapped or separated to prevent contact between them. Each completed package has to be correctly marked, labelled and accompanied with appropriate shipping documents (as applicable).

In case of substances that must be transported refrigerated or frozen, appropriate temperature label may be provided. A refrigerant (e.g. ice, dry ice) should be placed around the secondary packaging, which position has to be secured within the outer package (in case of melting of refrigerant). The primary

receptacle and the secondary packaging shall keep its integrity at the lower temperatures e.g. samples transported in liquid nitrogen should be placed in plastic primary receptacles, capable of withstanding very low temperature. In case of dry ice the outer packaging has to allow the release of carbon dioxide gas. In case of using ice, the outer packaging should be leak-proof. In case of usage of dangerous refrigerant (e.g. solid carbon dioxide, cryogenic liquids) appropriate information about it shall be included on outer package. Moreover in case of use solid carbon dioxide additional packaging instruction has to be followed (PI003 or PI945). The outer package need to be additionally marked with label (Fig. 2) number UN 1845, and information *CARBON DIOXIDE, SOLID, AS COOLANT* with the net quantity of dry ice in kilograms.

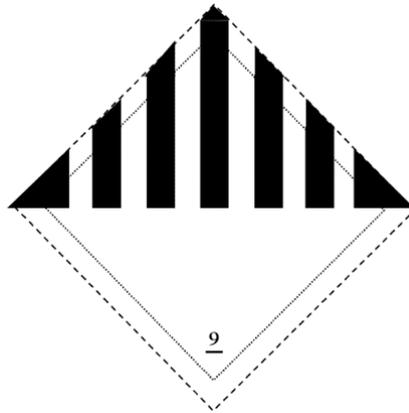


Figure 2. Hazard label for miscellaneous dangerous substances e.g. for solid carbon dioxide, solid (dry ice) (UN 1845) (Image adapted from WHO Guidance on regulations for the transport of infectious substances 2015–2016, WHO/HSE/GCR/2015.2, World Health Organization, Geneva, 2015)

Several of the same type of packaging can be combined together to form one unit – an ‘overpack’, which is then sent to the same destination. Overpacks can contain dry ice, if needed. In the case of using ice, the overpack should be leak-proof. All markings given on outer packages should be clearly placed (repeated) on overpacks. This information includes the shipper name, the telephone number of responsible person, the recipient’s name and address, the proper shipping name with the United Nations number.

Due to various hazard levels, detailed packing instructions are different for infectious substances Category A (UN 2814 and UN 2900) and for infectious substances category B (UN 3373). The main differences concerning types of packaging, labelling and documentation are discussed below.

3.4.1. Category B substances

The samples under UN 3373 are subjected to transport and packing instructions P650. The package for category B should consist of three components, as mentioned before. The package has to be strong enough to resist the shocks, temperature and humidity changes, vibrations, loadings normally encountered during transport. The primary or secondary package needs to resist differential pressure 95 kPa without leakage. For air transport the outer package need to be rigid. Typical packaging and labelling is shown in the figure below.

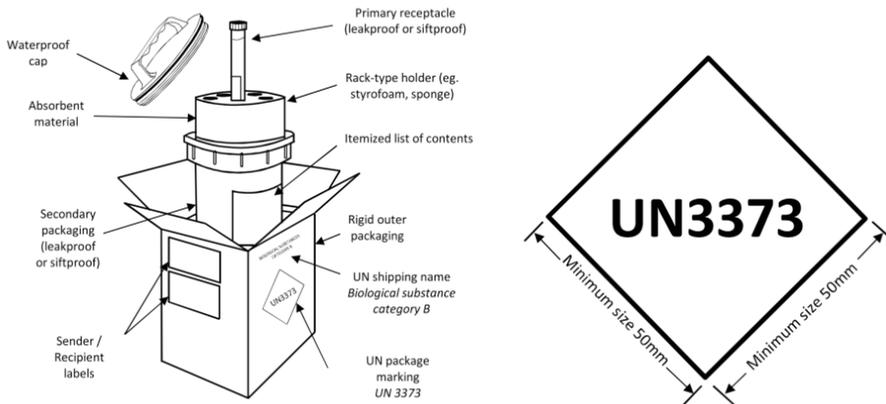


Figure 3. Example of package for category B infectious substances. Marking rhombus sign for category B infectious substances (Image adapted from WHO Guidance on regulations for the transport of infectious substances 2015–2016, WHO/HSE/GCR/2015.2, World Health Organization, Geneva, 2015)

There is no maximum quantity per package, in case of surface transport. Whereas in case of an air transport for liquid substances the primary receptacle should not exceed 1 litre and the outer packaging must not exceed 4 litres. For solid substances the outer packaging must not contain more than 4 kg (with exception to organs, body parts, whole bodies). The outer packaging should contain the following information:

- Sender's details (name, address, telephone number);
- Recipient's details (name, address, telephone number);
- Information that the package contains biological material – shipping name 'BIOLOGICAL SUBSTANCE, CATEGORY B' adjacent to the appropriate rhombus sign with the 'UN 3373' wording used for marking category B infectious substances (Fig. 3).

3.4.2. Category A substances

Category A substances (UN 2814 and UN 2900) have to be transported in triple packaging system produced and certified against special UN class 6.2 requirements. These include 9 m. drop test, a puncture test, a stacking test and internal pressure test of primary or secondary receptacle (without leakage in differential pressure not less than 95 kPa and temperatures in the range -40°C to $+55^{\circ}\text{C}$). Finally package need to be labelled by manufacturer with United Nations packaging specification marking. Packaging need to follow P620 instruction. Likewise category B, for transport by roads, rail or ship there are no limits regarding quantity or weight per package, but there are limitations of content for air transport: 50 ml or 50 g for passenger aircraft, 4 litres or 4 kg for cargo aircraft. Both primary receptacle and secondary packaging have to leak-proof, whereas outer package has to be rigid. If there is more than one of inner packaging, they should belong to subclass 6.2, other materials and dangerous goods are granted in limited amounts only for maintaining the viability, stabilizing or preventing degradation or neutralizing the hazards of the infectious substances. Substances that can be transported at room temperature or higher, should be placed in primary receptacle made of glass, metal or plastics with leak proof sealing. Typical packaging and labelling is shown in the figure below (Fig. 4).

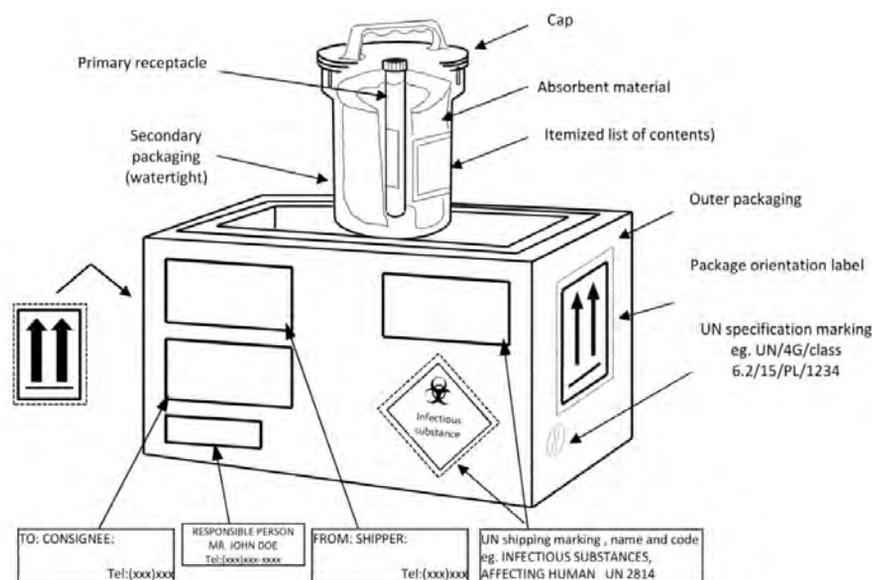


Figure 4. Example of triple packaging system for the packaging and labelling of Category A infectious substances (Image adapted from WHO Guidance on regulations for the transport of infectious substances 2015–2016, WHO/HSE/GCR/2015.2, World Health Organization, Geneva, 2015)

Between the secondary packaging and the outer packaging, a detailed list of the contents shall be enclosed. For air transportation, the shipper's Declaration of Dangerous Goods has to be attached, also indicating other than 6.2 subclass of dangerous goods e.g. solid carbon dioxide. If the transported substance is unknown, but there is a reasonable suspicion that meets the criteria for inclusion in category A, it should be indicated with the words Suspected category A infectious substance (in documentation but not on the package).

The outer packaging should contain the following information:

- sender details (name, address, telephone number);
- telephone number of the person responsible for shipment;
- recipient's details (name, address, telephone number);
- information that the package contains Category A biological material
- shipping name: *UN 2814 INFECTIOUS SUBSTANCE, AFFECTING HUMANS* or *UN 2900 INFECTIOUS SUBSTANCE, AFFECTING ANIMALS ONLY*, adjacent with the appropriate rhombus with international biohazard sign used for marking category A infectious substances;
- Additionally for air transportation of volumes over 50 ml, orientation label (double arrows) has to be placed to indicate position of primary receptacles (Fig. 5).



Figure 5. Safety marks for Category A infectious substances (10 cm x 10 cm or 5 cm x 5 cm) and orientation arrows indicate position of primary receptacles (size A7) (Image adapted from WHO Guidance on regulations for the transport of infectious substances 2015–2016, WHO/HSE/GCR/2015.2, World Health Organization, Geneva, 2015)

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4. METHODS OF SAMPLING

4.1. Introduction

The principal objective of sampling for biological agents is to provide the requirement for unambiguous agent identification in environmental media, food, water, or clinical samples.

The primary objective for sampling is that the strategy provides representative samples, that a sufficient number of samples are taken and that the sampling regime is in line with the physical properties of the suspected agent as well as the environment being sampled (e.g. soil, water, air etc.). Where to sample is also dependent on the weather conditions which should be measured and documented. The relevant samples taken should of course support accurate identification and quantification of the agent at appropriate concentrations that reflect both acute and chronic exposure dosages.

There is no standard procedure, which is recommended and described exactly, how a sampling team should perform its task.

Sampling may be needed in order to:

- Identify the biological agent;
- Confirm the presence of a specific agent;
- Identify the source;
- Verify the occurrence of a crime;
- Formulate an appropriate response, for example support for medical personnel.

4.2. Sampling plan

A sampling plan or strategy is crucial for an efficient, secure and well documented sampling that is a prerequisite for an analysis that need to withstand forensic and legal requirements. The sampling methodology and procedures should be robust and repeatable.

A sampling plan should include:

1. Input values: commands, limitations, infrastructure, terrain, immediate actions, purpose with sampling;
2. Risk assessment: threat scenario, agents, amounts, climate, population;
3. Implications: What samples should be collected, how to sample, protective measures, plan of evacuation, routines, scenario.

In addition to sampling equipment, portable devices which can record factual information during sample collection. Such equipment includes audio recorders, video cameras, GPS, and maps. It is also important to use weather stations to measure wind direction and temperature etc.

The essential protective equipment shall consist of:

- Protective clothing, first aid kits, antidote kits, antibiotics, and individual decontamination equipment;
- Biological detector kits where available;
- Biological warfare agent-related vaccinations or chemoprophylaxis, where available.

Canisters should be capped and placed into a plasticizer-free bag (or container). After expelling excess air, the bag should be closed and then sealed by taping.

4.3. Sampling Sources

During the sampling process should be select samples in areas which exhibit wet stains, powders, or particulate matter on surfaces, vegetation, water surfaces, or the ground. Less preferred sampling points are those exposed to direct sunlight and high temperatures and not exhibiting any visual indicators.

There are several types of samples or sample matrixes that can be encountered. Sampling may be performed from water, soil, sediments, air, vegetation, powder, liquid, dust, food and feed. In addition sampling may be performed in industries, upon detection or directly on suspicious goods.

Appropriate samples should be taken of the following categories:

Category 1. Unusual powders, liquids, defused munitions (including residues or fragments), NBC protective equipment, in particular respirator canisters and clothing, and (suspected) contaminated non-porous surfaces.

Category 2. Environmental samples including air, vegetation, soil, snow and water samples in the vicinity of the attacked area including food and drinking water.

Category 3. Biomedical samples, including physiological fluids such as blood, urine and saliva from presumed casualties or blood/tissue/organ samples from human and/or animal corpses. Arthropod vectors of disease such as mosquitoes, ticks etc. especially if noted in particularly large numbers and/or unusual geo-climatically sites.

4.3.1. Aerosol samples

As aerosol we mean a suspension of solid or liquid particles (of the size of several microns) in a gaseous medium, usually air. It is formed in the process of aerosolization, which results in the formation of the so-called aerosol cloud.

Aerosols are one of the commonly used forms of application for a variety of agents of biological, chemical or radiological weapon used in a terrorist attack. The appearance of aerosols, smokes or sprays coming from aircraft, vehicles, shells or other ammunition is one of the indicators of the presence of biological, chemical or radiological factors on the potential battlefield. In addition, there may be a hidden attack because the diluted aerosol clouds of the agent may initially be undetectable by senses and may be used to contaminate of drinking water or food supplies. This hidden action may be a part of a terrorist strategy in sabotage attempts. Recognition of signs of aerosol formation may be crucial in a given emergency situation, but the hidden use of chemical, biological, or radiological (CBRN) agents is generally not possible for early detection.

Aerosols are one of the types of typical environmental samples. Aerosol samples should be taken when there is a strong presumption of residual airborne contamination, which must be confirmed with available CBRN detection equipment. NATO has developed procedures for sampling and identification of biological, chemical and radiological agents (SIBCRA). According to general scenarios developed by NATO, aerosols may contain chemical agents (mainly nervous and blistering) or biological agents (toxins, viruses and bacteria) and may enter the body through the skin, eyes or by inhalation route. Aerosol sampling procedures are performed in three operational phases after an attack: immediate, urgent and late. Sampling is required in the first two phases, while in the third phase is optional. Depending on the type of aerosol, guidelines for protective actions for sampling staff have also been developed. These actions include the provision of personnel equipment such as a manual chemical detector, low level oxygen and carbon monoxide portable monitors, skin protective clothing, HEPA and charcoal filters for respiratory protection, and self-contained breathing apparatus required at very high concentrations of factor in the air or oxygen depletion.

Sampling of aerosols in the immediate operational phase (during the first minutes to one hour after the onset of attack) is one of the method of evaluating the attack area (established by the emergency cordon) in terms of airborne contamination. For chemical agents, this assessment can be accomplished by simply observing of visible signs of contamination in combination with use of simple hand-held detection equipment or by sampling and immediate analysis in a field laboratory. For biological agents, airborne contamination can be assessed by observing of visible signs, using available detection equipment, or by sampling and immediate analysis in a field laboratory. For radiological agents, this assessment may be accomplished using appropriate procedures or by using an

impromptu air sampling method. Detection or air sampling should be continued if the changes in the aerosol cloud or the release of a new agent will occur. If airborne contamination is detected outside the area of attack, the boundaries of the emergency cordon should be adjusted accordingly.

Aerosol sampling in an urgent operational phase (from several hours to several days after the onset of an attack) is intended to determine the type of CBRN agent that is present in the material and to assess the changes of location of the airborne contamination clouds. This, in turn, enables the military and political authorities to take effective protective actions and countermeasures to counter further direct exposure or inhalation of contamination and to initiate the process of attack site recovery.

Agents released in the form of aerosol or gas may leave little or no physical evidence of attack with their use (no potential casualties). However, useful samples may be taken from water, vegetation, and other materials such as protective equipment in the immediate attack area, where a particular agent may be absorbed. Sampling should be avoided in areas that are shielded by obstacles, as poor deposition of the agent may occur in these places. For biological and chemical agents, sampling should also be avoided in areas exposed to direct sunlight and high temperatures as these conditions promote rapid degradation of agents, especially biological agents. Ideally, when samples are obtained from shaded areas, and sometimes from buildings.

General guidelines for air, smoke and aerosol sampling.

The objective of air, smoke and aerosol sampling is to assess airborne contamination by CBRN agents, to assess exposure levels, verify of contamination cloud modelling, and implement of protective actions.

Filters and media for air samples may be evaluated in field laboratories deployed in the attack area or in laboratories remote from the attack site, respectively. Field laboratory analyses can provide confirmed agent identification and precisely determine the agent-specific levels in the air. Analyses in remote laboratories can provide unequivocal identification of the agent and determine agent-specific levels in the air. Samples may be collected using large volume air samplers (for all CBRN agents) or by personnel carried small volume air samplers (for chemical and radiological agents).

The sampling staff must be aware of the hazards that may be encountered in the field and take the necessary precautions. Never perform any field activities without proper safety equipment and know how to use it. All monitoring activities are conducted to maintain the lowest level of exposure to the agent. Members of the sampling team must be aware of the possibility of regression of contamination. Monitoring teams must refrain from eating, drinking or smoking in any contaminated area or in areas where monitoring activities are carried out.

The general scheme of air, smoke and aerosol sampling is as follows: collection of appropriate equipment, preliminary activities (steps 1, 2), location of the large and small volume air sampler (step 3), sampling with large and small volume air sampler (steps 4, 5, 6), packaging and administration (step 7) and contamination control/recycling (steps 8, 9).

Supplies and equipment for sampling.

Supplies and equipment for sampling include common equipment for all types of samples and equipment for large and small volumes air sampling. The large volume air sampling equipment includes:

- High volume air sampler (at least 100 Lpm or 30 cfm);
- Sampler collection head compatible with filter size (e.g. 47 mm diameter), clean/sterile;
- Air sampler tripod (one per sampler);
- Filters containing sample collecting media. Depending on the type of medium, there are several types of filters: glass fibre or quartz fibre filters with an initial collection efficiency of 97–99%, low pressure loss, high flows and good loading capacity, difficult to dissolve, with high alpha self-absorption, can be used to fission product monitoring; polystyrene filter with initial collection efficiency of 99%, moderate pressure loss, high flows and good loading capacity but low mechanical strength; PVC membrane filter with a pore size of 0.03–8 μm , initial collection efficiency of 99%, moderate pressure loss, useful for silica, carbon black and quartz particles; cellulose filter with a pore size of 1–3 μm , initial collection efficiency of 99%, high pressure loss, easy dissolution but fast loading, deteriorate with high moisture, serves to monitor of alpha radiation; polycarbonate membrane filter with a pore size of 0.2–5.5 μm , initial collection efficiency of 99%, very high pressure loss, excellent surface collection but poor strength and fast dust loading, is used for air sampling in the breathing zone;
- Plastic bags or glassine envelopes (i.e. impermeable) for the sample collecting media (enough for one filter per bag);
- Portable power supply (generator) and fuel;
- Extension cord 20 feet long;
- Tweezers.

Small volume air sampling equipment (for chemical agents and radiological agents in the breathing zone) includes:

- Manual or electric pump to draw the medium through sample tubes (for gas, vapours, liquid aerosols) or filters (for smokes and solid aerosols);
- Sample tubes with end caps and adsorbent material, e.g. Chromosorb 106 or Tenax;
- Air sampling bags such as Tedlar type;
- Sampler collection head compatible with filter size (e.g. 20 mm diameter);

- GF/A Whatman filter paper, size is compatible with the collection head.

Other equipment includes plasticizer free, sealable plastic bags or glassine envelopes (1 per sample), stopwatch and air sample forms (enough for each filter with collecting medium, C-3 for radiological samples and C-8 for biological and chemical samples).

Preliminary activities.

Step 1

- Calibration of the air sampler according to the manufacturer's instructions;
- Check the sterility of a large volume air sampler for biological sampling;
- Receive preliminary instructions and assignments from Command;
- Obtain the appropriate equipment;
- Check the performance of the equipment;
- Check the radio;
- Check a GPS.

Step 2 (according to instructions from Command)

- Wrap the instruments (except air samplers) in plastic to prevent contamination (except for the detector window if fitted);
- Set alarm levels of direct-reading dosimeters and dose rate meters in case they need to be used;
- Use of appropriate protective equipment, such as agent blocking drugs, protective clothing, protective masks etc.

Sampling with a large volume air sampler.

Step 3 (Location and set-up)

- Find an open-air position – away from any objects that could cause turbulence. As a practical rule it is assumed that the air sampler should be located as far away from the building as at least twice the building's height;
- Mount the air sampler and secure it with a tripod or other mounting device;
- Adjust the height of the sampler so that its inlet is approximately 1.5 m (5 ft) above the ground;
- Properly position the air sampler inlet with the source of the suspect airborne material;
- Record the location of the sampler (ideally in the form of geographical coordinates). Use the appropriate form for air samples.

Step 4 (Installation of a new filter)

- Remove the filter retention frame or filter holder;
- Obtain a clean, weighed filter and record its number;
- Placement of a new filter on the sampling head;

- Reinstallation of the retention ring or frame.

Step 5 (Collection of air samples)

- Record sampler data (e.g. sampler ID number, sample number, sampler serial number, etc);
- Placement of a small -xII sign (in pencil only) on the outer edge of the exposed side of the filter;
- Turn on the sampler and record the start time and the flow rate on the prepared sample form (either as a rotameter or a similar flow measurement device read and as a corrected flow rate calculated from the calibration chart attached to the air sampler). **Do not stand in front of the sampler while work!**

Step 6

- Record the rotameter reading and corrected flow rate at the end of the desired collection time (minimum volume is 100:l for biological and chemical agents and 8,000:l for radiological agents);
- Turn off the sampler and record the end time and other data on the sample form. **Warning! Before proceeding with changes of filters, make sure the sampler device is turned off!;**
- Remove the retention ring of the filter or gasket of the filter faceplate using gloves;
- Remove the filter paper from the air sampler by grasping the filter edges using tweezers. For large filters, fold the paper lengthwise with the exposed side to the inside;
- Place the filter paper in a glassine envelope or plastic bag and seal it after removing as much air as possible from the bag. **Warning! Turn off unused battery-powered devices to avoid flat batteries, but only when away from the cloud area!**

Sampling with a small volume air sampler.

Step 3 (Location and set-up)

- Locate within 1–2 m of the contaminated surface at the downwind edge of a contaminated area;
- Properly position the air sampler inlet to the source of suspect airborne material;
- Record the location of the sampler (ideally in the form of geographical coordinates). Use the appropriate form for air samples.

Step 4 (Installation of a new filter or adsorbent tube)

- Remove the paper retention frame (in case of filter) or clamp (in case of tube)
- Record the filter or tube number;
- Place a new filter on the sampling head or a new tube in the sampler orifice;
- Reinstall the retention ring or frame (in case of filter) or clamp (in case of tube).

Step 5 (Collection of air samples)

- Record the sampler data (e.g. sampler ID number, sample number, sampler serial number etc.);
- Place a small sign (in pencil only) on the outer edge of the exposed side of the filter or at the inlet side of the tube;
- For manually operated pumps: starting pumping and recording the final sample volume on the previously prepared form for air samples. The standard flow rate should be at least 100 ml/min and the desired volume of the air sample should be at least 1 l;
- For mechanically operated pumps: turning on the sampler and recording the starting time and the flow rate on the prepared sample form (either as a rotameter or a similar flow measuring device reading and as a corrected flow rate calculated from the calibration chart attached to the air sampler). The standard flow rate should be at least 100 ml/min and the desired volume of the air sample should be at least 1.5:l. Recording the rotameter reading and corrected flow rate at the end of the desired collection time (minimum collection time is 15 min). Turning off the sampler and recording the ending time and other data on the sample form. **Do not stand in front of the sampler while working!**

Packaging and administration

Step 6

Warning! Before proceeding with filter changes, make sure the sampler device is turned off!

- Remove the retention ring of the filter or gasket of the filter faceplate or tube using gloves;
- For filters: remove the filter paper from the air sampler by grasping the filter edges using tweezers. Place the filter paper in a glassine envelope or plastic bag and seal after removing as much air as possible from the bag;
- For tubes: tight closure of tubes using the end caps. Placing the filter paper in a glassine envelope or plastic bag and sealing after removing from the bag as much air as possible.

Step 7 (Completion of the sample delivery form)

Contamination control/recycling.

Step 8

- Visually inspect the sampler, and if necessary, repair or cleaning/sterilization;
- Clean/sterilize the filter head with fluid from a clean source and load a new filter paper if necessary. Clean the tweezers if they are not disposable;
- Refuel the generator for the power supply;
- Make sure that the sample form for the air is properly completed, include the sample and date and time of start and end of the sampling, total sampling time and volume of the sample according to standard conditions.

Step 9

Finally, check personnel and equipment (contamination control) using A-4 and/or A-8 procedures respectively.

4.3.2. Water and liquid sampling

The purpose of water and liquid sampling is to assess the CBRN hazards in drinking water, crops irrigated with water or livestock/wildlife utilizing water in case of a possible attack.

One should record the location of sampling, as well as the environmental conditions at the time of sampling, such as weather conditions, water temperature and flow rate, a kind of a water source etc. If R agents are suspected, the ambient gamma dose should be measured and recorded.

Equipment

Equipment that is or might be needed for sampling (depending on the purpose, environment and the agent):

- Equipment common to all sampling protocols (such as: sample position markers, indelible ink pens/writing pad, CBRN hazard labels, disposable plastic sheeting, tissues/paper roll (for cleaning purposes), solvent, alcohol or de-ionised water (for cleaning/decontamination purposes) – in litres, protective clothing, first aid kit, antidote kit, antibiotics and individual decontamination equipment, GPS, maps);
- Equipment specific to biological/chemical/radiological sampling;
- Overshoes, boots, waders;
- Water filters and sampler head for water filters, connective hoses (for B agents);
- Adsorbent cartridges (for R or C agents extracted from water).

For small volume sampling:

- 50–100 ml pipettes (with a balloon pump), syringes (one per sample);
- 50–100 ml sample bottles with lids (one per sample);

For large volume sampling:

- a bucket or a similar container;
- plastic funnels and cylinders;
- a peristaltic pump or other similar device;
- a precipitation sampler;
- tape measure and a stop watch to measure the liquid flow.

Location for sampling and sample size.

- Reservoirs that can be a source of useful water samples are for instance: ponds, lakes, rivers, streams, puddles; any water source in the nearest area of a suspected attack. For all water and liquid samples, the surface samples are most

preferable. Liquid/water can be taken as a sample as such, or the agents can be extracted according to proper protocols.

– Two sets of samples may turn out to be advantageous for confirmatory second analysis.

– Sample size depends on the suspected CBRN hazard. If the agent concentration is too low, bigger sample volume may be required. On the other hand, big neat agent doses may pose a threat and a safety risk. Typical size of water and liquid samples are given in a Table 1.

Table 1. Typical size of water and liquid samples

Sample type	Agent types	
	C, B, R	C, B
Surface or drinking water	1-4 l	50- 100 ml
Neat or dissolved agent		10-50 ml

Sampling

Small scale, direct sampling

1. One pipette or one syringe should be used per sample;
2. Immerse the tip of the syringe or of the pipette just below the liquid surface;
3. Take at least 50 ml of the liquid;
4. Transfer the sample into a clean sample bottle;
5. Close the bottle tightly and proceed to step 2.4.

Small scale, extractive sampling

1. Install a new filter or adsorbent cartridge prior to collecting the sample:
 - a) remove a paper retention frame (filter) or clamp (cartridge);
 - b) write down the filter or cartridge number;
 - c) place the new filter on the sampler head or put the cartridge in the sampler or syringe orifice;
 - d) reattach the retention ring or frame (filter) or clamp (cartridge).
2. For collecting the sample use one syringe or one filter per sample.
3. Immerse the pump inlet or the tip of the syringe needle just below the surface and with minimum disturbance.
4. Aspirate minimum 50 ml of liquid.
5. Remember not to use too much pressure to force the liquid through the extractive device!
6. Write down the extracted volume.
7. For filters: carefully remove the wet filter with the tweezers and put it in a sterile capped bottle.

8. For cartridges: cover with end caps and place in a glassine envelope or a plastic bag and seal, trying to release as much air as possible.

9. Extracted water/liquid should be collected in a clean, sterile bottle, closed tightly.

Large scale, direct sampling

1. Immerse a collection container (e.g. a bucket) and the sample container in the water and rinse both of them. If using a peristaltic pump, pump the water long enough so it can rinse the inside of the whole system accurately.

2. Wear gloves and waders when collecting samples with a bucket or a pump. Carefully submerge them in the water again and let them fill in slowly and continuously but not to the absolute top (samples for a biological analysis require vigorous shaking before taking portions). Avoid collecting bottom sediment, vegetation or small fish, also try to avoid surface disturbance.

3. In case of using a sampler, empty its content slowly into the actual main container using a new, clean funnel. Pour the liquid with minimal disturbance.

Preservation of the sample

1. The containers should be capped tightly and their exterior surface should be wiped down.

2. Write the sample ID on the sample container using an indelible pen.

3. Preserve the sample – preservation will depend on the agent:

- In case of R agents, the hydrochloric acid (11 M) must be added to sample bottles, 10 ml per liter of sample, either prior or after sampling, in order to avoid adsorption of radionuclides on the container walls. It is especially important when storage time before analysis may extend (AEP-66, NATO Handbook).
- In case of B and C agents, the protocols are more specific and require more detailed recommendations. Samples must be preserved during the entire mission, including transport to the laboratory in such a way that live agents are not inactivated (e.g. keep cool, airtight containers). Handling techniques must be sterile to avoid cross-contamination.

4. Store the sample bottles at 2–8°C.

4.3.3. Environmental samples

Solids

Solid environmental samples should be packed in plastic bags or containers. Place each sample in an individual bag. Remove excess void space, seal the bag (self-closure) and further seal by taping. The amount of sample

required depends upon each specific case, even a very small sample may be of great value.

Soils. Samples should be taken from a surface area of about 10 cm x 10 cm by scraping a sample from the surface not exceeding 2 cm depth. Plants, seeds, and debris, when present, should be included. Soil samples from an area suspected of having been contaminated should be collected as soon as possible from the centre of the contaminated area or as close to it as possible. Collect samples from the ground near the bodies of any fallen casualty or dead animal paying particular attention to areas contaminated with fluids expelled from the animal after death. Place soil samples in clean bags, expel excess air and seal the bags. Clean, sterilized, non-breakable glass jars, metal tins or other Teflon-lined containers that can be sealed are also satisfactory.

Stones. Should stones be selected for collection, then these should be of moderate size (about 0.5–2 cm), to a total volume of 200–300 ml. Place samples in plasticizer-free bags in the same manner as soil samples.

Snow. Samples should be collected from the layer believed to be exposed to a biological attack. Take samples from an area of 10 cm x 10 cm to a depth of 2 cm. New snow covering the exposed layer would preserve the agents, but should not be collected. The samples should be placed in clean sample bottles and closed with a Teflon-lined lid. Be careful to clean the threads before closing the bottles to prevent melted snow leaking out.

Vegetable matter. At least two litres of vegetation should be collected, preferably from several locations in the target area. Vegetation is an excellent source of absorbed agent especially if sampling is conducted soon after the suspected attack. Leaf samples, particularly from plants that are withering, are preferred. Broad-leafed vegetation is desirable as well as samples of grasses, bushes, and grains. Avoid touching the surface of the leaves with gloves or sampling equipment while gathering the samples. Store vegetation in bags, expel excess air, seal the bags and chill the sample.

Fragments of munitions, canisters, and NBC protective clothing. Munitions remnants and fragments, respirator canisters from protective masks and items of individual NBC protective clothing are highly desirable sources of agent samples. Canisters are particularly useful since they collect particulate agents. It is noted that the location where a canister is found may not be the contaminated site, because the user may have moved after being contaminated.

Non-transportable solid items. Samples from immovable objects such as buildings, walls, paved surfaces or vehicles. These can be sampled by scraping or swabbing the contaminated surface with swabs (cotton, Dacron™, polyester, rayon, and foam). Shafts can be comprised of either wood or plastic. Generally, synthetic swab tips with plastic shafts are recommended because they are not of biological origin and will not interfere with DNA-based detection systems. Swabs may be used dry or wetted with distilled water or phosphate buffered

saline (PBS) buffer solution. In general, studies have shown that wet swabs have higher collection efficiency than dry swabs. For sampling from the surface we can also use Agar Plates. Agar plates, also known as 'sticky plates', can be used to sample biological agents.

Control Samples. Control samples corresponding to each type of contaminated sample should be taken. The control samples should be packaged and transported in the same manner, along with contaminated samples, ensuring that the possibility of cross contamination is eliminated.

Table 2. Biological Sampling

Type of Sample	Instrumentation	Quantity of Sample	Further Details
AEROSOL	Sterile biological aerosol sampling unit or high volume cyclone	As produced (min 100 l)	Only when positive indication is received from detection equipment
SOLID	Tongs/forceps/scoop	Stones. Only those of moderate size (about 0.5–2 cm) should be selected to a maximum volume of 200-300 ml	Stones, plastics, metals etc. Sizes of sample – a range of 0.5–2cm
SNOW	Scoop	An area of 10 cm x 10 cm x 2 cm deep	Collect only layer exposed to attack
SOIL	Scoop	An area of 10 cm x 10 cm x 2 cm deep	Sandy soil is easier to analyse
WATER	Pipettes, syringes, & vacuum containers	100 ml	Surface liquid
LIQUID	Vacutainers, pipettes, syringes	10 ml	Neat agents
CANISTERS, NBC CLOTHING	Tongs/forceps, scissors and scalpel	Any amount to be placed in 500 ml bags	Canisters trap agents

Table 2. cont.

Type of Sample	Instrumentation	Quantity of Sample	Further Details
UN-TRANSPORTABLE SOLID ITEMS	Dry cotton wool/cotton wool soaked in distilled water or PBS/swabs with transport media	Any numbers	Samples taken by scraping and/or swabbing
VEGETATION	Tongs/forceps, scissors & secateurs	21	Broad-leafed plants are preferred
URBAN SAMPLING	Sterile dry rayon/cotton swabs or cotton tipped sticks in a storage tube with transport medium, cloth discs or filter paper wetted with sterile water	Area for smear 100 cm ²	Flat surface swabs

Biomedical Samples

Dead Animals. If the carcass is small, collect and ship it complete, refrigerated where possible. Place samples in plasticizer-free bags or sterilized containers. If the dead organism is large, useful samples may be taken from the spleen, kidney, the liver, the lungs, the skin, the muscles, the blood from the heart, the lymph nodes, small bones and the brain. Arthropod vectors of disease such as mosquitoes, fleas, ticks, lice etc. should be collected in large numbers.

Human Casualties. Normally, casualties of suspected biological attacks would be taken to medical facilities where appropriate equipment is available to collect samples of physiological fluids, tissues, etc. Therefore, such equipment has not been included in the field sampling kits.

The following samples may be taken from human casualties:

1. Blood. Blood samples from casualties should be taken by vacuum syringe (Vacutainer). The sample tubes must be sterile; i.e. hermetically sealed with a plastic plug which can be pierced readily. Blood samples should be kept

cold but NOT frozen. The following blood samples should be taken from each casualty:

- 1 x 10 ml into an uncoated sterile tube;
- 2 x 10 ml into tubes with sodium heparin;
- 1 x 10 ml into a tube with EDTA (disodium Salt);
- 1 x 10 ml into a tube with sodium citrate.

2. Urine. Urine samples of 100–200 ml volume should be taken into sterile glass or plastic containers. Collection of urine over an extended period (up to 24–48 hours) is also desirable. Biomedical samples (blood/urine) should be taken as soon as possible after the exposure to agents, preferably within 72 hours.

3. Skin. Swab samples from skin, small samples of the affected area should be taken, along with fluid from any blisters. Skin is best stored on ice.

4. Mucous Membranes. Swab samples from the mucous membranes and the nasopharynx should be taken.

5. Saliva, Vomit and Excrement. Since samples of this type are usually of little value, they should not be taken unless there is a particular reason for it. Samples should be taken into sterile glass containers.

Table 3. Biomedical sampling

Type of Sample	Instrumentation	Quantity of Sample	Details of Sample
DEAD ANIMALS	Tools	Whole carcass or relevant parts thereof.	
HUMAN BLOOD	Venojet or vacuum syringe system	1 x 10 ml into a sterile tube. 2 x 10 ml coated by sodium by medical corps heparin. 1 x 10 ml in tube coated with EDTA. 1 x 10 ml in tube coated in sodium citrate.	Only to be taken by medical corps personnel.
HUMAN URINE	Sterile glass or plastic container	100–200 ml	
SKIN	Swabs	Swab samples	
MUCOUS MEMBRANES	Swabs	Swab samples	Eyes, naso-pharynx.
SALIVA, VOMIT AND EX-CREMENT	Sterile glass container	volume possible to download	

4.4. Biological Sampling Equipment

Use of Sampling Equipment in the Field

Samples should be collected and handled with clean (in the case of biological agents: sterile) forceps, spatulas, scoops, scissors, scalpels, and other sampling instruments. Sufficient quantities of handling devices are to be available, to avoid re-use of the instruments in the collection of more than one sample. This minimizes the risk of contamination transfer from gloves to samples or from one sample to another.

Sampling equipment should be scrupulously clean (sterile for biological agents) and it should be stored in clean (sterile) bags prior to the mission.

General equipment:

- 60 x sealable zip bags (various sizes);
- 10 x 50 ml sterile wide necked glass or plastic bottles (air tight);
- 6 x 250 ml sterile wide necked glass or plastic bottles (air tight);
- 4 x transport bags (50 l);
- 1 x tissue container;
- 50 x sheets for description of samples;
- 5 x pairs NBC gloves;
- 1 x bottle of sodium hypochlorite solution (IZ-15% chlorine) or similar disinfectant;
- 1 x bottle of distilled water;
- 1 x bottle of phosphate buffered saline (PBS);
- 10 x swabs;
- 1 x roll sealing tape (parafilm);
- 2 x marker pens (waterproof);
- disinfectant-impregnated absorbent material for transport packaging;
- 1 x bag sealer (portable);
- 1 x portable refrigerator unit powered by battery and capable of maintaining 2–8°C.

Additional Equipment for Environmental Sampling:

For sampling solids:

- Sterile tongs, secateurs and scissors (large);
- Sterile scalpels and spare blades;
- 12 x sterile spoon spatulas;
- 5 x sterile disposable forceps;

- Spade/shovel (small);
- Disposable scoops (plastic; e.g., 100 ml, 200 ml);
- 1 x roll aluminium foil;
- 50–100 ml sample bottles, with Teflon-lined, airtight lids (for snow samples, one per sample);
- Large, sealable bags or glassine envelopes or 2–4 l plasticizer free plastic jugs with cap (for solids and objects; one per sample);
- Environmental sample forms and sample custody forms (one per sample).

For sampling surfaces:

- 10 x sterile dry rayon swabs;
- 10 x sterile swabs in microbiological transport medium;
- 100 ml phosphate buffered saline.

For sampling liquids:

- 12 x sampling needles (18 gauge blunt);
- 12 x pipettes (50–100 ml).

For sampling air:

- 10 x aerosol sampling filters assemblies, capacity 20 litres/minute (5–10 cm diameter);
- or hand-held aerosol impinger of the same air capacity;
- 1 x bottle of distilled water.

For sampling surfaces:

- 10 x sterile dry rayon swabs;
- 10 x sterile swabs in microbiological transport medium;
- 100 ml phosphate buffered saline.

For sampling liquids:

- 12 x sampling needles (18 gauge blunt);
- 12 x pipettes (50–100 ml).

For sampling air:

- 10 x aerosol sampling filters assemblies, capacity 20 litres/minute (5–10 cm diameter);
- or hand-held aerosol impinger of the same air capacity;
- 1 x bottle of distilled water.

Additional Equipment for Biomedical Sampling

- 1 x small tool kit;
- 1 x thermometer (digital);
- 1 x water sampling kit consisting of

- 50 x 10 ml evacuated flasks (sterile, silicone coated);
 - 50 x double-ended sterile needles (18 gauge fitted with safety caps);
 - 6 x sterile blunt ended needles for insertion into sampler tubing;
 - 6 x sterile sampler tubing (weighted) – (3 x 25 cm; 3 x 100 cm);
 - 1 x rack for evacuated tubes.
- 1 x medical sampling kit for taking of pathological specimens;
 - 1 x entomological kit;
 - 1 x blood sampling kit consisting of:
 - 50 x vacutainer (10 ml);
 - 100 x un- and pre-coated tubes;
 - 50 x double-ended sterile needles fitted with safety caps;
 - 6 x sterile blunt ended needles for insertion into sampler tubing;
 - 6 x sterile sampler tubing (3 x 25 cm, 3 x 100 cm);
 - 1 x rack for evacuated tubes.

4.5. Labelling and documentation of samples

The samples should be labelled and forwarded for analysis as expeditiously as possible through appropriate national channels.

Labels should be affixed to each sample container. On each should appear a code number which clearly and uniquely refers to the accompanying the nature and circumstances of collection. If on-site contamination checks reveal the presence of a toxic substance, this should be clearly indicated on the sample containers by means of colour coding and/or a highly visible internationally recognized hazard symbol. Labelling should not contaminate the sample and should be sufficiently resistant to degradation, decontamination.

All aspects of sample collection, packaging, storage and transportation must be documented (Data sheet).

4.6. Sampling based on autonomous or remote controlled ground or aerial vehicles

The crucial point in eliminating or reducing the number of casualties and reducing the spread of CB warfare contaminations is how quickly they are detected. The key issue is also safety of sampling. Current biological detection system can detect only a limited number of biological agents and only after exposure. Sensitivity, selectivity and durability of these detection technologies demand of perfection.

The goals of detection system include the following:

- 1) to detect agents in time to warn, protect and minimize the number of casualties;
- 2) to identify agents in time to initiate medical therapies on casualties;

3) to collect samples for verification.

The overall goal of a detection system should be: detection of agents in sufficient time to warn, protect and minimize the number of casualties among those who would be exposed to the agents, identification of agents and possibility for independent verification of identification. The generic model for a point detection system will include four elements; a collector, a trigger, a detector, and an identifier. The trigger component provides non-specific detection of the presence of possibly harmful biological material. The trigger component should give a rapid indication of the likely presence, but not the identity, of biological material and normally bases this indication on a change in the background conditions.

The detector component determines the presence of categories of biological agents, but may not provide sufficiently specific information on which to base protective or treatment decisions. The identifier component, as the name implies, identifies the specific BW agent to the degree necessary to allow commanders to initiate appropriate protective measures.

The groundwork of proper identification and possibility of use of suitable method is sampling. Biological sampling is normally targeted at living organisms, so the sampling technique must preserve and not harm the collected sample. Most biological detection and presumptive and confirmatory identification technologies require a liquid sample, so the collection must be from an aerosol or particulate onto a liquid. The liquid sample must be highly concentrated and available for analysis rapidly.

The word 'sampling' is usually taken to mean the collection of a large volume of air and concentrating the particulate matter in either an air or fluid medium so as to prepare a 'sample' for further investigation and analysis. Hundreds of litres of air are normally required to obtain an adequate sample of material, and the collection device must be able to gather that much air and aerosol.

4.6.1. Sampling based on unmanned ground vehicle

Unmanned ground vehicle (UGV) is a vehicle that operates without human presence on the board and in contact with the ground. It can be used in many applications especially in dangerous environment or when human operator presence is impossible. UGV can be used as an autonomously or remote controlled devices. An autonomous UGV is essentially an autonomous robot that operates without the need for a human controller. The vehicle uses its sensors to develop some limited understanding of the environment, which is then used by control algorithms to determine the next action to take in the context of a human provided mission goal. This fully eliminates the need for any human to watch over the menial tasks that the UGV is completing. Autonomous

UGV travel between way points without human navigation assistance. It can also help to aim according to GPS data.

For biological sampling autonomous UGV can be used to take a sample of aerosol after triggering and detection of aerosol cloud. On the platform of UGV should be mounted devices for triggering and detection of biological agents (i.e. Fluorescence and Particle Sizing, Size and Shape Analysis, Pyrolysis/Gas Chromatography/Ion Mobility Spectrometry, Chemical Luminescence, Flame Photometry/Gas Chromatography, Flow Cytometry, Mass Spectrometry). Proper taking of aerosol samples for identification of an agent requires the use of a cyclone-based working device. Aerosol particles are collected in the liquid phase and transferred to the lab.

4.6.2. Sampling based on remotely operated ground vehicles

Remotely operated ground vehicle (ROGV) needs human operator who radio-manages the action observing it on the monitor. ROGV has devices for sampling of air, ground, water and other little objects like stones or suspicious tanks installed on the board. Contamination of air can have forms of aerosol or mist. Aerosol is dispersion in air of solid particles of microscopic size. Mist is dispersion in air of liquid droplets usually large enough to be seen by the naked eye. For air sampling commonly instruments working on the base of cyclone are used. One-time this instrument aspirates about 10 m³ of air and suspends solid particles in 30–50 ml of liquid phase. The sample is transferred for tests carried out by devices on ROGV or in the stable laboratory after it's back. Results of examination carried out on the ROGV are send by radio to the basic laboratory.

Liquid samples (water or other liquids) are taken by special devices mounted on ROGV working as a pump. These samples should be taken to sterile containers and examined by devices on ROGV or/and after back in stable laboratory. Required volume of liquid sample – c.a. 100 ml.

On the platform of ROGV a special device working as a digger can take a sample of ground. The sample is transferred to sterile plastic bag and transported to the basic laboratory. For taking other samples like stones, vegetables, small dead animals, ROGV should be equipped with special claws enabling of drawing such material and packing it to special plastic bags. ROGV should be equipped with facilities for disinfection and ability for self-disinfection before back to the basic laboratory. Required weight of soil sample - c.a. 100 g.

4.6.3. Remote Controlled Aerial Vehicles (RCAV) – Drones

UAVs (Unmanned Aerial Vehicles) are a component of Unmanned Aircraft Systems (UAS), which consist of a UAV, a ground-based controller,

and a system of communications between the two. UAV may operate under remote control by a human operator or autonomously by on-board computers. Drones may be used for aerosol sampling only if there is a possibility to install a miniaturized cyclone-type device to suspend the aerosol particles in liquid. The sample should be taken to the basic laboratory. Drones must be disinfected in the place of landing. Drones are dispatched directly to the aerosol cloud (detected by other methods) and sample of 1 cubic meter taken by cyclone apparatus seems to be adequate for identification of a biological agent in laboratory.

4.7. Field exercises – environmental sampling for the presence of biological weapons

Location for solids and soil sampling

The best location for sampling will be where casualties have occurred or where there may be wilted or discoloured plants or an unusual number of dead animals such as fish, birds or rodents. Soil, snow, vegetation and solid samples should be from a highly contaminated area or from near a casualty.

4.7.1. Sampling of stones

Collecting the sample:

- Select stones of moderate size (about 0.5–2 cm) should be selected to a maximum volume of 200–300 ml;
- Use clean/sterile tweezers or tongs to place the stones in plasticizer free, clean/sterile bags or in plasticizer free jugs;
- Seal the bag, or close the jug airtight;
- Store the sample preferably at 2–8°C.

4.7.2. Sampling of soil

Collecting the sample:

- Use one disposable scoop per sample or use a cleaned spade/shovel;
- Take a sample over a surface area of about 10 cm x 10 cm by scraping material from the surface, not exceeding 2 cm depth. Plants, seeds, and debris, when present, should be included;
- Transfer the soil sample into a clean (sterile) bag or sample bottle;
- Seal the bag, or close the sample bottle airtight with a Teflon lined lid;
- Store the sample preferably at 2–8°C.

4.7.3. Sampling of snow

Collecting the sample:

- Use one disposable scoop per sample or use a cleaned spade/shovel;
- Collect a sample from the layer believed to be exposed to an attack. Take samples from an area of 10 cm x 10 cm to a depth of 2 cm;
- Transfer the snow sample into a clean (sterile) sample bottle;
- Close the sample bottle airtight with a Teflon lined lid;
- Store the sample preferably at 2–8°C.

NOTE. New snow covering the exposed layer would preserve the agents, but should not be collected.

4.7.4. Sampling of vegetable matter

Collecting the sample:

- Use one disposable scoop per sample or use a cleaned spade/shovel;
- Collect at least two litres of vegetation, preferably from several locations in the target area. Broad-leaved vegetation as well as samples of grasses, bushes and grains should be collected;
- Transfer sample material into a clean (sterile) bag or sample bottle;
- Seal the bag, or close the sample bottle airtight with a Teflon lined lid;
- Store the sample preferably at 2–8°C.

NOTE. Avoid touching the surface of leaves with gloves or sampling equipment while gathering the samples.

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5. METHODS OF BIOLOGICAL SAMPLES FIELD ANALYSIS

5.1. Introduction

Biological agents that can be used as biowarfare agents (BWA) are the most dangerous threats, and can have an influence on large numbers of both humans and animals. In terms of health protection, as well as prevention of BWA attacks, rapid diagnostics are the most important step in the case of sudden suspicious situations. According to AEP-66 NATO protocols, three detection levels are required: Provisional – one of the following criteria must be met (immunological assays, detection of nucleic acids, culturing); Confirmed – two of the listed criteria must be applied (immunological assay, molecular method, culturing), and Unambiguous – all of the listed criteria must be applied (immunological assay, molecular method, culturing and animal model testing, if possible/necessary). Two of the levels (Provisional and Confirmed) can be determined in the field by a sampling team at the Provisional stage, or at a mobile laboratory (Confirmed), if available at the time of the field research. Immunoassay mainly includes rapid immunochromatographic tests that return results in 10–15 min. These tests do not require specialized equipment and skilled personnel. However, the major limitation is the sensitivity of the tests, as even negative results do not exclude the presence of the biological agents tested for. Samples must then be transferred to a mobile laboratory or stationary reference laboratory for further analyses. Molecular techniques are one of the most commonly in detection of biowarfare agents, in labs and in the field. Several instruments and reagents allow for rapid, accurate and specific identification of biological agents using Polymerase Chain Reaction method (PCR). Nowadays, classic PCR is not routinely used and has been replaced with real-time PCR. This method is more sensitive and need the short time for experiment, the fact that they can be run in multiplex format, and their ease of use after just a short training period. Finally, most of the immunological tests dedicated to environmental screening are not subject to *in vitro* diagnostics, including clinical samples.

5.2. Immunoassays (IA)

Immunoassays methods are based on detection of antibodies or antigens which may be found in number of different samples, e.g. clinical samples (animal and human serum samples), environmental samples such as water, soil, powder, swipes from the abiotic surfaces or carcasses. IA methods allow testing various of them on site in the field conditions as well as in the mobile laboratory. Most commonly used tests are the rapid immunochromatographic assays and enzyme-linked immunosorbent assay (ELISA). In case of rapid chromatographic test they are designed for field detection and are prepared in vacuum-foiled bags with appropriate sample buffers, consumables and practical instruction how to prepare samples. Large number of test may be adopted in case of rapid field detection of biological agents concern biowarfare (Table 1). The results are available within after about 10–15 minutes.

ELISA is another immunological method that allows detection of antibodies antigens. It can be done only in laboratory conditions, also in the field laboratory which may be equipped with ELISA readers and others instruments required for sample preparation workflow. Number of samples of different origin can be tested with ELISA: animal and serum samples, environmental samples (soil, water, powders, swipes). However this method characterizes with moderate sensitivity and specificity, especially in case of environmental samples where chemical inhibitors may be found, it is routinely used in samples diagnostic scheme at the Confirmed stage.

5.2.1. Rapid Immunochromatographic Assay

Rapid immunochromatographic tests, or SMART (Sensitive Membrane Antigen Rapid Test) belong to modern and rapid detection technologies for biological agents. This technique applies mono-, or polyclonal antibodies tagged with colloidal gold immobilized on a nitrocellulose membrane. It is a rapid and cost-effective preliminary detection and identification method used routinely in the field.

In the SMART method, two types of antibodies directed against the antigen are used: one is immobilized on nitrocellulose membrane, while the other is labelled with colloidal gold and penetrates through the test surface. If the sample is positive antigen binds to the colloidal gold, hence to the appropriate antibodies. This complex is moves along the membrane until an immobilized on the membrane antibodies. This effect is visible as a line. If there are not antigens in sample, colloidal gold labelled antibodies don't bind to the antigen and anti-antigen antibody. Control line is visible when colloidal gold labelled antibody connects with control antibodies.

These tests allow biological weapons agents factors detection for example: anthrax, ricin toxin, botulinum toxin, plague, or SEB (Staphylococcal enterotoxin B) and others biological factors (Table 1).

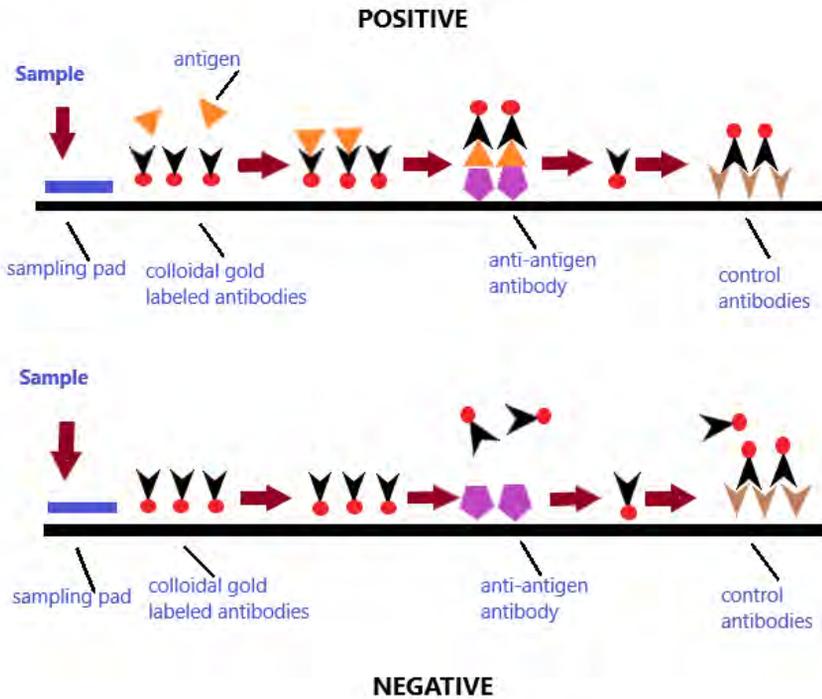


Figure 1. Basic principles of SMART immunoassay tests (Author: Patrycja Głowacka)

Table 1. Commercially available immunoassay tests

Biological agent	Lateral flow assays		
	Category A	Producer	Test name
Anthrax (<i>Bacillus anthracis</i>)		New Horizons Diagnostic	SMART II
		ADVNT Biotechnologies	BADD
		BIOFIRE	Biothreat Panel
		Response Biomedical	RAMP
		Tetracore	BTA
		Alexter Technologies	Biodetect Tests and RAID
		Environix	ENVI

Table 1. cont.

Biological agent	Lateral flow assays	
Botulism (<i>Clostridium botulinum</i> toxin)	ADVNT Biotechnologies Tetracore Response Biomedical Alexter Technologies Environix	BADD BTA RAMP Biodetect Tests and RAID ENVI
Plague (<i>Yersinia pestis</i>)	ADVNT Biotechnologies Tetracore BIOFIRE Alexter Technologies Environix	BADD BTA Biothreat Panel Biodetect Tests and RAID ENVI
Smallpox (<i>Variola major</i>)	Response Biomedical Alexter Technologies	RAMP RAID
Tularemia (<i>Francisella tularensis</i>)	New Horizons Diagnostic ADVNT Biotechnologies BIOFIRE Tetracore Alexter Technologies Environix	SMART II BADD Biothreat Panel BTA Biodetect Tests and RAID ENVI
Ebola virus	BIOFIRE	Biothreat Panel
Marburg virus	BIOFIRE	Biothreat Panel
Category B		
Brucellosis (<i>Brucella species</i>)	ADVNT Biotechnologies Tetracore BIOFIRE Genomix Biotech Alexter Technologies	BADD BTA Biothreat Panel (<i>B. meli-</i> <i>tensis</i>) Brucella Antibody Test Kit Biodetect Tests and RAID
Epsilon toxin of <i>Clostridium perfringens</i>	Thermo Fisher Scientific	Epsilon Toxin Rapid Test Kit
<i>Salmonella</i>	New Horizons Diagnostic	SMART II
<i>E. coli</i> O157:H7	New Horizons Diagnostic	SMART II
<i>Shigella</i>	Meridian Healthcare	Rapid-VIDITEST <i>Shigella</i> spp., <i>S. dysenteriae</i>

Biological agent	Lateral flow assays	
Glanders (<i>Burkholderia mallei</i>)	BIOFIRE	Biothreat Panel
Psittacosis (<i>Chlamydia psittaci</i>)	Bioplus	<i>Chlamydia</i> antigen
Q fever (<i>Coxiella burnetii</i>)	BIOFIRE	Biothreat Panel
Ricin toxin	ADVNT Biotechnologies Response Biomedical Alexter Technologies Environix	BADD RAMP Biodetect Tests and RAID ENVI
<i>Staphylococcal</i> enterotoxin B	New Horizons Diagnostic ADVNT Biotechnologies Environix	SMART II BADD ENVI
Typhus fever (<i>Rickettsia prowazekii</i>)	ImmuneMed	Murine Typhus Rapid
<i>Vibrio cholerae</i>	New Horizons Diagnostic	SMART II
<i>Cryptosporidium parvum</i>	Thermo Fisher Scientific	<i>Cryptosporidium parvum</i> Rapid Test

One of the most important disadvantage is the range of detection limit between 1.5×10^4 to 8.3×10^8 spores/ml for *B. anthracis*, or for other biological agents about 1.0×10^5 CFU/ml. For bacteriological toxins limit detection is about 10-30 ng/ml, but for ricin toxin: 5 ng/ml in ENVI test. In relation to above limitations, negative results of IA tests should not be considered as 'negative' and samples must be forwarded to stationary reference laboratory, or mobile laboratory for confirmatory tests.

5.2.2. ELISA

Enzyme-linked immunosorbent assay as a EIA (Enzyme Immunoassay) is adopted for detection the presence either antibodies produced in response to infection, or antigens from the infecting agents in examined samples.

In the ELISA assay, the targeted antigen is immobilized to the polystyrene plate directly by adsorption, or indirectly using the capture antibody attached to the plate. The direct and indirect antigen detection is carried out relatively using the enzyme-conjugates primary antibody or enzyme-conjugated secondary antibody with unlabelled primary antibody.

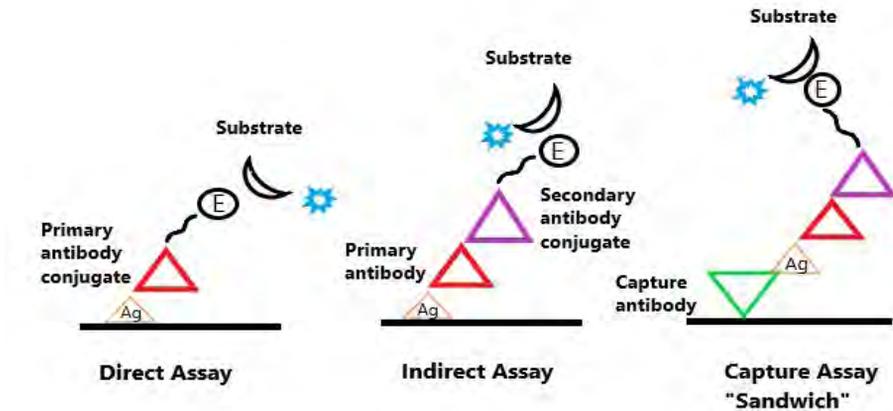


Figure 2. The ELISA reaction (Author: Patrycja Głowacka)

For bioterror detection the directed ELISA method to antigens is implemented. In case of particular antigen detection presented in environmental samples (suspension of solid material e.g. powder, soil or liquids or swipes) the specific antibodies are used. These antibodies may be adopted for reaction with different protein epitopes. The unknown antigen should be affixed to the surface of polystyrene microwells, and the binding of antigen and specific antibody should be revealed by applying the antibody covalently linked to the enzyme. Between each step for removing the residual components, not specifically bounded antigens, or antibodies, the washing with detergent solution should be applied. In final step the enzyme converts the special chemical substrate to visible signal which is detected spectrophotometrically. The intense of coloured signal indicates the quantity of antigen in examined samples.

ELISA can be used as convenient tool for screening large numbers of small-volume examined samples. The use of a monoclonal antibody in immunoassay results in high specificity of the reaction and it has low level background. However, the polyclonal antibodies can increase the range of the assay to detect multiple isolates belonging to different species of bacteria, virus, or fungi.

For the detection of biological bioterror agents (and simulants e. g. *Bacillus globigii*) in environmental samples BioThreat Alert® ELISA Kits are available (Tetracore). Additional, this method is preferred for complex sample matrices belonging to category A (according to CDC) bioterrorism agents (*Bacillus anthracis*, *Clostridium botulinum* toxin, *Yersinia pestis*, *Francisella tularensis*), and category B (*Brucella* spp., *Burkholderia* spp, *Vibrio cholerae*, Staphylococcal Enterotoxins (SEB), Ricin toxin from *Ricinus communis* and abrin toxin from *Abrus precatorius*) (<http://tetracore.com/elisa-kits/index.html>). All of the tests

are recommended for samples with complexed matrices. ELISA preparation workflow requires specific instruments, including ELISA readers and washers for reaction preparation and results data analysis what makes this method available only in the laboratory conditions (stationary or mobile).



Figure 3. The Tetracore system (BTICC)

pBDi Detector (Bruker)

ELISA for detection of biothreats can be automated. Portable instrument that is designed to use directly on-site for detection and identification of biological agents and it is pBDi Detector (Bruker). Its capabilities allow rapid as well as specific detection of biological warfare agents and toxins (*Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, *Brucella melitensis*, *Burkholderia mallei*, Orthopox viruses (Smallpox), botulinum toxin A, B, C, D, E, F, *Staphylococcal* enterotoxin A and B, ricin, abrin) with easy-to-use workflow. One of the advantage is it can be operated by non-skilled personnel and may be adopted in hot zone (www.bruker.com). The specifications of this detector can be found on www.bruker.com.

5.2.3. Immunofluorescence (IFA)

Immunofluorescence (IFA) – is a laboratory technique to identify the presence of antibodies bound to specific antigens with use fluorescent dyes. There are two types of IFA: direct and indirect. In direct IFA only primary antibody labelled with appropriate fluorescent dye against targeted antigen is present. In case of indirect method, two classes of antibodies are used – primary and secondary, where secondary antibody (labelled with fluorescent dye) attach to primary antibody. Results can be seen only when using fluorescent microscope what makes this method available only in laboratory and require skilled personnel. The most commonly use dye include fluorescein isothiocyanate (FITC) which after excitation emits green and tetramethylorodine isothiocyanate (TRITC), which after excitation emits a red colour.

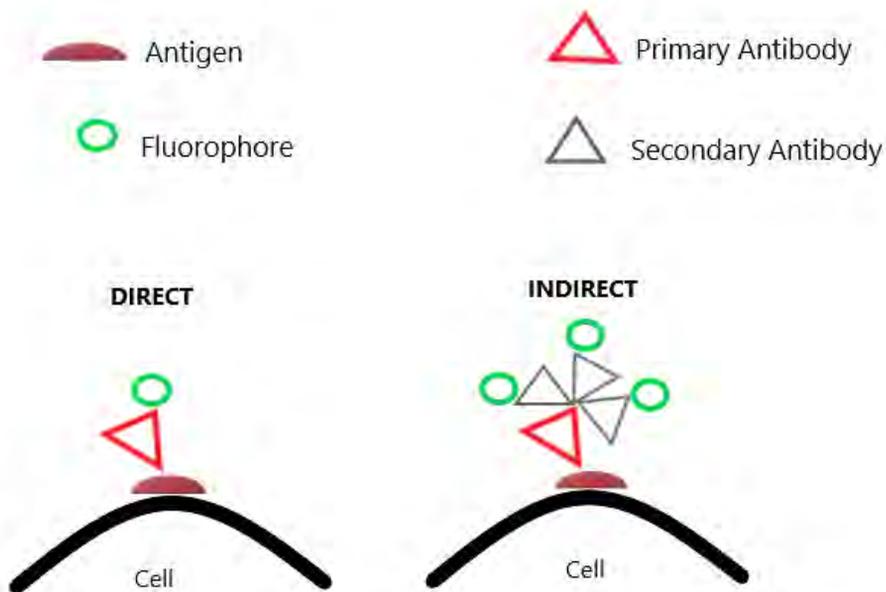


Figure 4. Direct and Indirect Immunofluorescence (IFA) testing
(Author: Patrycja Głowacka)

In an indirect IFA, human antibodies associated with the microorganism are detected by fluorescein-labelled anti-human antibodies. In direct IFA the antigen reacts with the dye labelled antibody to give the antigen-antibody complex.

Finally, there are no IFA tests for bioterror agents in the case of detection of environmental samples. Number of tests are dedicated for diagnostics of clinical samples.



Figure 5. Fluorescent microscopy (BTICC)

5.3. Molecular methods

For the identification of BW agents the several genetic based assay methods are available. The methods are based on the molecular taxonomy of bacteria using the analysis of genetic sequences of subunits 16S or 23 S rRNA. The nucleic acid hybridization is based on the DNA or RNA complementary to synthesized unique sequence labelled with enzyme or fluorochrome or radioactive compounds. The techniques dedicated to DNA or RNA detection are called Southern blot and Northern blot, respectively. However, they are not routinely used because of the time of experiments and highly skilled laboratory personnel.

The most common method for detection and identification of biological agents is PCR and its modification real-time PCR. These methods allow detection a DNA from a single bacterial cell or RNA from viruses, thanks to modification called Reverse Transcriptase real-time PCR (RT real-time PCR). Real-time PCR method involves using a molecular probe labelled with fluorescent dye and quencher (this modification is also known as TaqMan® probe). Positive results are revealed as a fluorescence curve curves. This method does not require agarose gel electrophoresis. However, it requires highly skilled personnel and basic knowledge about molecular biology. Despite this inconvenience, real-time PCR has become a standard method in most laboratories around the world. Other

probes like: HybProbes®, molecular beacons and Skorpion® probes can also be used however they are expensive and designing process is very difficult and time consuming are routinely used for scientific research.

Some modifications of real-time PCR identification techniques are dedicated to military use and represent closed versions of the instruments (<http://www.biofireidx.com>).

The development of DNA amplification makes possible to extract the thermostable DNA polymerase from *Thermus aquaticus* bacterium (Taq polymerase). That microorganism lives in hot sources at 50–80°C of Yellowstone Park in USA. PCR technique was developed by Kary Mullis in 1983 (in 1993 received Noble Prize for his discovery). Quantitative polymerase chain reaction (qPCR) use the fluorescent dyes and serves as technique that enable monitoring of PCR amplicons with the fluorescent dyes. Due to this the procedure the analysis is relevant, less-labour intensive, accurate and eliminates electrophoresis gel process for PCR products' detection. The method measures the quantity DNA in real – time process.

Reverse transcriptase PCR (RT-PCR) is a method of converted RNA molecules into complementary DNA (cDNA). Reverse transcriptase is an enzyme used for this purpose. Newly synthesized cDNA is amplify in standard PCR. Techniques are useful to amplify viral RNA. This method can used in classic or real-time PCR format as well, but it takes more time because of. The advantage is a single tube reaction format, wherea template the RNA is added, not necessary cDNA. It is convenient and saving time for other analysis.

Last decade has showed a rapid development of other real-time PCR methods. One of them is Loop mediated isothermal amplification PCR (LAMP PCR). Contrary to standard PCR and real-time PCR methods that must be conducted on thermocyclers with use of temperature range (between 50–95°C), LAMP PCR is performed at constant temperature and does not required thermocyclers. It can also be combined with reverse transcriptase enzyme for detection viral genetic material (RNA). In the LAMP-PCR method target nucleic sequence is detected using two or three pairs of primers where molecular probes or DNA incorporating dyes may be added as an reaction indicators. In case of SYBR Green dye (incorporating dye), UV light emitted lamp is sufficient for LAMP results visualization. Because of simple reaction format, rapid result (up to 1 hour), high specificity and reproducibility LAMP PCR has become an interesting molecular tool for detection of number biological, including biowarfare agents.

5.3.1. Real-time PCR SYBR Green

The most common real-time PCR technique is based on the non-specific fluorescent dyes that intercalate with double stranded DNA. The PCR products' detection is monitored by measuring the increase in fluorescence

throughout the cycle. The disadvantage of SYBR Green PCR is detection of all double-stranded DNA (not only targeted DNA). It is necessary to carefully optimize the reaction and additionally determine the melting curve analysis of PCR products.

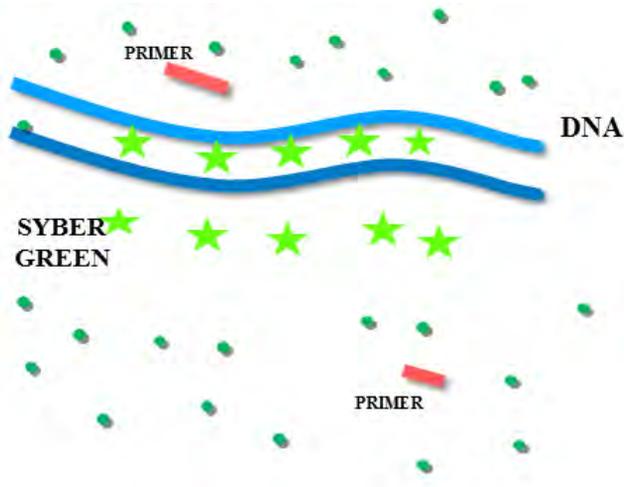


Figure 6. Real-time PCR using SYBR Green (Author: Agata Bielawska-Drózd)

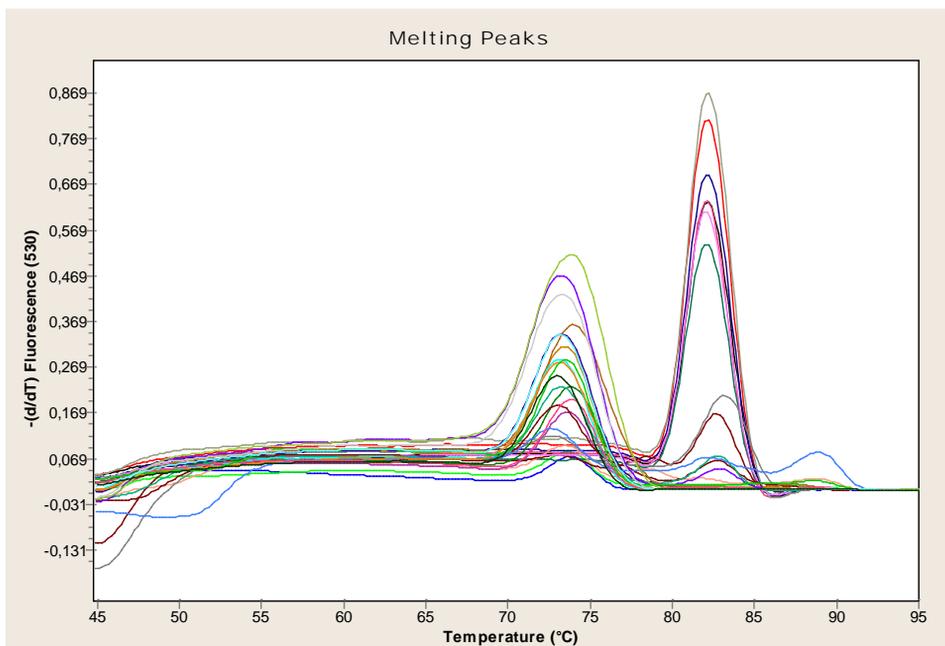


Figure 7. Melting curve analysis for targeted PCR products using SYBR Green

5.3.2. HybProbe real-time PCR

The specificity and sensitivity of PCR reaction may be utilized using by fluorescent-labelled target-specific probes. Fluorescence is contingent on the hybridization of both oligonucleotides so fluorescence measuring is performed at the annealing step of the PCR cycle.

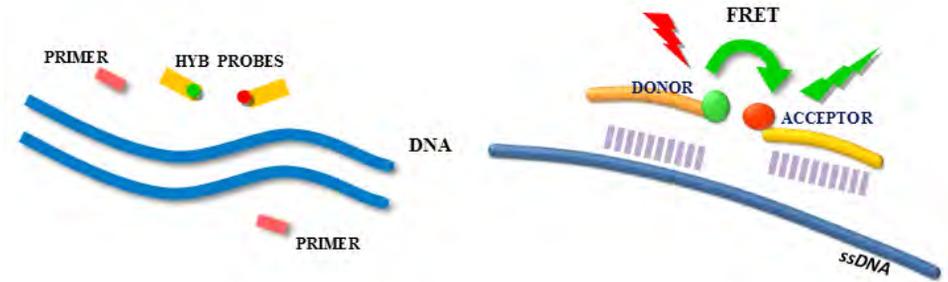


Figure 8. HybProbe real-time PCR (Author: Agata Bielawska-Drózd)

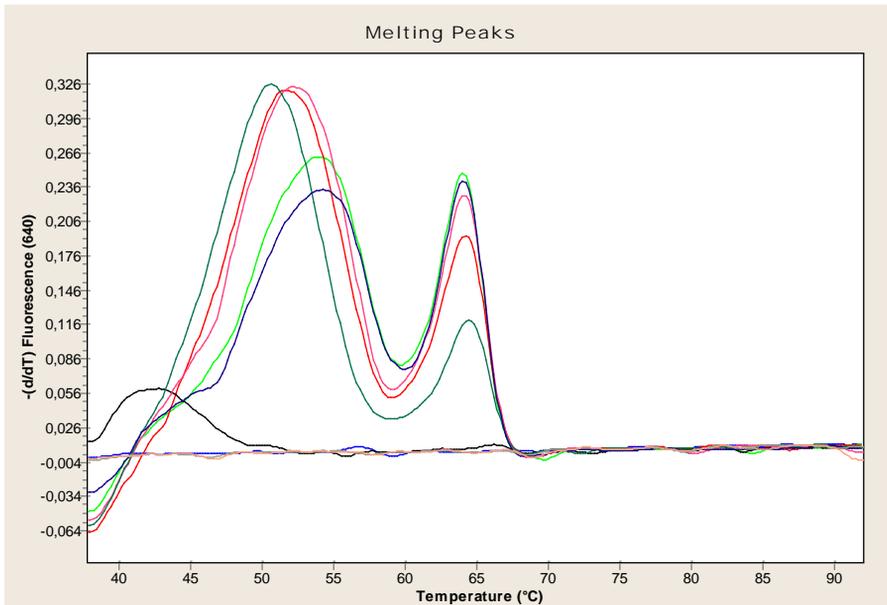


Figure 9. Results of real-time PCR using hybridization probes

The activity of hybridization probes is based on FRET (Fluorescence Resonance Energy Transfer) – the interaction between two dyes molecular

probes in close proximity causes the transfer of energy from donor to acceptor. The acceptor fluorophore emits longer wavelength which may be measured in specific channels.

5.3.2. TaqMan real-time PCR

TaqMan real-time PCR uses TaqMan probes with 5' nuclease activity. They are short oligonucleotides of a double – labelled fluorophores. The reporter dye (e.g. FAM, HEX, ROX) and quencher dye (e.g. TAMRA, DABCYL, BHQ) are attached to the 5' and 3' ends, relatively. The 5' nuclease PCR enables to produce specific PCR products by cleavage of a double-fluorogenic labelled probes using Taq polymerase activity.

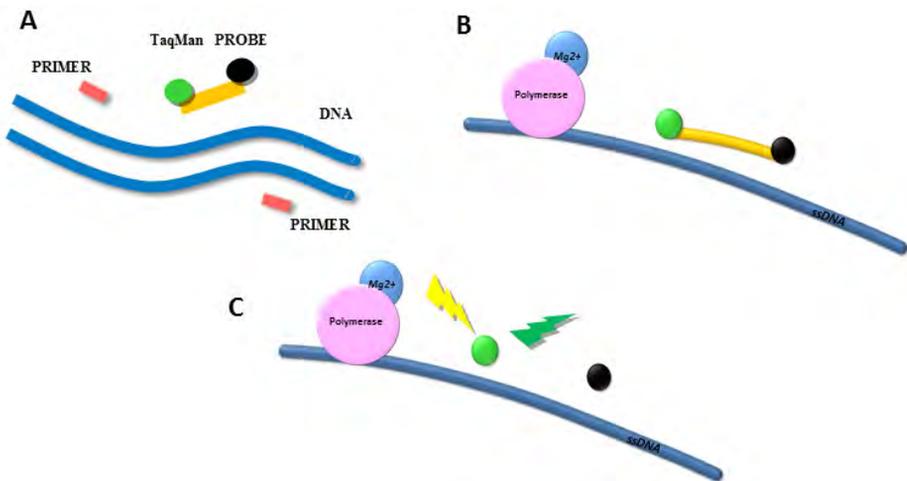


Figure 10. TaqMan real-time PCR (Authors: Agata Bielawska-Drózd, Piotr Cieřlik)

The hydrolysing probes in the TaqMan method reveal the quenching of the reporter fluorescence. During hybridization to the target PCR product the probe is cleaved by the 5'–3' nuclease activity of Taq DNA polymerase. In the figure above, A is the composition of real-time PCR reaction, B is the probe bind complementary to single-strand DNA (ssDNA), and C is the released reporter dye emits specific wavelength.

5.3.3. Available tests and instruments

CFX96 instrument (BioRad)

Tube Multiplex Real-Time PCR for detection of pathogen genes by TaqMan® technology. There is a wide range of variety of PCR platforms to identify biowarfare agents (viruses, bacteria). Multiplex tests are able to detecting up to

four pathogens in one tube, and up to 33 in a single patient sample. *E.coli* verotoxin, *Coxiella burnetii*, *Brucella* spp., *Burkholderia mallei/pseudomallei*, MERS-CoV, Dengue virus, West Nile Virus, Yellow fever virus, Ebola virus, CCHFV (Crimean Congo haemorrhagic fever virus), Zika virus, Hanta virus, Chikungunya virus (<http://www.labgene.ch>). CFX96 instrument is only for laboratory use (also in the mobile laboratory).



Figure 11. CFX96 Real-time system

LightCycler (Roche)

Roche molecular offers the multiplex PCR kits with TaqMan PCR for various bacterial and viral agents: set for *E. coli* (VTEC), *Vibrio* (seeds PCR) rods; cholera bacteriological examination of feces for detection *Vibrio cholerae* and Set LightMix Kit Dengue Virus (Types 1–4), LightMix Kit Yellow fever, TaqScreen West Nile Virus test and COBAS West Nile Virus, and LightMix® Zika rRT-PCR tests. Tests for biological agents that may cause haemorrhagic fever are also available. Instrument software allows design custom-made sets of primers and probes in

case of interested research area. The military upgrade of LightCycler instrument, R.A.P.I.D LightCycler (BioFire), is a portable tool, that is dedicated for mobile laboratories. The range of biological agents detection covering: BioThreat Screening Kit Tests for detection *B. anthracis*, *F. tularensis*, *Y. pestis*, pathogen Test Kit Tests for: *Listeria monocytogenes*, *E. coli* O157, *Salmonella* spp., *Campylobacter* spp., *Brucella* spp. Other biological factors can also be detected: *Cryptosporidium*, variola (Small Pox), ricin, Avian Influenza H5 subtype, avian Influenza H5 Subtype, influenza A.



Figure 12. Light Cycler 2.0 detection system (BTICC)



Figure 13. R.A.P.I.D LightCycler detection system

RAZOR EX BioDetection System (BioFire)

The system enables to detect and identify biological agents in field use. The application of BioFire's patented pouch system (integrated freeze-dried reagents in special cartridges) increase reliability DNA based results. That system is convenient for the most common biothreats. This instrument because of small size and possessing own power supply (battery) can be taken directly on field or be used in the laboratory. On the producers website, instruction movie about sample processing and instrument preparation steps is available (www.biofire.com).

The Biothreat screening kit includes assays for: *B. anthracis*, *F. tularensis*, *Y. pestis*, *Brucella species*. For training on the R.A.P.I.D. instruments, kits for many different pathogens are available (*Listeria monocytogenes*, *E. coli* O157, *Salmonella species*, *Campylobacter spp.*, *Clostridium botulinum* type A, *Cryptosporidium sp.*, Variola virus or ricin toxin).

FilmArray (BioFire)

The Film Array BioThreat offers the BioThreat panel of 16 threat pathogens which may be detected in environmental samples within an hour, including samples genetic material extraction. The panel includes: *Bacillus anthracis*, (detects 3 genetic targets – chromosomal, pXO1 and pXO2), *Brucella melitensis*, (detects 2 genetic targets), *Burkholderia*, (detects 2 genetic targets), *Clostridium botulinum*, *Coxiella burnetii*, (detects 2 genetic targets), *Ebola virus* (Zaire), *EEE virus*, *F. tularensis*, (detects 2 genetic targets), *Marburg virus*, (detects 2 genetic targets), *Ricinus communis*, *Rickettsia prowazekii*, (detects 2 genetic targets), *Variola virus*, *VEE virus*, (detects 2 genetic targets), *Yersinia pestis*, *Orthopoxvirus* (detects 2 genetic targets). The FilmArray system is also integrated with freeze-dried reagents in special vacuum-sealed cartridges.

FilmArray is a fully automatic instrument with genetic material extraction as a part of the real-time PCR process. Sample loaded into the pouch is transferred in the special cassette where small beads (white colour) are used for disruption of bacterial, viral and non-organic particles. Serial washes with appropriate buffers eliminate residues and nucleic acids (DNA and RNA) are then subjected for real-time PCR process. This process utilizes nested real-time PCR with use incorporation dye as an indicator.

Among Gastrointestinal Panel FilmArray offers detection of: *V. cholerae*, *Shigella/Enteroinvasive E. coli* (EIEC), *Enteroaggregative E. coli* (EAEC), *Enteropathogenic E. coli* (EPEC), *Enterotoxigenic E. coli* (ETEC), *Shiga-like toxin-producing E. coli* (STEC) *stx1/stx2 E. coli* O157 with biothreat potency. The Biothreat screening kit includes: *B. anthracis*, *F. tularensis*, *Y. pestis*, *Brucella spp.*



Figure 14. The FilmArray[®] multiplex PCR system (BTICC)

Using this instrument it is possible to detect other biological agents described in following panels: Respiratory Panel (detection and identification of viruses: Adenovirus, Coronavirus HKU1, Coronavirus NL63, Coronavirus 229E, Coronavirus OC43, Human Metapneumovirus, Human Rhinovirus/Enterovirus, Influenza A, Influenza A/H1, Influenza A/H3, Influenza A/H1-2009, Influenza B, Parainfluenza Virus 1, Parainfluenza Virus 2, Parainfluenza Virus 3, Parainfluenza Virus 4, Respiratory Syncytial Virus; bacteria: *Bordetella pertussis*, *Chlamydomphila pneumoniae*, *Mycoplasma pneumoniae*); Blood Culture ID and Meningitis Encephalitis Panel. All of the mentioned panels are dedicated for clinical samples (www.biofire.com), nonetheless they can be used by mobile laboratory.

The system enable to detect and identify biological agents in field use. The application of BioFire's patented pouch system (integrated freeze-dried reagents in special cartridges) increase reliability DNA based results. That system is convenient for detection of the most common biothreats, and also in the case of clinical samples.

LAMP-PCR (Tetracore)

This method offers compact real-time PCR instrument for rapid and specific detection of several biological factors. The T-COR 8 thermocycler may be used in the field as well as in the laboratories (including mobile laboratories). The commercial sets are not dedicated for diagnostic purpose (system is dedicated only for research use).

The system include sample – processing devices at the point of care ‘collect-to-test’. The multiplex identification of biothreat agents as: *Bacillus anthracis* – lethal factor (pXO1), *Bacillus anthracis* – capA (pXO2), *Brucella* spp., *Yersinia pestis*, *Francisella tularensis*, *Burkholderia* spp., Venezuelan Equine Encephalitis Virus (VEE), Ortopox virus, African Swine Fever Virus, Capripox, genes encoding of botulinum toxins, ricin, abrin and *Staphylococcus aureus* enterotoxins (SEB).

Field detection of biological agents is a highly rigorous and complex process in case of diagnostic purpose. It requires knowledge concerning biology of infectious agents, highly-skilled and trained sampling team (which may be consisted by different specialists: doctors, medical physicians, biologists, chemists etc), appropriate sampling protocols and equipment. Above mentioned methods and instruments may be used not only in military purpose but can also be adopted as a support for other public health service forces including police, fire departments, border guard etc.

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MODULE IV

DETECTION AND LIQUIDATION OF TOXIC CHEMICAL AGENTS

1. INDIVIDUAL PROTECTION EQUIPMENT

This section presents a basic overview of modern personal respiratory and skin protection equipment.

1.1. Personal respiratory protection equipment

Depending on the task in hand, the following types of respiratory protection equipment are available:

- Cleaning equipment – for protection from harmful substances in the air (dusts, gases, vapours etc);
- Isolation equipment – for oxygen-deficient atmospheres ($-17\% \text{O}_2$);
- Escape equipment.

1.1.1. Cleaning equipment

Cleaning equipment purifies contaminated air of gases, aerosols, dust and chemicals. It does this using:

- Filters – to stop particulates (dust) and aerosols;
- Absorbers – to suspend chemicals in a gaseous state;
- Combined filters – with both filtering and absorbing actions.

Filters, absorbers and combined filters are used in various types of masks and respirators, which come in two varieties: half-masks (Fig. 1) and full-face masks (Fig. 2 and 3).



Figure 1. Half-mask with combined ABEKP filters (author's photo)



Figure 2. MP-5 gas mask used by the Polish army (author's photo)



Figure 3. MP-6 gas mask used by the Polish army (author's photo)

Depending on need, absorbers and combined filters are further divided into various types, as shown in Table 1 below.

Table 1. The different types of mask filters, with the symbols and colour codes used to designate their intended use

Type	Colour	Main application
P	white	Dust, particles
A	brown	Gases and vapours of organic compounds with boiling point > 65°C
AX	brown	Gases and vapours of organic compounds with boiling point ≤ 65°C
B	grey	Inorganic gases and vapours, e.g. chlorine, hydrogen sulfide, hydrogen cyanide (excluding carbon dioxide/monoxide)
E	yellow	Sulphur dioxide and other acidic vapours and gases
K	green	Ammonia and ammonia derivatives and gases
Hg	red	Mercury vapours

Figures 4–8 show the markings used by absorber and filter manufacturers:



Figure 4. A1B1E1K1 Absorber – Type ABEK, Class 1 (author's photo)



Figure 5. K1 Absorber – Type K, Class 1 (author's photo)



Figure 6. A2 Absorber – Type A, Class 2 (author's photo)



Figure 7. A2 Absorber – Type A, Class 2 (author’s photo)



Figure 8. A2B2E2K2HgP3 combined filter – Type ABEKHg, Class 2 filter (P2) Class 3 (P3) (author’s photo)

Apart from categorising absorbers by the kinds of substances they protect against, they also have one of three class divisions:

- Class 1 – Low-absorbent sorbent, designed to protect against gases or vapours in airborne concentrations of up to 0.1%;
- Class 2 – Medium sorption, designed to protect against gases or vapours with airborne concentrations of up to 0.5%;
- Class 3 – Highly absorbent sorbent, designed to protect against gases or vapours in airborne concentrations of up to 1%.

The type and class of each absorber of combined filter type determines its minimum protection time (Table 2).

Table 2. Minimum protection (breakthrough) time in minutes

Type, class	Test substance	Minimum breakthrough time in minutes	Concentration of test substance in air (%)
A1	cyclohexane	70	0.1
B1	Chlorine/hydrogen sulfide/ hydrogen cyanide	20/40/25	0.1
E1	Sulfur dioxide	20	0.1
K1	Ammonia	50	0.1
A2	cyclohexane	35	0.5
B2	Chlorine/hydrogen sulfide/ hydrogen cyanide	20/40/25	0.5
E2	Sulfur dioxide	20	0.5
K2	Ammonia	40	0.5
A3	cyclohexane	65	1.0
B3	Chlorine/hydrogen sulfide/ hydrogen cyanide	30/60/35	1.0
E3	Sulfur dioxide	30	1.0
K3	Ammonia	60	1.0

Filters are also classified by their efficiency of filtration, in their case against sodium chloride aerosols and paraffin oil mist (DEHS):

- Class 1 (P1) – filtration efficiency of 80%. Protects against low toxicity solids for which the WEL long-term value is $\geq 2 \text{ mg/m}^3$;
- Class 2 (P2) – filtration efficiency of 94%. Protects against low and medium toxicity and solid particles for which the WEL long-term value is $\geq 0.05 \text{ mg/m}^3$;
- Class 3 (P3) – filtration efficiency of 99.95%. Protects against solids and liquids with high toxicity for which the WEL long-term value is $< 0.05 \text{ mg/m}^3$.

Filter half-masks

Filters for half-mask respirators are also classified into one of three protection classes:

- Class 1 (FFP1) – filtration efficiency of 80%. Protects against solids and liquids with low toxicity for which the WEL long-term value is $\geq 2 \text{ mg/m}^3$ at maximum concentrations of 4x the WEL long-term value;

- Class 2 (FFP2) – filtration efficiency of 94%. Protects against solids and liquid particles with low and medium toxicities for which the WEL long-term value is 0.05 mg/m^3 at maximum concentrations of 10x the WEL long-term value;

- Class 3 (mark FFP3) – filtration efficiency of 97%. Protects against solids and liquids with high toxicity, for which the WEL long-term value is $< 0.05 \text{ mg/m}^3$ at maximum concentrations of 20x the WEL long-term value.

- The following designations are used by the manufacturers:

- S – half mask designed for filtration of particulate matter (dust, smoke) only;

- SL – half mask designed for the filtration of particulates (dust, smoke) and liquid particles (aerosols, mist);

- D – additional requirement for dust absorption against dolomite dust;

- C – additional dust requirements for carbon dust.

1.1.2. Isolation equipment

Isolation suits provides a supply of clean air from an independent source (oxygen tanks), so that the user is completely cut off from contaminated air, even under reduced oxygen conditions. Isolation equipment is divided into:

- Stationary (with a compressed air hose and generator);

- Autonomous (with a tank or recycling system).



Figure 9. Isolation breathing apparatus (author's photo)

1.1.3. Escape equipment

The third kind of protective respiratory gear is considered 'escape equipment', as it is intended not for prolonged working use, but simply for emergency use in the fast evacuation of hazardous areas. A good example of escape equipment is the protective hood and cover shown in figures 10 and 11, below.



Figure 10. Protective cover (author's photo)



Figure 11. KO-1 protective hood (author's photo)

1.2. Personal skin protection equipment

Skin protection equipment is used to protect the wearer's body surface from the harmful effects of poisonous substances. Based on the materials used and the purpose of the equipment, the protective layers of such clothing are designed to:

- Isolate harmful substances;
- Filter out harmful substances.

1.2.1. Isolation suits

As the name implies, isolation suits provide an impermeable barrier against contaminated environments. This type of clothing is usually worn by sub-units of chemical military troops, chemical disaster rescue teams, and medical and chemical rescue services. They provide excellent protection against dangerous chemicals (including Chemical Warfare Agents (CWAs)), and biological agents.



a)



b)

Figure 12. Examples of lightweight clothing a) one-piece Tychem b) two-part LIO Maskpol (author's photo)

Gas-tight suits are also used by specialised chemical rescue workers, and can take the form of one-piece suits with a gas-tight lock. They provide the highest level of protection and are designed for use in particularly hazardous conditions, such as with CWAs, Toxic Industrial Chemicals (TICs), and low oxygen levels. They are designed for use with breathing apparatus, and most importantly, can be decontaminated after use.



Figure 13. Examples of gas-tight suit type 1 (author's photo)

1.2.2. Filter clothing

Filter clothing is made with the use of absorbent materials. It protects against gaseous substances, but is not very effective against liquid substances (aerosols etc.). Filter clothing passes the wearer's heat and humidity (sweat) out, thus

enhancing user comfort. Due to its low protection value against liquids, the shoes and gloves of protective filter clothing are made of isolating materials. Unlike isolation suits, filter clothing cannot be decontaminated after use.



Figure 14. FOO-1 Filter Cloth (author's photo)

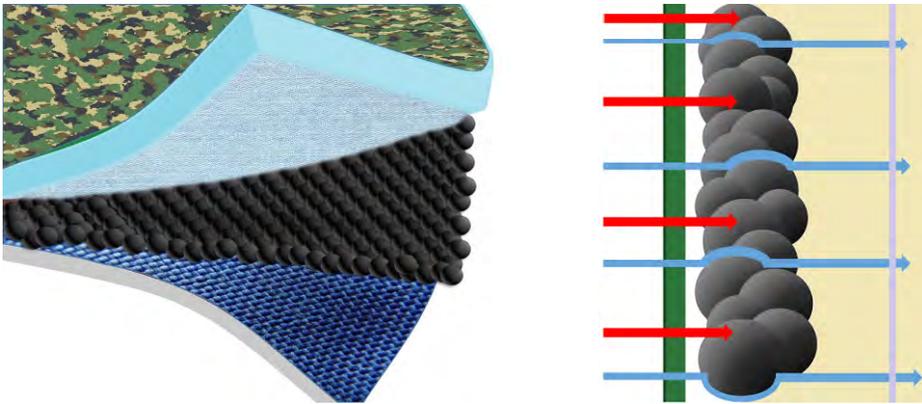


Figure 15. SARATOGA material with spherical, activated carbon

1.3. Selection of appropriate protection equipment

The following environmental conditions at an incident scene involving chemicals will determine the selection of appropriate personal protective equipment:

- Amount of oxygen in the air – If the oxygen content is below 17%, use only isolation equipment (compressed air from a generator or from oxygen tanks). Keep in mind that the local oxygen content may also suddenly decrease, for example in confined spaces such as tanks, wells, ducts etc, or when oxygen could be displaced by another gas.

- Type of hazardous substance present – The first priority is to identify the harmful substances at the incident scene. If this cannot be determined, only isolation equipment can be used; do not use cleaning equipment.

- Type of contamination – Determine whether the hazardous substance(s) take the form of water-based mists or aerosols (use particle filters (S)); dispersed liquid aerosols (use particle and liquid filters (SL)); gas and steam (use absorbers); combinations of aerosols, vapours and gases (use combined filters).

- Concentration of the hazardous substance.

- Work intensity – The harder or more intensely the suit/mask-wearing operators are expected to work, the more clean air they will need to breathe.

- Working time – The usable time of respiratory protective equipment is sometimes limited by the specifications of the equipment itself. For example, oxygen tanks contain only a limited amount of air; filters can only filter so much hazardous material before they need replacing; very high concentrations of hazardous substances reduce the usable time of an absorber, and so on. When long working hours are expected, it is important to use high-quality equipment that is comfortable to use. Use of a system with an airflow should also be considered.

– Ambient temperature, humidity – Elevated temperatures at an incident scene will lead to increased effort by the operators. This then leads to high humidity from sweating inside a full-face mask or suit, especially when combined with high-intensity work. Also, be aware that elevated temperatures and high humidity can shorten the duration of work of air suckers.

– Visibility – All types of respiratory protection reduce their wearer's field of vision. This is especially true of full-face masks, so use of those with the largest possible lenses is highly recommended.

– Communication – Masks and hoods deform the wearer's voice, but clear communication is still possible in relative quiet over short distances. Some masks have a built-in vocal membrane to facilitate conversation.

– Mask tightness – it is very important to ensure a close fit to the wearer's face. Masks and half-masks are available in different sizes, so it is important that wearer's carefully check the tightness of their particular mask before entering the hazardous environment. For men with particularly thick facial hair, there are, for example, masks with extra airflow systems that produce some hypertension in the face (Fig. 16 and 17).



Figure 16. Mask with an extra airflow system (author's photo)



Figure 17. Mask with an extra airflow system (author's photo)

1.4. Practical exercises in the use of personal protection equipment

Practical exercises in the use of personal protection equipment include:

- Testing of and familiarisation with personal respiratory and skin protection equipment;
- Selecting the appropriate size mask, filter type and protective clothing;
- Correctly fitting masks and testing for leaks;
- Practising putting on protective equipment safely and rapidly (for emergency situations).

1.4.1. Selecting the appropriate size mask, filter type and protective clothing

For masks:

1. Measure the height of the face (from the bridge of the nose to the base of the chin) and use the result to select a mask based on the manufacturer's suggested sizes.
2. Check the surface of the mask for mechanical damage.
3. Surfaces in contact with the skin should be disinfected.
4. Install a new, suitable filter, an absorber or combined filter for the mask after identifying the type of hazardous substance involved (based on type and class (see Section 1, above)).

5. Always make sure that absorbers have been tested and approved for use with the hazardous substance involved.

6. Put the mask on, so that no hair comes between the mask and the skin.

7. Adjust the mask so that it adheres to the contours of the face.

For clothing:

1. Choose a size of suit appropriate for the wearer's height and chest circumference.

2. In the case of suits made of several elements, make sure that they are securely connected (Velcro, zip fasteners, elastic bands etc.).

3. Check that the hood is properly attached to the face part of the gas mask.

1.4.2. Correctly fitting masks and testing for leaks

To check the fit of a mask, use a PORTACOUNT device. This measures the number of naturally-occurring particles in the air and the number of particles under the mask (after passing through the filter/combined filter etc.

- Attach a mask of the correct size;
- Connect one of the filter ports on the filter or combined filter casing to the air sampler tube (Fig. 18);
- Have the user breathe calmly and normally for a minute;
- The PORTACOUNT unit simultaneously counts the number of particles in the air around the mask, and under the mask. It calculates the ratio of surrounding particles to the number of particles under the mask, giving the FIT FACTOR, a properly fitting mask will have a FIT FACTOR of at least 10,000.



Figure 18. Checking the fit of a mask, using a PORTACOUNT (author's photo)

Using a PORTACOUNT device, you can quickly determine if a mask fits the wearer's face properly by checking total internal leakage from the mask. The mask's user is located on a treadmill in a sealed chamber (Fig. 19 and 20). The interior of the chamber is filled with a sodium chloride aerosol. The user then performs a series of exercises to simulate the use of the mask in real-life conditions (fast walking, turning their head up and down and side-to-side etc.). Using a spectrophotometer, the concentration of sodium chloride in the chamber and under the mask is determined and the internal leakage is thus calculated.



Figure 19. Checking total internal leakage (author's photo)



Figure 20. Checking total internal leakage (author's photo)

1.4.3. Practising putting on protective equipment safely and rapidly

In case of a rapid threat of contamination, operators should don the appropriate masks as soon as possible. The ideal time to correctly attach a mask is max. 9 seconds. After the filter's lifetime has expired, it must be replaced with a new one – keeping in mind that there may still be hazardous substances in the environment! To replace a mask filter in a hazardous environment:

1. Take a deep breath;
2. Hold that breath;
3. Unscrew the filter casing while beginning to very slowly and continuously exhale;
4. Insert the new filter and re-attach the filter casing;
5. Inhale. Remember, do not inhale without a properly installed filter!

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2. DETECTION OF CHEMICAL CONTAMINANTS

2.1. Chemical Warfare Agents (CWA)

2.1.1. The History of Chemical Warfare (CW)

Chemical warfare can be defined in various ways for the purposes of historical review, but primarily as the use of the toxic properties of chemical substances as weapons.

In western literature the first mention of CW is in Greek mythology, in which Hercules was said to have poisoned his arrows with the venom of a Hydra. In *The Iliad*, Homer claimed that in the Trojan War, both sides used poisoned arrows. There are also many other documented uses of early CW from ancient times. The timeline below shows not only recorded uses of chemical warfare, but also the attempts to ban it.

600 BC – The Athenian military taints the water supply of the besieged city of Kirrha with poisonous hellebore plants.

479 BC – Peloponnesian forces use sulphur fumes against the town of Plataea.

1845 – During the French conquest of Algeria, French troops force more than 1,000 members of a Berber tribe into a cave and then use smoke to kill them.

1914, August – During the first month of World War I the French deploy tear-gas grenades, first developed in 1912 for police use.

1914, October – German forces fire 3,000 shells containing dianisidine chlorosulfate, a lung irritant, at the British army at Neuve-Chapelle. The British are unaware that they have been subjected to a chemical attack because the chemical is incinerated by the explosive charge.

1915, January – The Germans fire 18,000 shells filled with the irritant xylyl bromide at Russian troops at Bolinow. The Russians are unharmed because the extreme cold keeps the liquid from vaporizing.

1915, April 22 – The German military launches the first large-scale use of chemical weapons in war at Ypres, Belgium. Nearly 170 metric tons of chlorine

gas in 5,730 cylinders are buried along a four-mile stretch of the front. In the end more than 1,100 people are killed by the attack and 7,000 are injured.

1915, September 25 – The British military uses chemical weapons for the first time against the Germans at the Battle of Loos. They release chlorine gas from cylinders.

1915, December 19 – Six days before Christmas, Germans first use phosgene on Allied troops. More than 1,000 British soldiers are injured and 120 die.

1917, July 12 – Mustard gas is used for the first time by German forces; it causes more than 2,100 casualties. During the first three weeks of mustard-gas use, Allied casualties equal the previous year's chemical-weapons casualties.

1918, June – The Allies begin using mustard gas against German troops.

1918, October 13–14 – A young Adolf Hitler, an enlisted messenger in the trenches at Werwick near Ypres, is temporarily blinded during a gas attack. Hitler is evacuated to a military hospital in eastern Germany and spends the rest of the war recuperating.

1918, November 11 – World War I ends with 1.3 million casualties caused by chemical weapons, including 90,000 to 100,000 fatalities, primarily from phosgene.

1925 – The Geneva Protocol is adopted by the League of Nations. The treaty bans the use of chemical and biological agents in war but does not prohibit the development, production, or stockpiling of such weapons. Many countries sign the treaty with reservations permitting them to respond in kind if attacked with chemical weapons.

1935–1936 – Benito Mussolini drops mustard-gas bombs in Ethiopia to destroy Emperor Haile Selassie's army. Despite Italy being a signatory of the Geneva Protocol, the League of Nations does not stop its use of chemical weapons.

1936 – German chemist Gerhard Schrader completes the synthesis and purification of tabun, a potent nerve poison. His intention is to build a pesticide, not a chemical weapon. The chemical he creates is so potent that army researchers call it taboo, or Tabu in German, from which it takes its name.

1939–1945 – During World War II poison gases are used in Nazi concentration camps to kill civilians and by the Japanese army in Asia. Nerve agents are stockpiled by the Nazis, but chemical weapons are not used on European battlefields.

1953 – British serviceman Ronald Maddison dies of sarin poisoning after being purposefully exposed to the toxin at Porton Down military facility.

1961–1971 – The United States uses napalm and the herbicide Agent Orange during the Vietnam War, sparking national and international protest.

1963–1967 – Egypt uses mustard gas and a nerve agent in Yemen to support a coup d'état against the Yemeni monarchy.

1972 – The Biological and Toxin Weapons Convention is completed. Combined with the 1925 Geneva Protocol, the new accord bans the development,

production, and possession of biological weapons. The accord has no mechanism to ensure compliance.

1980s – During the Iran-Iraq war Iraq uses chemical weapons, including tabun, against Iran and Iraq's Kurdish minority. United Nations experts confirm Iraq's use of chemical weapons, but there is little international outcry. Iran initiates its own chemical-weapons program in retaliation.

1993 – The Chemical Weapons Convention is signed. Beginning in 1997, the disarmament agreement bans the development, production, stockpiling, and use of chemical weapons.

1994, June 27 – The first successful use of Chemical Warfare Agents by terrorist in Matsumoto.

1995, March 20 – Terrorist attack in Tokyo subway with sarin.

2013 – The Syrian military uses sarin gas against civilians during the Syrian Civil War; hundreds are killed. Bashar al-Assad's government relinquishes its arsenal of chemical weapons after threats of U.S. air strikes.

2017, February 13 – Kim Dzong Nam assassinated with VX.

Although chemicals had been used as tools of war for thousands of years, as can be seen above modern chemical warfare had its genesis on the battlefields of World War I.

The use of several different types of chemical weapons, including mustard gas (also known as yperite), resulted in 90,000 deaths and over one million casualties during the war.

Surprisingly, despite extensive preparations by all sides, CW wasn't used on a large scale in WW II. Historians are still debating why, with one explanation being the mutual fear of massive chemical counter-attacks. Some historians say that Hitler was adverse to using mustard gas since he had himself been a mustard victim in WWI.

Throughout history there have been several attempts to ban CW, with limited success. One example is an Italian gas attack in Ethiopia, when Mussolini dropped mustard bombs despite Italy having signed the Geneva Protocol.

A significant improvement came with the 'Convention on the Prohibition of the Development, Production, Stockpiling and Use of Chemical Weapons and on their Destruction', better known in its shorter version, the Chemical Weapon Convention (CWC). This is an arms control treaty that outlaws the production, stockpiling and use of chemical weapons and their precursors. Thanks to this Convention, a chemical weapon demilitarization process was begun in 1997. For the purposes of implementation and verification of the CVC, The Organisation for the Prohibition of Chemical Weapons (OPCW) was created in April 1997. By the end of 2016, 67,098 metric tonnes (90% of the world's declared stockpile of 72,304 metric tonnes of chemical agents), have been verifiably destroyed.

On October the 11th, 2013, the Norwegian Nobel Committee announced that the OPCW had been awarded the Nobel Peace Prize for "extensive work to eliminate chemical weapons".

2.1.2. Definition of CWA

In the previous section a short definition of Chemical Warfare was presented, and this section will look at Chemical Warfare Agents. Wikipedia states that “A chemical weapon agent (CWA) is a chemical substance whose toxic properties are used to kill, injure or incapacitate human beings”.

A similar definition is proposed by the Federation of American Scientists (FAS): “A chemical agent is a substance which is intended for use in military operations to kill, seriously injure or incapacitate people because of its physiological effects. Excluded from this definition are riot control agents, herbicides, smoke, and flame”.

A little wider definition is proposed by Satu M. Somani, a Professor of Pharmacology and leading expert in the field, who states that “Chemical Warfare Agents are chemicals that have direct toxic effects on humans, animals and plants”. A much broader definition, which by necessity is also much more precise (to avoid misunderstandings at the international level), is included in the CWC, in Article II. Definitions and Criteria:

1. “Chemical Weapons” means the following, together or separately:

(a) Toxic chemicals and their precursors, except where intended for purposes not prohibited under this Convention, as long as the types and quantities are consistent with such purposes;

(b) Munitions and devices, specifically designed to cause death or other harm through the toxic properties of those toxic chemicals specified in subparagraph (a), which would be released as a result of the employment of such munitions and devices;

(c) Any equipment specifically designed for use directly in connection with the employment of munitions and devices specified in subparagraph (b).”

To clarify what toxic chemical and precursor is:

2. “Toxic Chemical” means:

Any chemical which through its chemical action on life processes can cause death, temporary incapacitation or permanent harm to humans or animals. This includes all such chemicals, regardless of their origin or of their method of production, and regardless of whether they are produced in facilities, in munitions or elsewhere.

3. “Precursor” means:

Any chemical reactant which takes part at any stage in the production by whatever method of a toxic chemical. This includes any key component of a binary or multicomponent chemical system.

A full list of chemicals covered by the CWC is published in its ‘Annex on Chemicals’, in which all substances are classified into three ‘schedules’, with the first being the most dangerous.

Definitions of chemicals are often ‘open’, which means that only their properties or general structure are defined. For example, in Schedule 2.B.4 – Chemicals, apart from the items listed in Schedule 1, there is a phosphorus atom to which is bonded

one methyl, ethyl or propyl (normal or iso) group, but no further carbon atoms. The definition of this atom is so open, that the estimated number of possible chemicals that can be derived from it is countable in the millions.

2.1.3. Classification of CWA

CWAs possess different characteristics and belong to various classes of compounds with pronounced physicochemical, physiological and chemical properties. As such, they can also be classified by their physical state – gaseous, liquid or solid.

Based on their volatility, they are then classified as persistent or non-persistent agents. The more volatile an agent, the quicker it evaporates and disperses. The more volatile agents, such as chlorine, phosgene and hydrogen cyanide, are non-persistent agents, whereas the less volatile agents, such as sulphur mustard and Vx are persistent agents.

Chemical Warfare Agents with a boiling point below 130°C are usually regarded as volatile, those with boiling points between 150°C and 300°C as persistent. For example, Phosgene has a boiling point of 7.5°C and is considered volatile, while VS has a boiling point of 300°C and is considered highly persistent.

CWAs can also be classified based on their chemical structure as organophosphorus, an organosulfur or organofluorine compounds and arsenicals.

However, the best known classification system is based on their effect on humans, as follows:

1. Nerve agents;
2. Vesicants (blistering agents);
3. Bloods agents (cyanogenic agents);
4. Choking agents (pulmonary agents);
5. Riot-control agents (tear gases);
6. Psychomimetic agents;
7. Toxins.

Nerve agents

Nerve agents are named for their effect on the functioning of the nervous system. They do not occur naturally, but belong to the group of OP compounds (organic compounds containing phosphorus). Generally, nerve agents are more toxic than other CWAs. They are highly toxic and can cause death from within a few minutes to a few hours after exposure, depending on the concentration. The first known nerve agent, Tabun (GA), was first developed in the 1930s by the German chemist, Gerhard Schrader, in his research on the development of new OP insecticides. Following this, a series of nerve agents known as G-agents, which include Sarin (GB) and Soman (GD), were developed. Historically, Germany maintained stockpiles of nerve agent

munitions during World War II, but did not use them. A variety of nerve agents were then developed for military use, up to the 1960s. The focus was on increasing their potency and environmental persistence, which led to V-agents – more stable versions of G-agents. These include VX, a sulphur-containing OP, which is more potent than sarin, more stable, less volatile and less water-soluble, acts through direct skin contact and persists in the environment for up to several weeks after release.

The only known battlefield use of nerve agents was in the Iran-Iraq conflict in 1980–1998; Iraq reportedly used nerve agents against Iranian troops and later against members of its Kurdish population in northern Iraq.

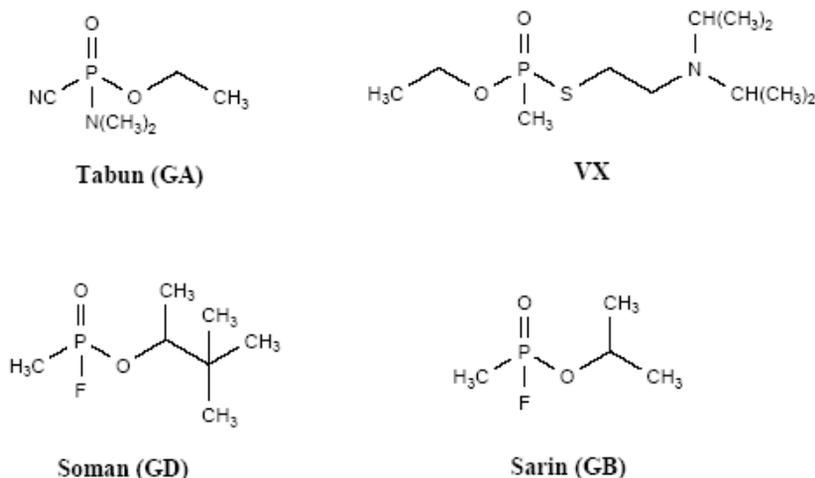


Figure 1. Chemical structure of nerve agents

	Tabun	Sarin	Soman	VX
Molecular weight	162.12	140.09	182.19	267
Vapor density (air = 1)	5.63	4.86	5.6	7.29
Liquid density (g/cm ³ , 25°C)	1.07	1.102	1.02	1.062
Melting point (°C)	-50	-57	-42	-51
Boiling point (°C)	248	147	198	298 (decom.)
Vapor pressure (mmHg, 25°C)	0.037	2.9	0.40	0.0007
Volatility (mg/m ³ at 25°C)	610	22,000	3,900	75
LD ₅₀ (skin, mg/kg)	1–1.5	24	10–15	<5
LCt ₅₀ (respiratory, mg-min/m ³)	135–400	70–100	70–400	30

Figure 2. Properties of nerve agents (figure used with permission under Creative Commons license)

Other examples of popular nerve agents include tabun, which is a stable, colourless to brownish liquid (depending on the purity level), and is odourless (but can obtain a faint fruity odour from impurities). Sarin is also a colourless to yellow-brown liquid that is either odourless or smells slightly fruity, and is about 20 times more volatile than tabun. Soman has a similar colour and odour as tabun and sarin, but is more persistent than sarin. When impure, it has a strong camphorous odour. VX is colourless and odourless, but can obtain an amber colour of formulated as an oily liquid. It has the lowest vapour pressure of all nerve agents and is therefore one of the more persistent chemical warfare agents.

Mechanism of action

The primary mechanism of toxicity results from inhibition of the enzyme acetylcholinesterase (AChE) at the neuromuscular junction, which causes overstimulation of the muscles through the excessive accumulation of the neurotransmitter acetylcholine (ACh). This over stimulation results in muscle paralysis.

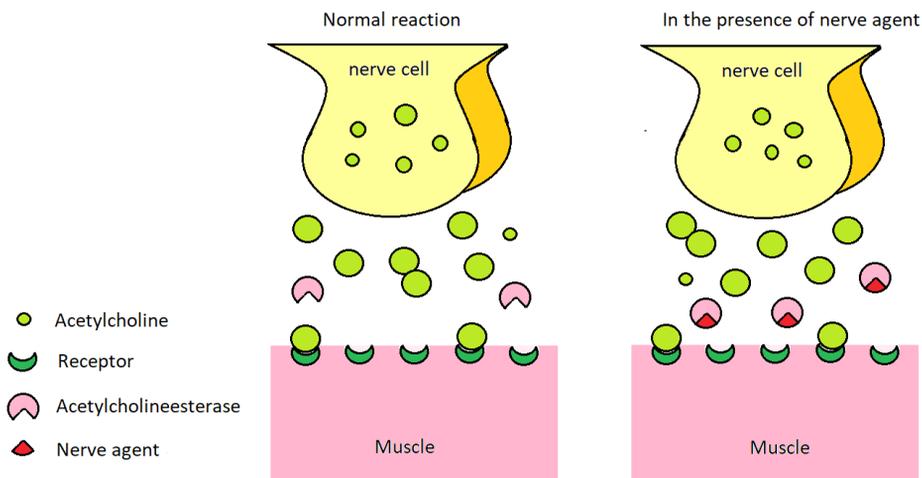


Figure 3. Mechanism of a nerve agent's action

Figure 3 presents a simplified picture of a cholinergic synapse, with the nerve in which acetylcholine is formed, and the receiving side (muscles, glands etc.) with receptors. Acetylcholine is formed and released from the nerve cell. On the other side of the synapse it binds to a muscle cell receptor for a split second. The signal to, for example, bend an arm or take a breath has now been transferred from the nervous system to the performing muscle. But in the presence of a nerve

agent, the enzyme acetylcholinesterase, which is responsible for breaking down acetylcholine, is inhibited. The receptor then keeps on sending signals to the muscle cell, and this leads to muscle cramp.

Blistering agents

Blistering agents, or vesicants, are toxic compounds that produce skin injuries resembling burns. Upon inhalation, these agents affect the upper respiratory tract as well as the lungs, producing pulmonary oedema. These agents can also cause severe eye injuries. There are two forms of vesicants: mustards and arsenicals. The most important substance in this class of CWA is sulphur mustard, otherwise known as the king of CW agents. Other members include nitrogen mustards (HN1, HN2 and HN3), and lewisites (L1, L2 and L3).

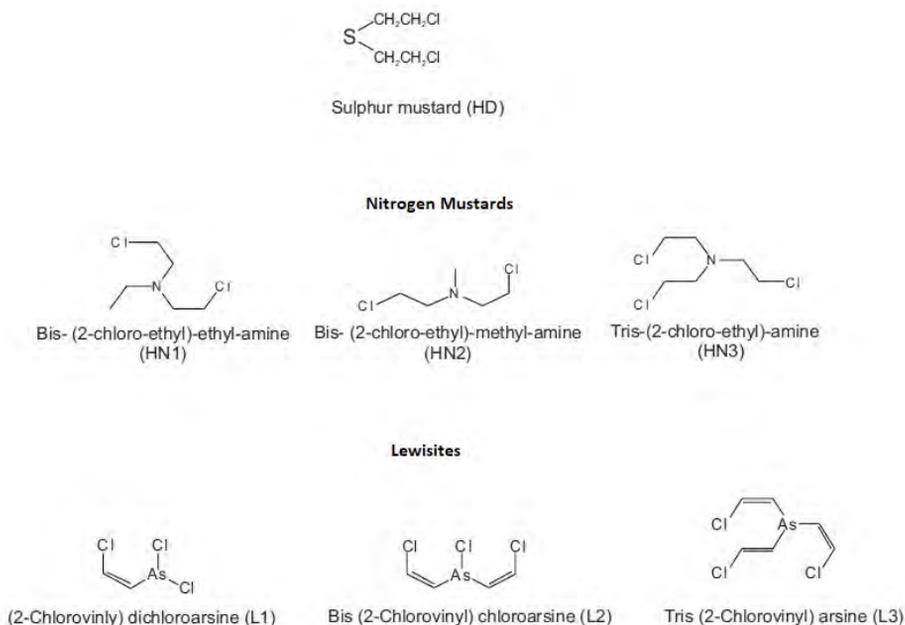


Figure 4. Chemical structures of blister agents

Mustard gas (yperite, sulphur mustard, H, HD) is odourless and colourless when pure, but in its impure form is a yellow-brown oily liquid with the odour of mustard or horseradish. Due to its high melting point of 14 °C it is not suitable for use during winter, but when mixed with Lewisite (63% Lewisite and 37% mustard), the melting point is lowered to -14°C.

Of the N-mustards (HN-1, HN-2, HN-3), HN-1 is suitable for use at lower temperatures. The odour of HN-1 and HN-3 is fishy, whereas higher

concentrations of HN-2 smell fruity. All mustards can easily penetrate rubber, textiles and leather, as well as porous materials such as bricks and concrete.

Lewisite is the main arsenic blister agent. When impure, this colourless liquid has a geranium-like odour.

Mechanism of action

The toxic effects of a mustard agent depend on its ability to covalently bind to other substances. The chlorine atom is spiked off the ethyl group and the mustard agent is transferred to a reactive sulfonium ion. This ion can bind to a large number of different biological molecules. Most of all, it binds to nucleophiles such as nitrogen in the base components of nucleic acids, and sulphur in SH-groups in proteins and peptides. Since mustard agents contain two 'reactive groups', they can also form a bridge between or within molecules. Mustard agents can destroy a large number of different substances in the cell by means of alkylation, and thereby influence numerous processes in living tissue.

Nitrogen mustards form cyclic aminium ions (aziridinium rings) through intramolecular displacement of the chloride by the amine nitrogen. This aziridinium group then alkylates DNA when it is attacked by the N-7 nucleophilic centre on the guanine base. A second attack after the displacement of the second chlorine forms the second alkylation step, which results in the formation of inter-strand cross-links.

Blood agents

Like the other groups of agents, blood agents derive their name from their effect. Blood agents are distributed *via* the blood, and generally enter the body *via* inhalation. They inhibit the ability of blood cells to utilise and transfer oxygen. Thus, blood agents are poisons that effectively cause the body to suffocate.

	Hydrogen cyanide (AC)	Cyanogen chloride (CK)
Molecular weight	27.03	61.48
Vapor density (air = 1)	1.007	2.1
Liquid density (g/cm ³ , 20°C)	0.687	1.18
Melting point (°C)	-13.3	-6.9
Boiling point (°C)	25.7	12.8
Vapor pressure (mmHg, 25°C)	742	1,000
Volatility (mg/m ³ at 25°C)	1,080,000	2,600,000
LD ₅₀ (skin, mg/kg)	100 (liquid)	No data available
LCt ₅₀ (respiratory, mg-min/m ³)	2,000	11,000

Figure 5. Properties of blood agents (figure used with permission under Creative Commons license)

Hydrogen cyanide is a transparent, colourless and easily water-soluble liquid that has a bitter almond odour that, due to genetic predisposition, cannot be sensed by everybody. Its very high volatility leads to difficulties in achieving lethal combat concentrations on the battlefield, but is still very dangerous in enclosed spaces. As such, it is mainly used as a terrorist weapon.

Cyanogen chloride is also a colourless liquid with a low boiling point, is highly irritant to the mucous membranes and causes severe tear flow. It also has an almond aroma and can penetrate the filter elements of a gas mask more readily than any other agent.

Mechanism of action

Cyanide has a very high affinity with iron in the ferric (Fe^{+3}) state. On entering the biological system, it readily reacts with trivalent iron of cytochrome oxidase (an end-chain enzyme of cellular respiration) to form a complex, thereby impairing the utilization of oxygen in the tissues. Eventually, death follows as a result of respiratory failure. The onset and intensity of symptoms depend on the concentration of inhaled toxic vapour and duration of exposure. Symptoms of exposure to low doses of HCN are weakness, giddiness, headache, confusion, and sometimes nausea and vomiting.

Choking agents

Choking agents mainly affect the respiratory tract, i.e. the nose, throat, and particularly the lungs. Choking agents were among the first CW agents produced in large quantities and were used extensively in World War I. They are generally heavier than air. Phosgene is used in many chemical industrial processes, making the control of these compounds difficult, and these can be used as a devastating low-tech weapon in the hands of terrorists. Phosgene is a colourless gas with the odour of cut grass or hay, and is highly volatile due its low boiling point. It is three times as heavy as air, and so accumulates in depressions, holes and cellars. Diphosgene is the colourless to yellow-brownish liquid form of phosgene, and has a higher boiling point.

	Phosgene (CG)	Diphosgene (DP)
Molecular weight	98.92	197.85
Vapor density (air = 1)	3.4	6.8
Liquid density (g/cm^3 , 25°C)	1.381	1.65
Melting point (°C)	-128	-57
Boiling point (°C)	7.6	128
Vapor pressure (mmHg, 25°C)	1,180	4.2
Volatility (mg/m^3)	4,300,000 (at 7.6°C)	45,000
LCt_{50} (respiratory, $\text{mg}\cdot\text{min}/\text{m}^3$)	3,200	3,000-3,200

Figure 6. Properties of phosgene and diphosgene (*figure used with permission under Creative Commons license*).

Mechanism of action

Phosgene is highly reactive and combines with the $-SH$, $-NH_2$ and $-OH$ groups of biological macromolecules, including enzymes, and this may account for its toxic effects. Poisoning is mainly attributed to acylation of certain tissue elements of the lungs, and increased permeability of the alveolar mucous membrane. This results in pulmonary oedema with consequent anoxia and death. Inhalation of low concentrations of phosgene produces rapid, shallow breathing, reduced respiratory volume, bradycardia and hypotension. Many cholinergic symptoms, such as increased salivation, nausea, micturition and defecation are also observed.

Riot-control agents

Riot control agents (RCA) are compounds that cause temporary incapacitation by irritation of the eyes (tearing and blepharospasm), causing them to close, and irritation of the upper respiratory tract. Technically, they are irritants, lachrymators and harassing agents, although they are general all considered 'tear gas'.

The classification of riot-control agents as CWAs is confusing. According to the Organisation for the Prevention of Chemical Warfare, state-level entities should declare their RCA stocks used for law enforcement purposes. Though their use is allowed for such, it is prohibited in warfare.

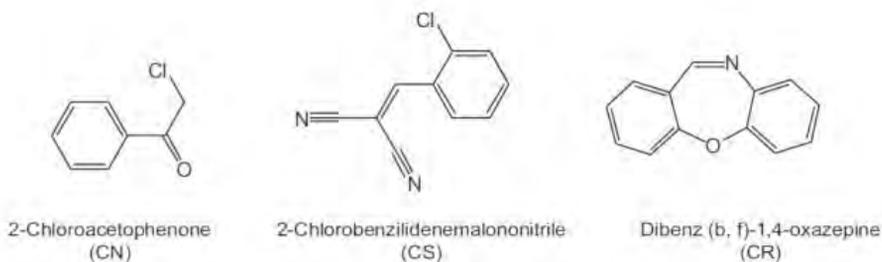


Figure 7. Chemical structures of riot control agents

	CN	CS	CR
Molecular weight	154.59	188.61	195
Vapor density (air = 1)	5.3	6.5	6.7
Melting point (°C)	54	94	72
Boiling point (°C)	248	310-315	335
Vapor pressure at 20°C (mmHg)	5.4×10^{-3}	3.4×10^{-2}	5.9×10^{-5}
Volatility at 25°C (mg/m ³)	34	0.71	0.63
L _C t50 (respiratory, mg-min/m ³)	7,000-14,000	61,000	Not available

Figure 8. Properties of RCAs (figure used with permission under Creative Commons license)

At room temperature, CN, CS and CR are colourless crystals that can be dispersed as aerosols. The compounds are thermally stable, resistant to detonation and can be mixed with other warfare agents.

Mechanism of action

For each of the acute symptoms of pain, the probable mode of action is a direct chemical attack on sensory receptors in the skin and mucosa that involves a nicotinamide adenine dinucleotide hydrogenase (NADH)-dependent enzymatic process. The peripheral nature of their site of action distinguishes these agents pharmacologically from other incapacitating agents that affect the central nervous system, such as psychochemicals.

Psychomimetic agents

This group of CWAs usually includes substances that, when administered in low doses (<10 mg), cause effects similar to psychotic disorders or other symptoms of damage to the the central nervous system (loss of feeling, paralysis, rigidity etc.).

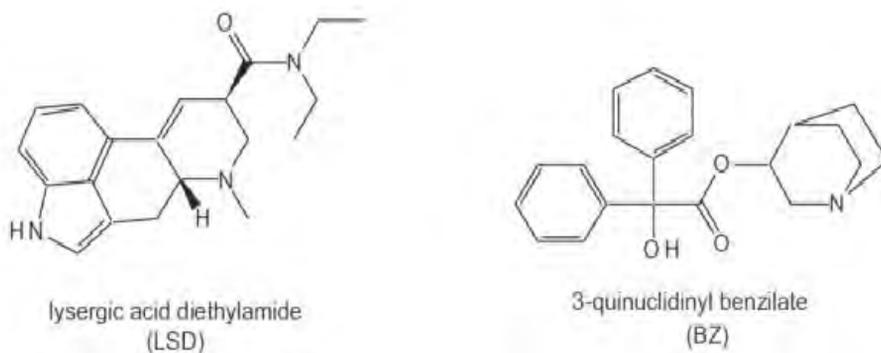


Figure 9. Chemical structures of psychomimetic agents

From a military point of view, BZ is the most important psychomimetic as it can be easily produced. BZ was weaponized during the 1960s. It is a nearly odour- and colourless crystalline powder with a high boiling point and low volatility.

Mechanism of action

BZ interferes with cholinergic nerve transmission at muscarinic sites, both in the peripheral autonomic nervous system and in the brain and spinal cord. BZ readily crosses the blood-brain barrier, and is distributed to all areas of receptors

as a competitor with the physiologically active transmitter acetylcholine. In the central nervous system, BZ bonds to all subtypes of muscarinic receptors, each of them having different functions in the brain.

Toxins

Poisonous chemical compounds synthesized in nature by living organisms such as bacteria, fungi, terrestrial and marine animals are called toxins. These are classified on the basis of their chemical nature, molecular weight, source, preferred targets in the body and mechanism of action. As such, there are two groups: protein toxins consisting of long, folded chains of amino acids, and non-protein toxins, which are generally small molecules with a complex chemical nature. Because of the hybrid nature of toxins, they have sometimes been considered CWAs and sometimes BWAs (Biological Warfare Agents). Based on their mechanism of action, they are classified either as cardiotoxins, dermatotoxins, hepatotoxins, neurotoxins, etc. The most potent toxins are neurotoxins, such as the botulinum toxin and tetanus toxin, but there are others potent toxins, such as the staphylococcal enterotoxin.

Origin	Toxin	LD ₅₀ (µg/kg) (approx.)	Mode of action
Bacteria	Botulinum toxin	0.0001	Neurotoxin
	Tetanus toxin	0.001	Neurotoxin
	Staphylococcus	2,000	Membrane damaging
	Enterotoxins B		Interference with regulation
Fungi	Tricothecenes	3,000	Inhibition of protein
	Aflatoxin	5,000	synthesis
			Inhibition of nucleic acid synthesis
Algae			
Cyanobacteria	Anatoxin	200	Neurotoxin
Dinoflagellates	Microcystins	40	Hepatotoxin
	Brevetoxin	10	Neurotoxin
	Saxitoxin	5	Neurotoxin
Plant	Ricin	0.01	Inhibition of protein
	Abrin	0.01	synthesis
			Inhibition of protein synthesis
Animal	Batrachotoxin	0.06	Neurotoxin
	Palytoxin	0.15	Cardiotoxin
	Snake venoms	0.20	Hemolytic or neurotoxin

Figure 10. Characteristics of several potent toxins (*figure used with permission under Creative Commons license*)

2.2. Toxic Industrial Chemicals

2.2.1. Description of Toxic Industrial Chemicals (TIC)

The term 'toxic industrial chemicals' covers a variety of chemicals used or produced by industry that can have a significant impact on human health if released into the air or water. According to the Chemical Weapon Convention, any TIC can be considered a chemical weapon if it is designed or used for military purposes. In the past, most CWAs consisted of TICs (chlorine, phosgene, hydrogen cyanide, chloropicrin, etc.), or were developed in the context of civilian research and development (for example, nerve agents). The main risk of TICs is that they are manufactured and stored in vast quantities and can be relatively easily used in international or domestic armed conflicts. A general limitation of the production and consumption of TICs is extremely difficult for various reasons. Toxic Industrial Chemicals include certain unusual compounds that are of interest to experts in chemical weapons. For example, dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) and its analogues. TCDD is well known as an impurity in herbicides (for example, Agent Orange), and is equally well known for its use in the Vietnam war. Experts also remember its part in a 1976 industrial accident in Seveso, Italy. It is, basically, an extremely toxic synthetic agent capable of alkylating various cellular polymers, including DNA. Some of the clinical symptoms of TCDD poisoning are similar to mustard gas. TCDD has a long latency period, and its effects are difficult to treat. It is ideal in ecological warfare, because it can cause long-term and dangerous contamination of large areas, even when used in relatively low concentrations.

2.2.2. Classification

Toxic Industrial Chemicals can be both organic and inorganic chemicals and have small or large molecular weight, so their classification is difficult. There have been many attempts to define a list of potential dangerous TICs. One of the most commonly used is based on the Hazard Index (HI).

Chemicals with $HI \geq 90$ are listed in the high risk group; those with HI between 36 and 89 are in the medium hazard group, and below 36 in the low hazard group. Use of toxic industrial chemicals is sometimes considered; chemical warfare without chemical weapons.' The Hazard Index is a good index for characterising overall, global danger from toxic chemicals. But it must be remembered that from even mildly toxic chemicals, much more hazardous materials can be extrapolated, deliberately or inadvertently. The 1984 Bhopal Disaster in Bhopal, India, is considered the most catastrophic CBRN incident ever, and had even worse consequences than the reactor meltdown in Chernobyl – and it was caused by Methylisocyanate, a medium-class TIC.

Toxicity (IDLH in ppm)	Index	State (Vapor Pressure in mmHg)	Index	Distribution ^a	Index	Number of Producers	Index
<1	5	Gas	5	5/6	5	>100	5
1 to 10	4	Liquid: >400	4	4	4	50 to 99	4
11 to 100	3	Liquid: 100 to <400	3	3	3	25 to 49	3
101 to 500	2	Liquid: 10 to <100	2	2	2	5 to 24	2
>500	1	Liquid: <10	1	1	1	<5	1

^a Number of continents in which production occurs.

Figure 11. Hazard Index Parameters (*figure used with permission under Creative Commons license*)

2.2.3. The Globally Harmonized System of Classification and Labelling of Chemicals (GHS)

The GHS defines and classifies the hazards of chemical products, and communicates health and safety information on labels and safety data sheets. The goal is to have the same set of rules for classifying hazards, and the same format and content for labels and safety data sheets (SDS), adopted and used around the world. An international team of hazard communication experts developed the GHS, which covers all hazardous chemicals and can be adapted to cover chemicals in the workplace, in transport, consumer products, pesticides and pharmaceuticals. The target audiences for GHS include workers, transport workers, emergency responders and consumers. The two major elements of GHS are:

1. Classification of the hazards of chemicals according to the GHS rules: the GHS provides guidance on classifying pure chemicals and mixtures according to its own criteria.
2. Communication of hazards and precautionary information using Safety Data Sheets and labelling.
3. Safety Data Sheets – GHS Safety Data Sheets have 16 sections, laid out in a set order, with space for the minimum prescribed information.
4. Labels – certain information will always appear on the label. For example, the chemical identity. Appropriate standardized hazard statements, signal words and symbols will also appear, as well as precautionary statements, if required by the relevant regulatory authority.

In the European Union, the GHS was implemented *via* the 2008 CLP (Classification, Labelling and Packaging) Regulation.

The GHS also introduces unified criteria for chemical classification, and introduces three classes of hazard:

- Physical hazards;
- Health hazards;
- Environmental hazards.

Within the GHS, two sets of pictograms are included: one for the labelling of containers and for workplace hazard warnings, and the other for use during the transport of dangerous goods. Either one or the other is used, but not both at the same time. The two sets of pictograms use the same symbols for the same hazards, although certain symbols are not required for transport pictograms. Transport pictograms also come in wider variety of colours and may contain additional information, such as a subcategory number.

Table 1. GHS hazard pictograms (non-transport)

Description	Pictogram	Hazard class and hazard category
Exploding Bomb		Unstable explosives Explosives of Divisions 1.1, 1.2, 1.3, 1.4 Self-reactive substances and mixtures, Types A,B Organic peroxides, Types A,B
Flame		Flammable gases, category 1 Flammable aerosols, categories 1,2 Flammable liquids, categories 1,2,3 Flammable solids, categories 1,2 Self-reactive substances and mixtures, Types B,C,D,E,F Pyrophoric liquids, category 1 Pyrophoric solids, category 1 Self-heating substances and mixtures, categories 1,2 Substances and mixtures, which in contact with water, emit flammable gases, categories 1,2,3 Organic peroxides, Types B,C,D,E,F
Flame Over Circle		Oxidizing gases, category 1 Oxidizing liquids, categories 1,2,3
Gas Cylinder		Gases under pressure: – Compressed gases – Liquefied gases – Refrigerated liquefied gases – Dissolved gases

Description	Pictogram	Hazard class and hazard category
Corrosion		Corrosive to metals, category 1 Skin corrosion, categories 1A,1B,1C Serious eye damage, category 1
Skull and Crossbones		Acute toxicity (oral, dermal, inhalation), categories 1,2,3
Exclamation Mark		Acute toxicity (oral, dermal, inhalation), category 4 Skin irritation, category 2 Eye irritation, category 2 Skin sensitisation, category 1 Specific Target Organ Toxicity – Single exposure, category 3
Health Hazard		Respiratory sensitization, category 1 Germ cell mutagenicity, categories 1A,1B,2 Carcinogenicity, categories 1A,1B,2 Reproductive toxicity, categories 1A,1B,2 Specific Target Organ Toxicity – Single exposure, categories 1,2 Specific Target Organ Toxicity – Repeated exposure, categories 1,2 Aspiration Hazard, category 1
Environment		Hazardous to the aquatic environment – Acute hazard, category 1 – Chronic hazard, categories 1,2

When done properly, the labelling of chemicals is a powerful tool for identification of chemicals and hazards. Transport labels are defined in more detail in the ADR (*L' Accord européen relatif au transport international des marchandises Dangereuses par Route*), which is another great source of information, especially for firefighters and HAZMAT Teams.

In addition, some very helpful tools are easily accessible to anyone with a smartphone (Android, iOS, Windows), in the form of the many apps available

that list all kinds of information about chemicals, identified by their UN numbers, including their toxicology, potential hazard, treatments etc.

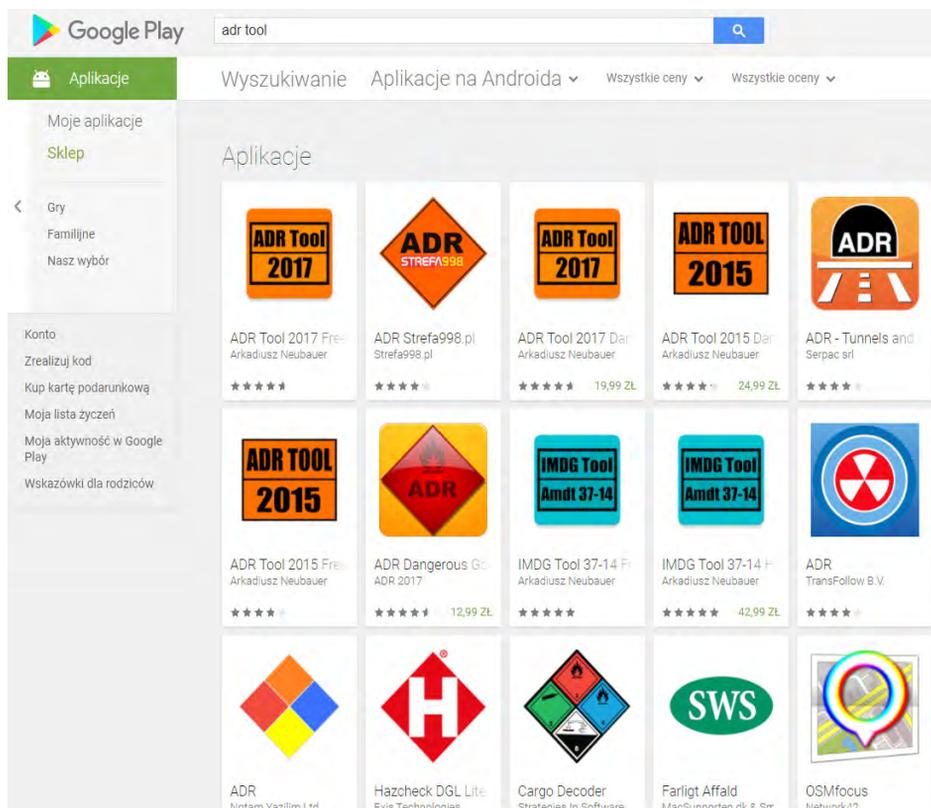


Figure 12. Some of the Android ADR apps

2.3. Chemical detection and identification techniques and equipment

2.3.1. Colorimetric detectors

Colorimetric detectors analyse the colour change resulting from the reaction between a targeted chemical and reagents. This technique has many different applications. One very well-known example of colorimetric detection is the use of litmus paper to test pH. Colorimetric detectors often take the form of badges and tubes, with which the signal (colour change) is detected by the naked eye, instead of with an electronic readout or device. Therefore, most colorimetric

detectors can be made very small and simple, are less costly to produce and do not need electric power for operation.

Detectors are made with a sorbent substrate, paper, paper tape or a glass tube impregnated with colorimetric reagent(s), which react with a specifically targeted chemical to form a particular colour. Colorimetric tubes can consist of finely impregnated beads, together with the necessary reagent ampoules.



Figure 13. Detection tubes (author's photo)

Detection papers are used for qualitative analysis, and only give information about the presence of a particular chemical (not a whole group or class of chemicals). Detection tubes can aspirate an amount of air and can be calibrated (a set of scales can be seen in figure 13 above), giving quantitative or semi-quantitative information about the concentration of the target chemical.

Summary

Strengths	Weaknesses
No need for electrical equipment	Can only measure one chemical at a time
Cheap and reliable	Operated manually
Low probability of false alarms	
Short detection time	

2.3.2. Flame Photometric Detector

Flame photometry is an atomic spectroscopy technique based on the light emission phenomenon of excited atoms, or clusters of atoms, as they return to lower energy states. This is a very important CWA detection technique that has been used successfully for a long time. The detection of characteristic light emission by excited sulphur and/or phosphorus atoms is used to identify CWAs. Most classic CWAs, such as the G-agents, V-agents, and sulphur mustard (HD), contain phosphorus and/or sulphur in their molecules. Detectors based on atomic spectroscopy technology can also be used to detect many TICs, if they contain phosphorus, sulphur, arsenic or other atoms that produce characteristic light emissions. Flame photometric detectors (FPDs) have been in use for as long as gas chromatographic and liquid chromatographic detectors. FPD instruments for field operations have portability and fast real-time analysis. Analysing a vapour sample with a handheld FPD does not require sample preparation, as it draws the sample directly from the surrounding air and provides a content analysis instantly. The only field FPD detectors available on the market are the AP4C and AP2C, made by the Proengin company.

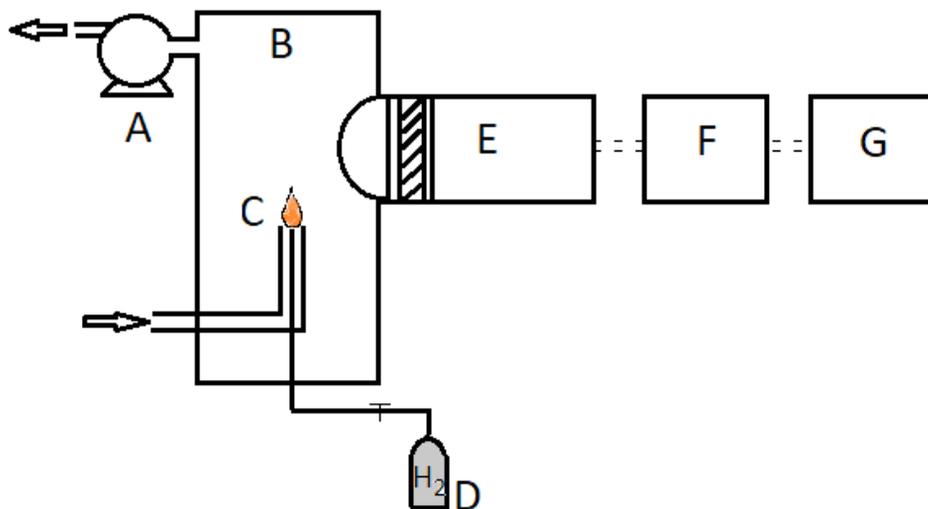


Figure 14. Principle of a Flame Photometric Detector's operation: A – pump, B – reaction chamber, C – flame, D – H₂, E – photometric sensor, F – electronics, G – visualization

Air samples are drawn in through an inlet and mix with hydrogen (D); at the end of the burner tip, the sample is burned. This causes CWAs or TICs containing phosphorus or sulphur to decompose and emit photons. These

photons then go through the filter to the sensor (E), where they are detected. A signal is processed (F), and the operator receives visual indication *via* a visible and/or audible signal.

Most FPD detectors are used in conjunction with separation techniques, such as gas chromatography (GC) and liquid chromatography (LC).



Figure 15. Agilent GC-FPD (author's photo)

Because of this, analyses are separated in time provide give additional parameters for identification – retention time or retention index. In the figure above, the FPD is equipped with a photomultiplier. This suggests that it only has one element detection capability (S or P). But if two photomultipliers are attached to the combustion chamber, both P and S could be detected simultaneously.



Figure 16. A Proengin AP4C FPD field detector, with an S4PE liquid sampler (author's photo)

Proengin's AP4C is the only field Flame Photometric Detector that can detect S, P, As and N chemicals. It is equipped with a sampler for liquids (or even solid surfaces, using wipes), for detection of persistent agents.

Summary

Strengths	Weaknesses
Low detection limits	Additional consumables are necessary – hydrogen canisters
Short detection time	High false alarm rate
Able to detect S, P, N and As agents	
Can analyse wet wipes	

2.3.3. Ion Mobility Spectrometry (IMS)

Ion mobility spectrometry is an analytical technique used to separate and identify ionized molecules in the gas phase, based on their mobility in the carrier buffer gas. Mobility is defined as:

$K = v/E$, where K is mobility [$\text{cm}^2/(\text{V} \times \text{s})$], v is drift velocity [cm/s], and E is the electric field gradient [V/cm].

Though heavily employed for military and security purposes, such as detecting drugs and explosives, the IMS technique also has many lab-based analytical applications, including analysis of both large and small biomolecules. IMS instruments are extremely sensitive stand-alone devices, but are often coupled with mass spectrometry, gas chromatography or high-performance liquid chromatography in order to achieve multi-dimensional separation. They come in various sizes, ranging from just a few millimetres to several meters, depending on the specific application, and are capable of operating under a broad range of conditions.

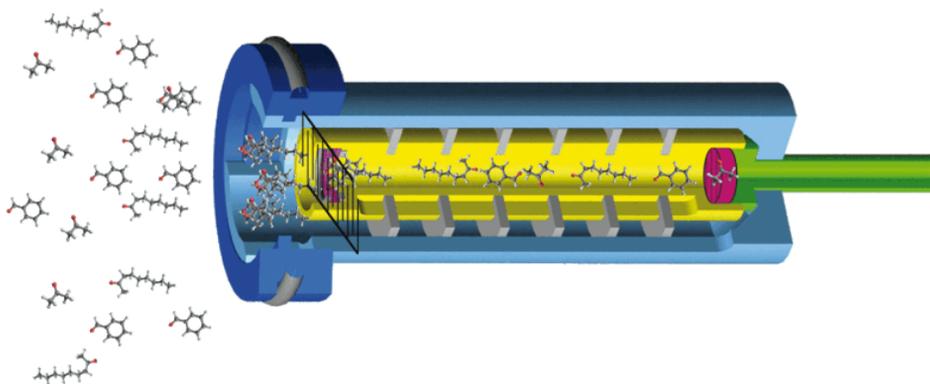


Figure 17. Principle of an Ion Mobility Spectrometry device's operation

Samples need to be in the vapour phase for detection by an IMS system. Vapour samples are analysed directly by drawing in sample flows containing the analyte of interest – CWAs, TICs and some volatile explosives can be detected in this way. Some samples, such as non-volatile explosives and many narcotic compounds, need to be heated to create enough vapour for detection. In this case, sample material released by thermal desorption is swept into the reaction region of the detector, usually by a small gas flow. IMS uses soft ionization techniques, such as ^{63}Ni or corona discharge, to form reactant ion species from the carrier gas employed in the system, normally air. Mixing these stable reactant ion clusters with vapour samples to be analysed can result in ionisation of the sampled materials, thus forming ion clusters characteristic of the sampled material. This ionisation process is generally referred to as Atmospheric Pressure Chemical Ionization (APCI).



Figure 18. RAID M-100 (author's photo)



Figure 19. PRS 1W – Differential Mobility Spectrometer for CWA and TICs detection, for mounting in vehicles and fixed installations (author's photo)

The small packet of ions thus formed is injected electrically into a drift region, where they pass to a collector electrode some distance away (typically a few centimetres), under the influence of an applied electrostatic field. Ions travel through the drift region at speeds characteristic of the size and shape of

the ion clusters. On arrival at the collector, each ion species generates a specific signal, and the ion current as a function of its arrival time is measured. A plot of ion current against K (mobility) forms an ion mobility spectrum, with an ion mobility band corresponding to each of the unique ionic species. The spectrum is a fingerprint of the parent compound. The measured IMS spectrum is then analysed and mobility information is extracted in real time, within the instrument. Comparison of measured mobility with the known mobility of threat compounds gives rise to an ALARM condition if there is a match.

There are many CWA detectors based on IMS technology. In fact, this technology can be divided into different subcategories (open loop technology, differential mobility spectrometry, and so on), but the basic principles are still the same. There are field, handheld, fixed, vehicle mounted and maritime IMS detectors available, for military and chemical safety applications.

Summary

Strengths	Weaknesses
Low detection limits	Moderate to low (DMS) false alarm rate
Short detection time	Low resolution power (except DMS)
Ability to detect CWA and selected TICs	
Simple operation and maintenance	

2.3.4. Infrared spectroscopy

Use of the IR technique for analysis and detection is based on the infrared absorption of the characteristics of a sample, at specific wavelengths. Function groups in a molecule vibrate at certain frequencies that fall within the IR's sensitivity. The molecule absorbs energy from IR waves that have the same frequencies, and so the vibrating function groups are excited to a higher energy level (specific function groups absorb IR radiation of specific frequencies). By determining the IR radiation frequencies being absorbed by the function groups composing the molecule, its molecular structure can be determined. The spectrum of a large molecule can then be matched with spectra from various databases.

Detectors based on IR technology have the advantage of reasonably high sensitivity and fast detection. Remote detection of chemical clouds can also be made using the infrared emission technique.

A sample is introduced to the spectrometer and exposed to IR radiation. The IR emission is filtered and certain well-defined wavelengths interact with the sample. When the sample contains targeted chemicals, absorption occurs. The intensity of radiation changes accordingly to the concentration of the chemical. By changing the wavelength of the radiation, the IR spectrum can be ascertained. Chemical

analysis is made by for the checking presence of absorption at certain wavelengths, or by analysing the entire spectrum (which is time consuming).

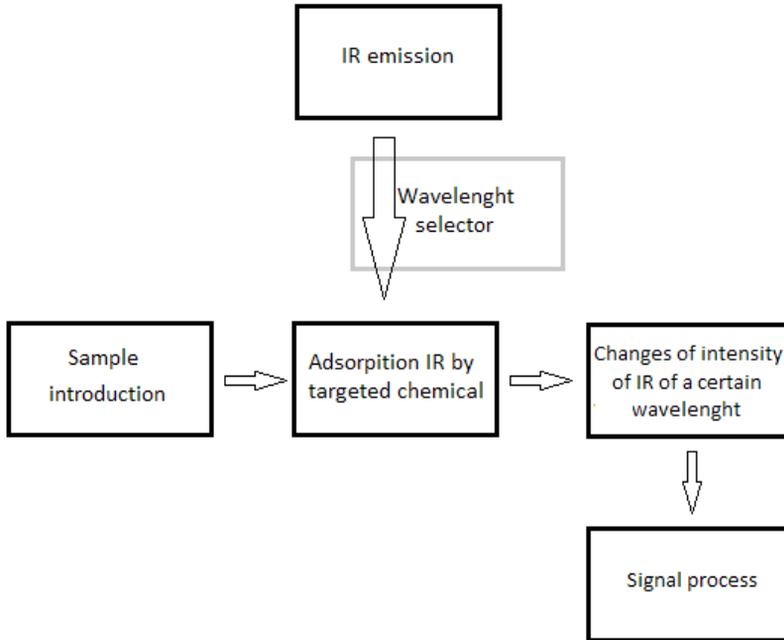


Figure 20. Block diagram of an IR detector

In more sophisticated detectors, known as Fourier transform infrared (FTIR) spectrometers, the entire IR spectrum can be collected and processed quickly. The heart of the FTIR detector is an interferometer (Michelson's is the most often used). The Michelson interferometer, illustrated in Figure 21, is composed of a beam splitter, a fixed mirror, and a moving mirror.

Incoming IR radiation is split into two beams that are reflected by the mirrors and then recombined before reaching the IR transducer. Changing the moving mirror's position enables the interference of the two beams to form an interferogram that is used to obtain the spectra. As Figure 25 shows, the IR beam (1) emitted from the IR source strikes the splitter, which then divides the beam into two beams (2 and 3). Due to the special properties of the splitter, about 50% of the incoming beam is reflected by the splitter toward the fixed mirror and the other 50% is allowed to penetrate the splitter to hit the moving mirror. These beams are reflected back by the fixed and moving mirrors toward the splitter. The splitter then reflects half of the beam from the moving mirror (4) to the transducer, and also allows the other half of the beam from the fixed mirror (5) to penetrate it. These two beams then recombine (6) and

pass through the sample cell. The rest of the radiation that is reflected from both the moving and fixed mirrors penetrates the beam splitter in the direction of the radiation source. Therefore, only about half of the initial IR radiation goes through the sample cell and reaches the transducer. The recombination of the beams after the splitter and the sample cell results in the interferogram, which is a plot of the output power versus the difference (δ) in path lengths for the two recombined beams. The δ value depends on the relative position of the moving mirror. Since the radiation strikes the moving mirror and is then reflected back, δ is equal to two times the difference between the path lengths of the moving mirror and the fixed mirror. If the sample in the sample cell absorbs the incoming IR of certain wavelengths, a characteristic interferogram is generated. By applying the Fourier transform to the interferogram, a distinctive spectrum is then obtained.

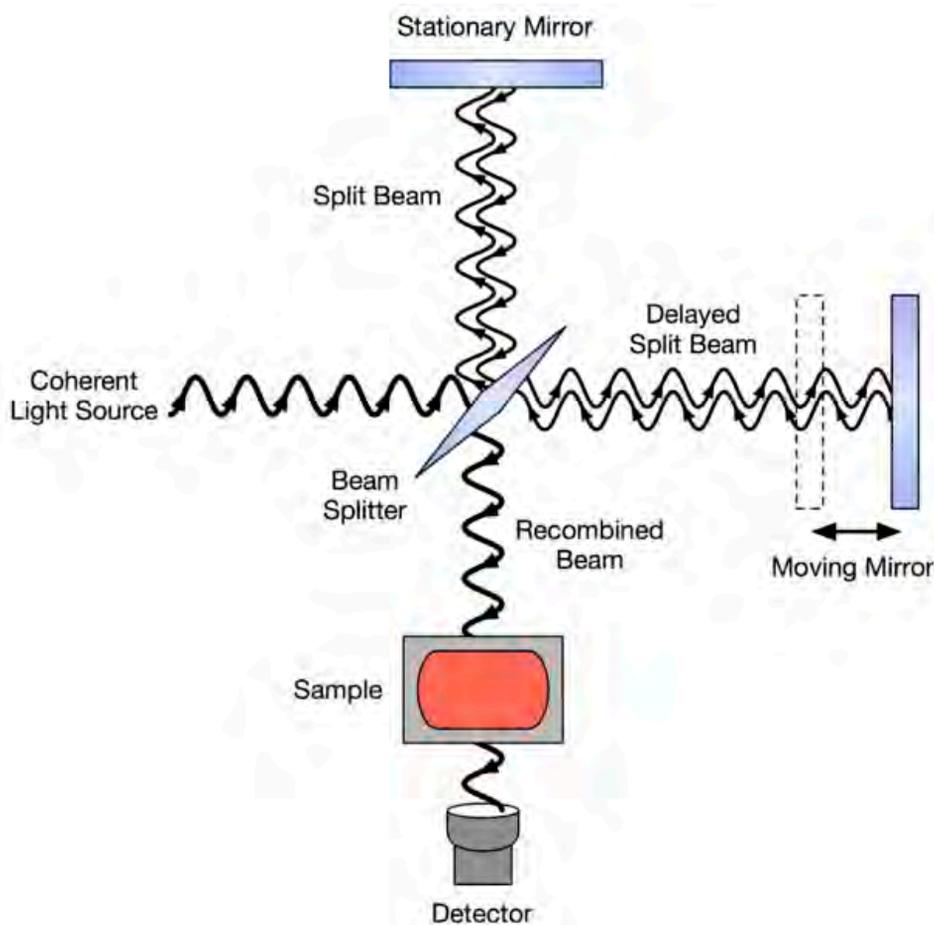


Figure 21. Schematic diagram of a Michelson interferometer

IR technique has huge potential and has applications, both in laboratories and in the field. IR detectors can also be used with gas or liquid chromatography for separation of targeted chemicals, and measurement of a single compound instead of a mixture. For military and chemical safety and security purposes, IR equipment is mainly used for identification of chemicals.



Figure 22. Spectrum BX FTIR Spectrometer (author's photo)



Figure 23. HazmatID detector (author's photo)

The design of field-use FTIR detectors allows for their decontamination, making them usable in hot zones. In most instruments, the user can create their own spectra library with chemicals of interest, to improving the instrument's applicability.

Summary

Strengths	Weaknesses
Rich-information spectra	Cannot analyse vapours and aerosols (except Gas ID, which can analyse neither liquids nor solids)
Ability to analyse solids and liquids	High cost
Strong identification capabilities	Requires a highly-trained operator if interpretation mode is used
High resolution power	
Low false alarm rate	

2.3.5. Raman spectroscopy

Raman spectroscopy is a technique that measures the energy of photons generated by the inelastic scattering of monochromatic excitation photons. During excitation, photons interact with the electron cloud of an analyte, and a small percentage of the photons undergo a change in energy as a result of the interaction. This energy change corresponds with molecular vibrations, rotations, and other low-frequency modes, and is plotted by instrumental operating software to produce a spectrum. Raman spectroscopy is often described as a complimentary technique to FTIR because both techniques are used to probe vibrational energy levels, although different selection rules allow FTIR and Raman to interrogate different sets of molecular vibrations. Raman scattering is an inherently low probability phenomenon, and as such the Raman scattering cross-section of a particular analyte will generally be several orders of magnitude less than the corresponding FTIR absorption cross-section. Practically, this means that the sensitivity of Raman spectroscopy is lower than FTIR spectroscopy. There are several advanced Raman spectroscopy techniques that generate a stronger Raman intensity when compared to spontaneous Raman scattering, such as Coherent Anti-Stokes Raman Spectroscopy (CARS), Resonance Raman Spectroscopy, Surface Enhanced Raman Spectroscopy (SERS), and Tip Enhanced Raman Spectroscopy (TERS).

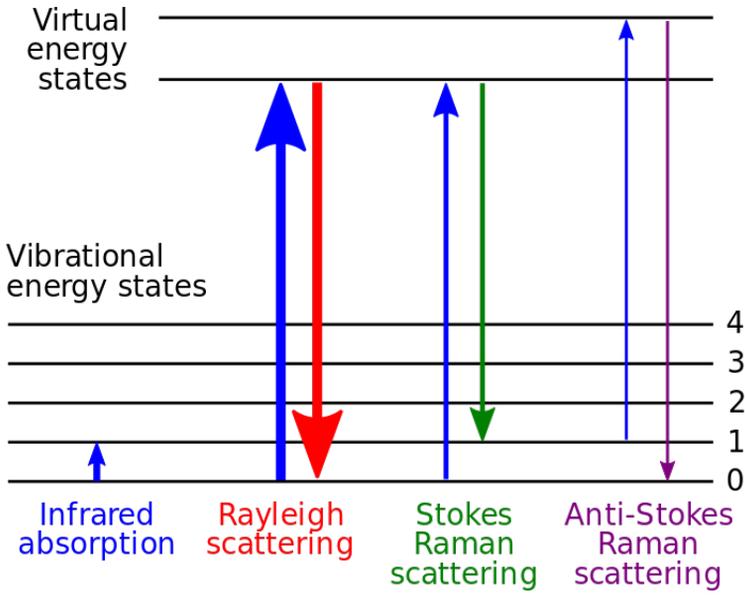


Figure 24. Raman Effect

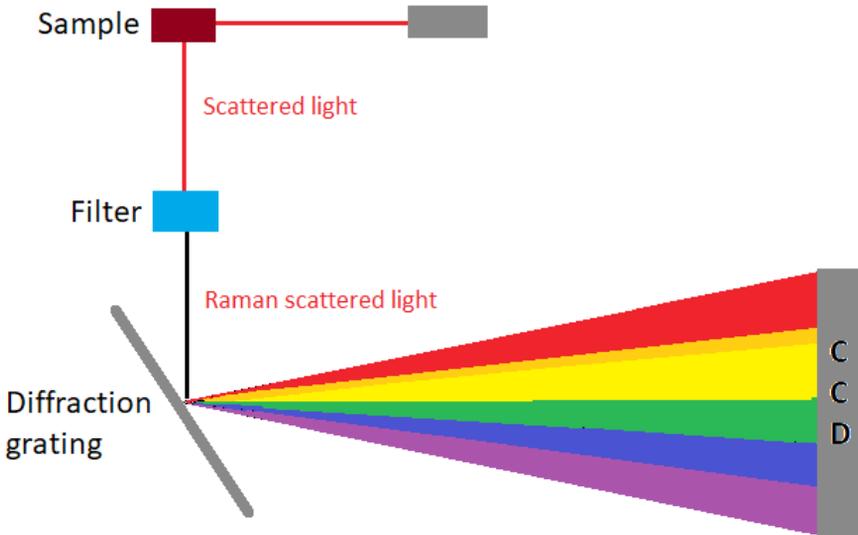


Figure 25. Simplified diagram of Raman spectrometer

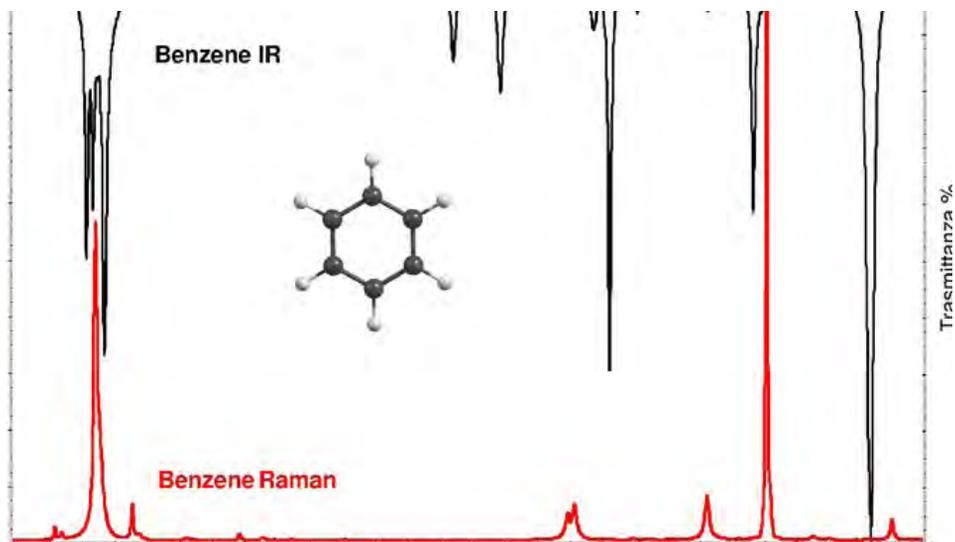


Figure 26. Raman spectrum of benzene



Figure 27. Bruker RamanScope III (author's photo)

A sample is irradiated with monochromatic laser light, which is then scattered by the sample. The scattered light passes through a filter to remove any stray light that may have also been scattered by the sample. The filtered light is then dispersed by the diffraction grating and collected on the detector. The spectrum thus obtained is then compared with a built-in library.

This technique has powerful identification potential, and one very interesting feature of Raman spectroscopy is its ability to analyse samples through the transparent wall of container (glass, plastic, amber glass). This is especially important when dealing with dangerous chemicals. On the down side, Raman spectroscopy uses a strong laser beam that can initiate explosions when applied to explosive material. For this reason, field Raman detectors have an option for delayed measurement, to give the operator time to hide in case of an explosion. And because of this, only small amounts of unknown samples should be analysed at a time.

Summary

Strengths	Weaknesses
Strong identification capabilities	High cost
Ability to analyse solids and liquids	High detection limits
Ability to analyse through transparent container walls	Probability of explosion initiation
Low false alarm rate	

2.3.6. Gas chromatography (GC)

Gas Chromatography is a commonly-used analytical technique in many research and industrial laboratories, and is used for quality control as well as identification and quantitation of compounds in a mixture. GC is also frequently used in many environmental and forensic laboratories, because it allows for the detection of very small quantities. A broad variety of samples can be analysed as long as the compounds are sufficiently thermally stable and reasonably volatile.

A mobile and a stationary phase are required for this technique. The mobile phase is comprised of an inert carrier gas, such as helium, argon, or nitrogen. The stationary phase consists of a packed column in which the packing or solid support itself acts as the stationary phase, or is coated with a liquid stationary phase (high-boiling polymer). Most analytical gas chromatographs use capillary columns, where the stationary phase coats the walls of a small-diameter tube directly (e.g. a 0.25 μm film in a 0.32 mm tube).

The separation of compounds is based on the different strengths of interaction of the compounds with the stationary phase (the 'like-dissolves-like'

rule). The stronger the interaction is, the longer the compound interacts with the stationary phase, and the more time it takes to migrate through the column (longer retention time).

In a gas chromatography analysis, a known volume of a gaseous or liquid sample is injected into the head of the injector column, usually with a microsyringe, solid phase microextraction fibres or a gas source switching system).

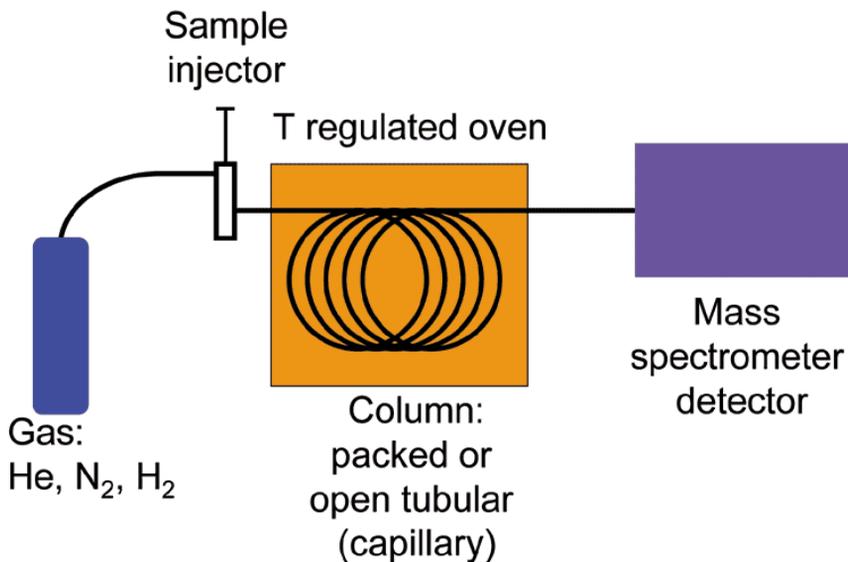


Figure 28. Principle of GC operation

As the carrier gas sweeps the analyte molecules through the column, this motion is inhibited by the interaction with the stationary phase. Since each type of molecule has a different rate of progression, the various components of the analyte mixture are separated as they progress along the column, reaching the end of the column at different times (retention time).

For example: If compound X interacts more strongly with the stationary phase than compound Y, it lags behind compound Y in its movement through the column. As a result, compound Y has a much shorter retention time than compound X.

At the end of the column, a detector monitors the outlet stream. Thus, the time at which each component reaches the outlet and the amount of that component can be determined. Generally, substances are qualitatively identified by the order in which they emerge (elute) from the column, and by the retention time of the analyte in the column. Quantitative information is derived from the intensity of the signal generated by the detector.

The amount of information generated by gas chromatography can be extended by use of different detectors. There are many types of GC detectors, and it is possible to connect them in parallel or series, with the restriction that the first one must not be destructive – like FPD or Raman.

Popular GC detectors for use in CWA analysis include:

- FPD – Flame Photometric Detector (P and S detection);
- NPD – Nitrogen-Phosphorus Detector (N and P detection);
- AED – Atomic Emission Detector (identifies atoms in molecules);
- ECD – Electron Capture Detector (detects electron-absorbing compounds, especially halogens);
- IR (FTIR) detector – Infrared detector (spectral technique for structural analysis, functional group identification and identification *via* library comparison);
- MS – mass spectrometer (spectral technique for structural and isotope analysis, and identification *via* library comparison (the largest spectra libraries are for mass spectra)).

GC is one of the most common chemical analytical techniques, and there are many types of GC systems. Most are designed for laboratory use, but there are also a significant number of mobile systems, some even mounted in military vehicles.



Figure 29. Micromass Autospec GC – HRMS (High Resolution Mass Spectrometer)
(author's photo)



Figure 30. Agilent S975T mobile GC-MS (author's photo)

Summary

Strengths	Weaknesses
Strong identification capabilities	High cost
Ability to analyse all types of samples after sample preparation	High maintenance cost
Low false alarm rate	Requires special gasses (helium, nitrogen, hydrogen)
Largest spectra libraries available	Requires highly-trained operators
Very sensitive	

2.4. Practical information for chemical mission preparations

2.4.1. Mission types

Knowing and understanding what you are facing is key to successfully preparing to respond to a CBRN incident. First of all, there are two kinds of CBRN incidents:

- Accidental – an event caused by human error or natural or technological reasons (spills, accidental releases, leakage, equipment failure);
- Intentional – deliberately caused events, including:
 - Criminal acts, such as deliberate dumping or release of hazardous materials to avoid regulatory requirements,
 - Malicious but not politically motivated poisoning of one or more individuals,
 - Terrorist acts inflicting serious harm on persons for political or ideological reasons.

In accidental CBRN events, the only hazard that is expected to be encountered at the scene is from the released chemicals. With an intentional incident, further hazards could be expected from the perpetrators. As such, external support from military or police units is needed to prevent further attacks and help contain the situation.

The next step after determining the cause of the incident is to classify the hazards involved, based on the nature of the agents used – whether Chemical, Biological, Radiological or Nuclear.

The third step in mission classification is determining the main purpose of the response. Is it a life-saving mission, or just a reconnaissance mission? The main difference between the two is time pressure. In life-saving missions, time is critical as hostages or injured and dying people are at stake. These kinds of missions tend to be fast-paced and require energy and stamina from the operators. In a reconnaissance or sampling mission, time is not such an important factor, and these missions are less energetic.

In either case, other than the time factor the safety of operators and security of samples taken, equipment and resources, etc, must also be taken into consideration. Forensic missions especially need to be well prepared and conducted with priority given to the quality of the evidence collected.

The type of mission being prepared for determines the clothing and equipment needed, as well as the amount of information that must be gathered before the mission begins, in order to better prepare for it.

2.4.2. Sources of information

There are many sources of information available to chemical teams before they arrive at the scene. Typically, a chemical reconnaissance team or Hazmat Team isn't the first on the scene – their first information is usually acquired from

interviews with rescue teams, firefighters, police officers, factory staff, victims and witnesses. Speaking with medical staff and ambulance crews can help about the symptoms of the injured can also help identify chemical agents, or agent classes.

As discussed earlier, chemical transportation labelling can also help quickly determine the nature of the threat. Information from UN numbers or pictograms on tanks and containers can identify the chemicals involved. In accidental CWA incidents, the relevant indicators include:

- Numerous dead animals;
- Lack of insect life;
- Mass casualties;
- Unexplained physical symptoms;
- Pattern of casualties;
- Illness in a specific, confined geographic area;
- Unusual liquid droplets;
- Areas of dead vegetation;
- Unexplained odours;
- Numerous people experiencing blisters;
- Low-lying cloud/fog-like conditions;
- Unusual metal debris/bomb-like materials.

Other relevant information can include the location, number of people involved or living in the vicinity, weather conditions and other agencies present at the scene. Note that these can also be important factors in non-chemical incidents.

2.4.3. Important detector parameters

The different types of missions and the variety of possible agents at a scene make it impossible to have one detector for all threats. There are many detectors on the market and they vary in cost, performance and reliability. Choosing the proper device for each mission can be quite challenging. Many factors should be considered when selecting which detector to take, but two of the most important are:

- Detection capability (selectivity, sensitivity, response time etc.);
- Performance (warm-up time, calibration needs, portability, power requirements, etc.).

In more detail, some of these factors are:

Selectivity is the ability of a detector to respond only to the targeted chemicals in the sample. When a detector is set to detect G-agents, it should only respond to G-agents detected in the sample, regardless of how many other chemicals are present. For example, colorimetric detectors are highly specific detection devices and have higher selectivity, as they respond to a certain number of specific chemicals that they were designed to detect with little or no interference from other chemicals.

More specific, selective detectors are limited in the number of compounds that can be added to their detection capability. Less-selective types of detectors can respond to more chemicals than selective detectors, but their responses cannot immediately be attributed to CWAs, TICs, or non-toxic substances.

Detectors working in a well-defined environment (i.e. a facility producing phosgene) can be more selective, because they are detecting a known chemical. In the case of checking for unknown agents, less selective devices could be a better option.

The **Sensitivity** of a detector or a method is the measure of its ability to discriminate between small differences in analyte concentration. The detector or method is more sensitive when a large change in signal intensity is observed for a small change in concentration.

Limit of detection (LOD) is the lowest concentration level that a detector can identify with a certain degree of confidence. There are many definitions of LOD, such as the concentration at which the response signal generated is three times the instrument noise level. Here, LOD is referred to as the minimum detection level (MDL) of concentration that will consistently cause the detector to ping. It is affected by background noise and blank signals, and may vary widely for different chemicals. Environmental and operational conditions can also drastically affect the LOD of a device. Manufacturers normally provide LOD information obtained under optimum conditions. Additionally, a good detector should provide a warning signal well before a dangerous concentration is reached, to permit proper retreat or evacuation. Each type of operational scenario can require a different LOD.

Response dynamic range is the concentration range between the limit of quantification (LOQ) and the limit of linearity (LOL). The LOQ is the lowest concentration at which quantitative measurements can be made. The LOL represents the concentration level at which the calibration curve departs from linearity. LOD and LOQ are different in that the LOQ is usually somewhat higher than the LOD. When the concentration of a sample exceeds the LOL of the detector, an increase in sample concentration may not be reflected in the observed or reported response. For an analytical instrument, response dynamic range is crucial. However, for a field instrument, simply detecting the presence of a toxic chemical in the air at a given concentration or higher, is more important.

Quantitative analysis capability is an important parameter for laboratory instruments, but fortunately, not so much for field equipment, as many factors (temperature, humidity, dust, wind speed) can influence the quality of measurements. For field use, semi-quantitative or just qualitative information about the presence of a target chemical above the LOD level is considered satisfactory.

False alarm rate. There are two types of false alarms. A false positive alarm (Type I error) occurs when a target chemical is detected that is actually absent

from the sample. These alarms can be caused by various factors, depending on the specifics of the technique used. For example, phosphorus and sulphur compounds, such as pesticides, would generate a false positive CWA alarm when a flame photometric detector is used. Detectors are always at risk of yielding false positive alarms, since no detector can be made 100% selective to only the targeted chemicals.

A false negative alarm (Type II error) occurs when there is no detection of a target chemical that is indeed present in the sample. These responses are viewed as more problematic than false positives, because failure to produce a necessary alarm can lead to dangerous or disastrous situations. Causes of false negative alarms include:

- Changing environmental conditions;
- Humidity;
- The presence of other chemicals that interfere with normal detection capabilities;
- Detector malfunctions, such as improper calibration and detection algorithm deviations.

Response time is the time required for the detector to respond to the targeted chemicals after an analysis cycle is begun. The elapsed time until an alarm should occur after the detector is exposed to the targeted chemical at different concentration levels is another important factor. Responses to higher-level concentrations must be fast and immediate, whereas somewhat slower response times for very low concentration levels are acceptable. Some detectors require a certain amount of time before they can respond with any alarm. For example, detectors with a sample pre-concentrator and/or a gas chromatography (GC) column in front of the sensor will require more time for analysis than detectors without such attachments. Response time is a function of vapour concentration for most direct-detection gross-level alarm devices.

Resistance to environmental conditions, including temperature, humidity level, dust concentration in the air, wind, and levels of contamination can all drastically affect the performance of a detector. For military operations, temperatures in the field can range from -30°C to $+50^{\circ}\text{C}$, with relative humidity levels ranging from less than 10% to 100%. It can also be very dusty, windy, and/or the air may contain diverse pollutants at varying levels. Ideally, a field detector must be operable under all environmental conditions, and should maintain its designated functions regardless of field conditions.

It should be noted, then, that all existing commercially-available detectors are affected to one degree or another by environmental conditions. Operating temperatures and humidity ranges are usually specified in the instrument documentation, or are available from the manufacturers. However, other factors are not always so readily available. Determining whether a detector can operate in the intended environment is crucial in the process of selecting which devices to take.

Setup time is the time needed to prepare the detector before it can even be powered up. For handheld detectors, the setup time is usually minimal because they are usually self-contained, with only the necessary batteries being separately serviceable parts.

Warm-up time is the time required for the detector to become ready for analysis after it has been turned on. This could range from a few seconds to half an hour or longer, depending on the ambient temperature and relative operational parameters of the detector type. In general, the device goes through an internal self-check protocol to satisfy pre-set parameter requirements before it is ready for analysis functions.

Recovery time is the time taken for a detector display to return to the baseline 'no response' value after being removed from the agent. With some instruments, the recovery time increases significantly after extended exposure to high concentrations of agent. Ideally a detector should recover in a short period of time (i.e. within minutes).

Calibration/verification of in field application – Proper detector functioning will often require verification of its capacity to perform upon each start-up of the device. For a chemical detector, the process is confirmed using known, non-toxic chemicals to simulate the compounds to be targeted. Ideally, the proper operation of the detector can be verified with simple simulant checks that do not require complicated correlation and calibration procedures before each use.

Portability, or how easily a device can be transported, includes the portability of any extraneous, supporting equipment required for the detector's operation. Field detectors must also be durable enough to withstand transportation from place to place by ground, rail, water and air.

Power requirements determine the amount of energy consumed by the detector and the ease with which it can be re-charged or have its batteries replaced. The most common power supplies for field-deployable detectors are batteries, however some detectors require specifically designed batteries, and finding replacements or recharging spent batteries in the field can be quite difficult. Ideally, a detector should be operable through the use of two or more alternative power sources, and battery life must be sufficient to last an entire mission.

Ongoing costs associated with a detector should be carefully explored, and should include equipment purchases, maintenance and consumables.

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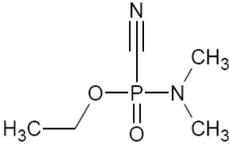
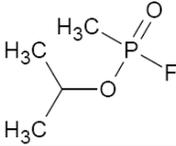
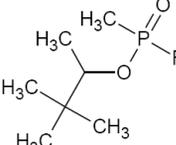
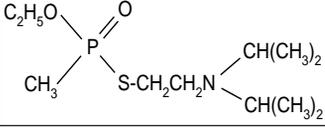
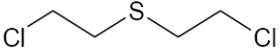
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3. DECONMTAMINATION OF CHEMICAL AGENTS

3.1. Properties of chemical agents

The physicochemical properties of chemical warfare agents (CWA) influence the spread and persistency of chemical agents in the environment, their absorption route and rate, and susceptibility to decontamination (Table 1).

Table 1. Properties of selected CWAs

Agent	Structure	Melt- ing point. [°C]	Boil- ing point. [°C]	Volatili- ty 25°C [mg/ m ³]	Water solubil- ity [g/l]	Log octa- nol/water partition coeff. K _{ow}
Tabun (GA)		-50	248	610	72	0.39
Sarin (GB)		-56	158	22000	miscible	0.3
Soman (GD)		-42	198	3900	21	1.78
VX		-39	298	11	30	2.09
Sulfur mustard (HD)		14,5	217	610	0.7	2.4

Because of their low melting points, most CWAs (an important exception is HD) remain as liquids in all environmental conditions. Consequently, even at low temperatures the evaporation of these compounds will occur, and toxic vapours will always emanate from contaminated surfaces. Comparison of volatilities (Table 1) and acute toxicity doses (Table 2) indicate that vapour concentrations in the atmosphere (especially GB), can be sufficiently high to be rapidly lethal.

Table 2. Toxic effects caused by chemical agents after 1 minute of inhalation

Agent	Effect and dose [mg·min/m ³]			NDS [mg/m ³]
	Threshold	Incapacitating	Lethal	
Sarin (GB)	2.5	30	100	0.1·10 ⁻³
Soman (GD)	0.4	10	30	0.03·10 ⁻³
VX	0.2	10	20	0.01·10 ⁻³
Sulfur mustard (HD)	25.0	100	1500	3.0·10 ⁻³

PD₅₀ – threshold dose: causing any noticeable effect in 50% of the exposed population.

ED₅₀ – effective dose: required for some effect in 50% of the exposed population.

ID₅₀ – incapacitating dose: amount incapacitating 50% of the exposed population.

LD₅₀ – median lethal dose: will kill 50% of the exposed population.

Intoxication with CWAs can occur percutaneously, by ingestion or by inhalation. The most likely exposure route is vapour inhalation (Table 3). Absorption of agents into the respiratory tract is very fast and efficient (80-90% of agents can be absorbed). Therefore, effective protection of the respiratory tract is the most important factor in defending against exposure.

Table 3. LD₅₀ values for various exposures

Exposure	LD ₅₀ [mg/kg]			
	Sarin	Soman	Tabun	VX
Intravenous	0.014	BD	0.014	0.0015
Percutaneously	24 - 28	18	14 - 21	0.086
Inhalation	0.05-0.10	0,7	0.15	0.005-0.015

3.2. Persistence

The persistence values given below in Table 4 are only approximate. CWA decomposition and evaporation rates depend on many factors: the lie of the land, the presence and type of plants, the ground and air temperatures, wind speed and direction, cloud cover, rain, snow, ground and air humidity, size of the agent's droplets, and the use of thickened agents. Therefore, in some cases the observed persistence can even be several times than the higher than the values shown in Table 4.

Table 4. Persistence of chemical agents

Agent	Summer	Winter
Tabun (GA)	1 h–24 h	2 h–3 d
Sarin (GB)	10 min–12 h	1 h–24 h
Soman (GD)	1 h–24 h	2 h–3 d
VX	2 d–1 week	2 d–weeks
Sulphur mustard (HD)	2 d–1 week.	weeks

3.3. Residual contamination

Decontamination is the process of making any person, object, or area safe by absorbing, destroying, neutralizing, rendering harmless, or removing chemical or biological agents, or removing radioactive material in or around the area. In practice, complete absorption, destruction, neutralisation, rendering harmless, or removal is impossible – the efficiency of the process will always be less than 100%, and so only reduction of contamination is possible. Remaining contamination is called residual contamination, the presence of which is unavoidable and must be considered when planning decontamination procedures and maintaining decontamination equipment. There are a few reasons for the formation of residual contamination, and understanding these can help operators to avoid mistakes during a decontamination process.

One very important reason for the presence of residual contaminations is the ability of chemical agents to be absorbed into contaminated materials. This occurs not only in obviously absorptive materials (e.g. concrete, wood, leather) but in polymers, and paints too. Elastomers, such as rubber and polymers with plasticizers, are especially susceptible.

Although decontaminants can remove free liquid remaining on a surface quite efficiently, they are not able to neutralize the absorbed agent, so part of the absorbed contamination will remain present after decontamination. Therefore, it is important to carry out decontamination as soon as possible. Subsequent desorption constitutes a continuing residual vapour hazard to personnel in the vicinity, in particular when ventilation is restricted. Besides this inhalation hazard, direct contact between bare skin and contaminated material is also highly dangerous, as chemical agents can be easily passed between them.

Another reason for residual contamination is the design of contaminated items – with the presence of gaps, cavities and joints, etc. Such features not only tend to hold contaminants and thus represent potential hazards to personnel, but are also difficult to clean adequately.

A third reason is improper application of decontaminants to contaminated surfaces. Omitting even a small fragment can result in residual contamination exceeding acceptable values. (Examples of acceptable values of residual contamination are given in Table 5 below).

Table 5. Acceptable values of residual contaminations

Agent	Acceptable residual contamination [mg/m ²]	
	Contamination on surface (not absorbed)	Reabsorption of agent vapours within 24 h
Sulfur mustard (HD)	50	420
Soman (GD)	0.01	19
VX	3	4.5

3.4. Decontaminants

The main requirements for a decontaminant are:

1. Rapid removal of dangerous substances from contaminated surfaces, either by detoxification of contaminants by chemical reaction, or the physical removal of the contaminants (with subsequent detoxification of harmful substances).
2. Ready to use (or with fast, easy preparation).
3. Versatility – can decontaminate various types of CWAs (especially blister and nerve agents), and TICs (Toxic Industrial Chemicals).
4. Non-toxic formula, with no further impact on the population and the ability to decontaminate large groups of people.

5. Low corrosiveness and high compatibility with decontaminated surfaces, equipment and infrastructure.
6. Decontamination of sensitive equipment.
7. Effective in a wide range of temperatures.
8. Usage of decontaminants on people must be ease, intuitive and possible without extensive training.
9. Long shelf-life of the means.
10. Environmentally safe – decontaminants should be environmentally benign and biodegradable.
11. Low risk of fire, explosion etc.
12. Low cost of usage.

Because of the difficulty of one decontaminant meeting all of these requirements, multiple different decontamination technologies have been created. As such, only selected decontaminants will be discussed here.

By applying the criteria of physical form and chemical composition, decontaminants can be categorised as follows:

- Liquid;
 - Water-based,
 - Organic,
- Solid decontaminants (powders);
- Gaseous decontaminants.

3.4.1. Use of water and aqueous solutions for decontamination purposes

The most accessible and cheapest medium is water, clean or with the addition of detergents. The main disadvantage of water is low reactivity towards CWA and poor solubility in water of some substances (e.g. sulfur mustard HD). The addition of surfactants improves the dispersal of sparingly soluble substances, increasing the efficiency of their removal. A beneficial effect on the effectiveness of removing contamination is also brushing the washed surface or using a high pressure water jet. An important improvement in the effectiveness of decontamination is also achieved by using a hot water jet under high pressure. Considering the use of water with the addition of detergents, it should be remembered that water removes contamination mainly physically, and the rate of chemical reaction in most cases is of no practical significance. Such treatments are accompanied by the formation of hazardous wastewater that may contain undestroyed CWA. There is also a risk of secondary contamination, especially when using a high pressure water jet (the ability to produce an aerosol containing toxic substances). The use of hot water increases the solubility of most substances, but also increases the evaporation of CWA, which can lead to the formation of an air CWA-contaminated cloud.

Table 6. Properties of decontamination means based on water and surfactants

Decontaminant	Water and water with detergents
<i>CWA decontamination:</i>	Physical removal of agent, moderately efficient. Poor removal of water insoluble substances (e.g. HD). Efficiency can be increased by addition of detergent and physical action, e.g. scrubbing. Low efficiency of concrete and other porous material decontamination. Formation of dangerous wastes containing chemical agent.
<i>BWA decontamination:</i>	Physical removal of agent. Formation of dangerous wastes containing biological agent.
<i>TIM decontamination:</i>	Mainly physical removal of contaminants.
<i>Application:</i>	<ul style="list-style-type: none"> – Building surfaces – Infrastructure – Non-sensitive equipment – <u>Humans, decontamination of whole body is possible.</u>
<i>Compatibility with materials:</i>	– Not compatible with sensitive equipment
<i>Safety to users:</i>	Safe
<i>Safety precautions:</i>	–
<i>Preparation to use:</i>	Detergent must be added to water before use or water can be used with concentrated solution of detergent in-line.
<i>Equipment:</i>	Standard equipment used for cleaning or used by firefighters.
<i>Training:</i>	Recommended.
<i>Environmental impact:</i>	Wastes containing agent or TIM can be harmful. Some detergents are dangerous to aquatic organisms.
<i>Shelf-life</i>	Limited by stability of detergents.
<i>Comments</i>	Useful at temperatures above 0°C.

Example of decontamination means based on water and surface-active means – SF-M

It is a decontamination mean for the preparation of deactivation solutions used for washing away radioactive pollutants from equipment and objects. The solutions are alkaline (pH 11). The solution consist of:

Table 7. cont.

Decontaminant	Hypochlorite
<i>BWA decontamination:</i>	Effective. A 6 log kill of <i>Bacillus subtilis</i> was achieved on hard, nonporous surface treated with sodium hypochlorite at pH 7 with a 60-minute contact time.
<i>TIM decontamination:</i>	Some materials are incompatible because of: <ul style="list-style-type: none"> – Release of chlorine or other dangerous gases – Formation of chlorinated organic compounds. – Formation of explosive compounds
<i>Application:</i>	2–5% solution: <ul style="list-style-type: none"> – Building surfaces – Terrain – Non-sensitive equipment Dilute hypochlorite solution (0,5%) can be used to decontamination of skin (not for mucous tissues).
<i>Compatibility with materials:</i>	<ul style="list-style-type: none"> – Highly corrosive – Oxidant – Incompatible with sensitive equipment
<i>Safety to users:</i>	<ul style="list-style-type: none"> – Destructive to tissues of the mucous membranes and upper respiratory tract – Toxic – Irritant – Liberates chlorine if mixed with acids
<i>Safety precautions:</i>	<ul style="list-style-type: none"> – Protective clothing, respiratory protection, gloves and eye/face protection should be used – Breathing of dust or aerosol must be avoided.
<i>Preparation to use:</i>	Dry substance must be dissolved in water – tank or other equipment is necessary
<i>Equipment:</i>	Standard sprayers can be used
<i>Training:</i>	Highly recommended
<i>Environmental impact:</i>	<ul style="list-style-type: none"> – Very toxic to aquatic organisms – Toxic wastes
<i>Shelf-life</i>	5 year (dry $\text{Ca}(\text{OCl})_2$ below 25°C, in darkness, properly sealed) Calcium hypochlorite rapidly decomposes on exposure to air. May decompose violently if exposed to heat or direct sunlight. Thermally unstable; decomposes at 177°C.
<i>Comments</i>	In temperatures below 0°C antifreeze additive must be used.

Currently, calcium hypochlorite is still used for decontamination, however, disinfectants containing hypochlorite release compounds are increasingly being used. Such compounds include dichloroisocyanuric acid.

Universal powder decontaminant (UOP)

The universal powder decontaminant UOP is used for decontamination of equipment, facilities and hardened surfaces contaminated with chemical warfare agents. It is intended for use in the form of solutions, aqueous suspensions, foamed emulsion, it can be sprayed with all available for decontamination spraying devices. Time to leave the agent on the contaminated surface is 30 min. The active ingredient of the disinfectant, i.e. the sodium salt of dichloroisocyanuric acid, also has decontamination properties, this compound is part of many decontamination means.

Universal powder decontaminant UOP consists of:

- 60% sodium salt of dichloroisocyanuric acid;
- 12% anhydrous sodium carbonate;
- 10% sodium tripolyphosphate;
- 17.8% rosulfulan LP;
- 0.2% cublene.

Although the decontaminant does not contain hypochlorite, but as a result of hydrolysis, dichloroisocyanuric acid is hydrolysed to release hypochlorous acid (Fig. 3).

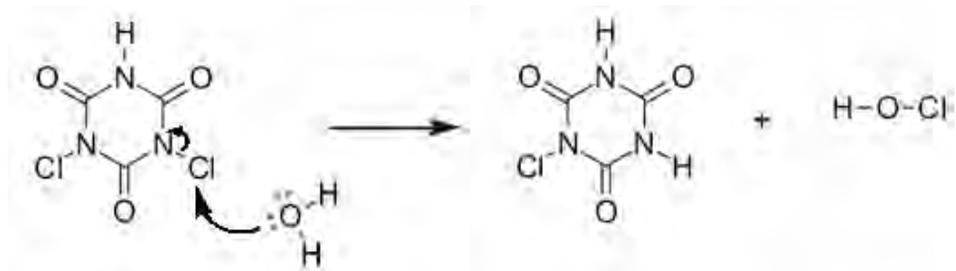


Figure 3. The reaction of hydrolysis of dichloroisocyanuric acid with the release of hypochlorous acid

For chemical decontamination purposes, UOP aqueous solutions are used with a concentration of 2% to 8% in the amount depending on the concentration of 0.6 dm³/m² do 2 dm³/m². Figure 4 shows the oxidation reaction of VX with the participation of dichloroisocyanuric acid.

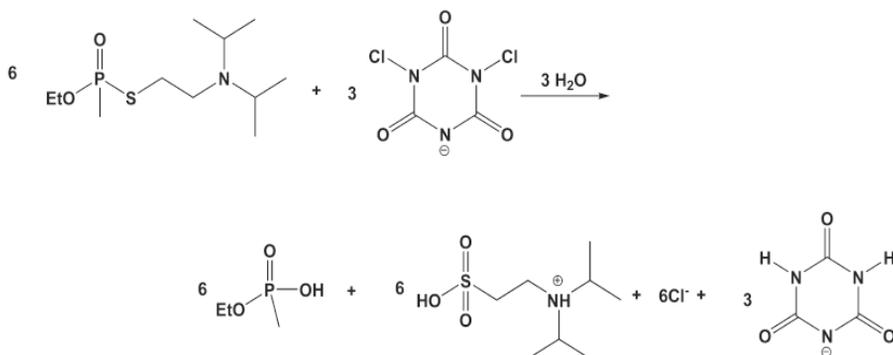


Figure 4. Oxidation of VX by dichloroisocyanuric acid

Standards for the use of UOP solutions for the decontamination purposes of weaponry and military equipment:

- solution of 2% – consumption of the solution ca. 2 dm³/m²;
- solution of 5% – consumption of the solution ca. 0.6 dm³/m²;
- solution of 8% – consumption of the solution ca. 0.6 dm³/m².

Solution for terrain/area decontamination:

- solution of 8% – consumption of the solution ca. 2 dm³/m².

3.4.3. Non-aqueous decontaminants

These decontaminants often contains alcoholates and aminoalcoholates.

Table 8. Properties of organic decontaminants

Decontaminant	Non-aqueous decontaminants
CWA decontamination:	Fast chemical reaction with nerve and blister agents (GA, GB, GD, VX, HD). Due to ability to dissolving of paints, greases etc. organic decontaminants can to decontaminate agents absorbed in such materials.
BWA decontamination:	?
TIM decontamination:	Not recommended.
Application:	Non-sensitive equipment.
Compatibility with materials:	- Corrosive - Damage of paints - Damage of plastics

Decontaminant	Non-aqueous decontaminants
<i>Safety to users:</i>	<ul style="list-style-type: none"> – Toxic – Caustic – Irritant – Flammable
<i>Safety precautions:</i>	<ul style="list-style-type: none"> – Protective clothing, respiratory protection, gloves and eye/face protection should be used. – Inhalation of vapours or aerosol must be avoided.
<i>Preparation to use:</i>	Ready to use
<i>Equipment:</i>	Typical sprayers can be used (high resistance to organic solvents is necessary).
<i>Training:</i>	Recommended
<i>Environmental impact:</i>	<ul style="list-style-type: none"> – Toxic wastes – Slow biodegradation
<i>Shelf-life</i>	10 year

Organic decontaminant ORO

It is designed for decontamination of weaponry and military equipment with the use of decontamination sets ZO-1, ZO-2, ZO-E, ZOd-2 i w Battalion Decontamination Set (BZLS). The solution is characterized by a weak smell of ammonia and hygroscopicity. It is a colourless or light yellow liquid that strongly irritates skin and eyes. It destroys most paint coatings. The ORO decontaminant should be applied to contaminated surfaces with density of 200 cm³/m², minimum time of leaving the solution on the contaminated surface is 30 min. The advantage of the disinfectant is the wide range of its application temperatures: od –20°C do 50°C.

ORO decontaminant consists of:

- 25% monoethanolamine;
- 45% diethylenetriamine;
- 28% ethyl alcohol;
- 2% sodium.

The active ingredients of the disinfectant are sodium ethoxide and sodium aminoethoxide.

Organic decontaminant R-18

This decontaminant is a colourless or light yellow liquid with an ammonia odour. It is part of the IPLS-1 individual anti-chemical package. The purpose of this decontaminant is to decontaminate metal elements of weapons and equipment.

The R-18 decontaminant consist of:

- 42.7% anhydrous ethyl alcohol;
- 40% dimethyl sulfoxide;
- 15.0% monoethanolamine;
- 2.3% metallic sodium (in form of sodium ethylate or ethylamine).

The R-18 decontaminant should be applied to contaminated surfaces with density of 250 cm³/m².

3.4.4. Sorbents

The great advantage of sorbents as disinfectants is their non-aggressiveness in relation to disinfected surfaces. This allows the use of sorbents for the decontamination of the skin and sensitive equipment. A major drawback is the lack of CWA decomposition, which means that the used sorbent may be a source of contamination. Therefore, when using sorbents, it is necessary not only to thoroughly cover the entire surface with the sorbent, but also to carefully remove the contaminated sorbent from the disinfected surface.

Table 9. Properties of decontaminants based on sorbents

Decontaminant	Sorbents
<i>CWA decontamination:</i>	Physical removal (absorption) of liquid agent. Can be applied to all liquid agents.
<i>BWA decontamination:</i>	-
<i>TIM decontamination:</i>	Physical, non-selective removal (absorption) of liquids.
<i>Application:</i>	<ul style="list-style-type: none"> – Building surfaces – Infrastructure – Sensitive equipment – Skin
<i>Compatibility with materials:</i>	Low reactivity Non-corrosive
<i>Safety to users:</i>	<ul style="list-style-type: none"> – Irritant if inhaled or applied on mucous membranes. – Used sorbent (with absorbed agent) must be considered as dangerous material. Vapours of agent can be emitted.
<i>Safety precautions:</i>	<ul style="list-style-type: none"> – Respiratory protection, gloves and eye/face protection should be used – Breathing of dust should be avoided.
<i>Preparation to use:</i>	Ready to use
<i>Equipment:</i>	Manual dispenser for small areas can be used.

Decontaminant	Sorbents
<i>Training:</i>	Recommended
<i>Environmental impact:</i>	Relatively small. Wastes of sorbent containing contaminant can be collected and disposed.
<i>Shelf-life</i>	In many cases not limited.
<i>Remarks</i>	N/A

Sorbent used in IPLS-1 decontamination set

This decontaminant is intended for the decontamination of exposed skin surfaces and individual equipment. It consists of:

- 82% magnesium oxide;
- 18% arsil (colloidal silicic acid).

3.4.5. Nanostructural sorbents

Decontaminants based on nanostructural sorbents make significant progress in sorbent decontamination technology. The technology of producing sorbents with high porosity and simultaneously with strictly defined and repeatable parameters of these pores (such as their size and shape) allowed to obtain a decon with parameters much higher than the previously used decontaminants based on sorbents. The new disinfectors are characterized by good wettability by CWA and rapid absorption of the removed substances inside the grains. As a result, the surface of the sorbent grains with the absorbed toxic agent is not covered with a liquid that can cause the contaminated sorbent grains to stick to the surface to be decontaminated. As a result, the removal of the contaminated sorbent is easier and more efficient. In addition, these properties of nanostructured sorbents reduce the release of CWA vapours from contaminated sorbents.

Table 10. Properties of decontaminants based on nanostructured sorbents

Decontaminant	Nanostructured sorbents
<i>CWA decontamination:</i>	Improved effectiveness of physical removal (sorption) of liquid agent. Nanostructured sorbent can be applied to all liquid agents. Fast decomposition of absorbed agents is possible.
<i>BWA decontamination:</i>	–
<i>TIM decontamination:</i>	Physical, non-selective removal (absorption) of all liquids.

Table 10. cont.

Decontaminant	Nanostructured sorbents
<i>Application:</i>	<ul style="list-style-type: none"> – Building surfaces – Infrastructure – Sensitive equipment – Skin (almost whole body can be decontaminated)
<i>Compatibility with materials:</i>	Low reactivity Non-corrosive
<i>Safety to users:</i>	<ul style="list-style-type: none"> – Irritant if inhaled or applied on mucous membranes. – Immediately after use, sorbent (with absorbed agent) must be considered as dangerous material, subsequently fast self-decontamination of sorbent occurs. – Low pressure of vapours over nanostructural sorbents results in small emission of toxic substances from contaminated sorbent.
<i>Safety precautions:</i>	<ul style="list-style-type: none"> – Respiratory protection, gloves and eye protection should be used – Breathing of dust should be avoided.
<i>Preparation to use:</i>	Ready to use
<i>Equipment:</i>	Manual dispenser for small areas can be used.
<i>Training:</i>	Recommended
<i>Environmental impact:</i>	Small. Wastes of sorbent containing contaminant can be collected and disposed.
<i>Shelf-life</i>	Not determined (new product). Predicted time over 10 year.
<i>Comments</i>	N/A

Sorbent AC10K type

AC10K type sorbent is synthetic sorbent based on aluminium-magnesium hydroxalcite. It is characterized by a proper surface ca. 170 m²/g.

3.5. Decontamination sets

3.5.1. ZO-1 decontamination set

Decontamination set ZO-1 is designed to carry out decontamination process of outer surfaces of vehicles and other equipment by mean of ORO decontaminant.

Minimal density of decontaminant ensuring effective decontamination process is $200 \text{ cm}^3/\text{m}^2$. Amount of decontaminant contained in ZO-1 set (4 dm^3) ensures decontamination of 20 m^2 of external surface (e.g. an off-road vehicle, or a howitzer). The minimum application density of the mean for effective decontamination is $200 \text{ cm}^3/\text{m}^2$.

Technical and tactical data of ZO-1

Set weight:

- unfilled 10–11 kg
- filled with decontamination mean 14–15 kg

Device weight:

- unfilled 1.5 kg
- filled with decontamination mean 3.5 kg

Decontamination mean filling unit 4 dm^3

Working pressure in the container 0.35–0.4 MPa;

Working pressure workout – by hand pump as an integral part of decontamination device.

Decontamination device working volume 2 dm^3

Thorough decontamination time period of the surface up to 20 m^2 up to 20 min.

Working temperature range -20°C – 50°C .

Storage temperature range 0°C – 20°C .

Composition of a ZO-1 set

Inside the interior of metal box (Fig. 5) there are placed: 2 dm^3 decontaminant containing container, 2 dm^3 spray container, rope set with handle, telescope lance with flat nozzle, spare parts and user manual of the device.



Figure 5. Interior view of a ZO-1 set (author's photo)

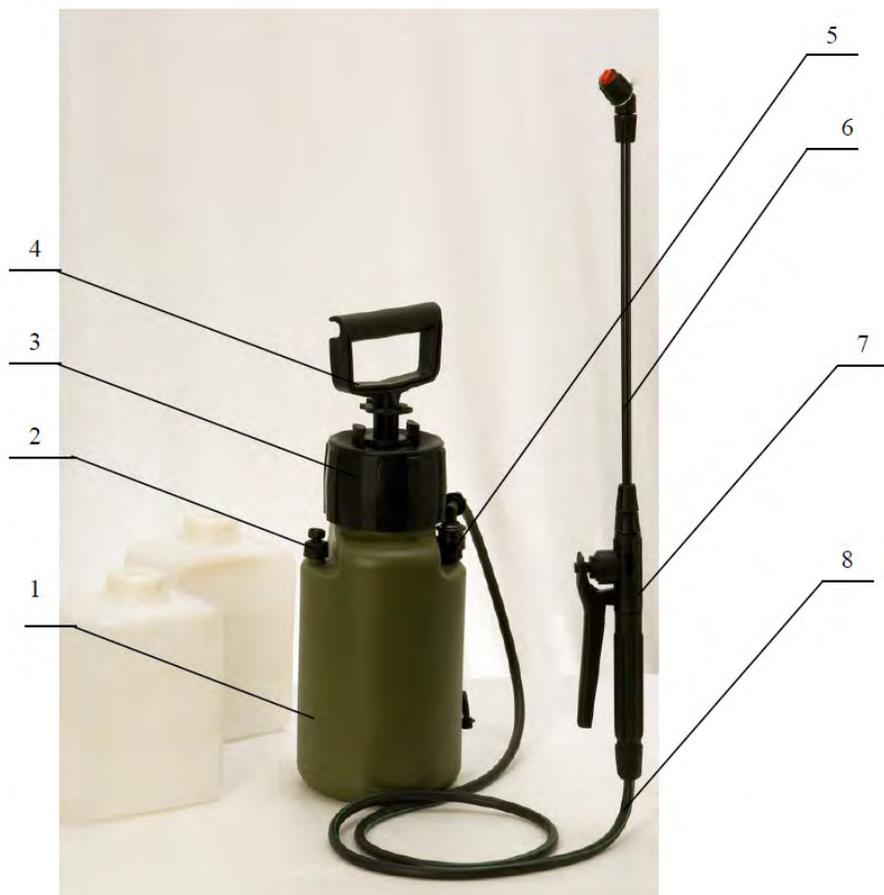


Figure 6. Spraying device for application of decontaminants as part of a ZO-1 set, a) 2 dm³ container (1) equipped with head (3), quick coupler (5), safety valve (2), threaded cap, b) hose set (8) with handle (7), c) telescope lance with flat nozzle (6), d) manual pump (4)

Decontamination set spray uses energy of compressed air by using hand pump placed in nozzle head. At the time of pressing the handle lever of shut-off valve pressurized air poses decontamination mean through flat spraying nozzle.

Preparation of the set to work

Preparation of fully completed set to work (i.e. filled with decontamination mean) lies on unscrewing the yellow cap and placing hand pump, placing quick fastener hose in the socket on the container body and pumping the container the air till the pressure reaches the safety valve cut-off.

Use of the set

After the preparatory procedure is done according to above mentioned one can start decontamination procedure by spraying decontamination solution on contaminated surface. Lance end ended by nozzle should be in distance ~ 10 cm of contaminated surface. One should be careful to ensure the density of the decontamination mean on the surface ~ 200 cm³/m².

3.5.2. ZO-2 decontamination set

Design and composition of ZO-2 set is analogue to ZO-1 set. Main differences are: bigger volume of decontamination solution (8 dm³) and resulting raised weight and surface able to be decontaminated (40 m²).

Differences in the technical and tactical data of a ZO-2 set compared to a ZO-1

Set weight:

- unfilled 12–13 kg
- filled with decontamination means 20–21 kg
- Decontamination mean filling unit 8 dm³

Thorough decontamination time period of the surface up to 40 m² up to 30 min.

Rules of operation, preparatory action and handling of ZO-2 set are analogous to described above relating to ZO-1 set.

3.5.3. ZO-E decontamination set

Decontamination set ZO-E is designed to carry out decontamination process of parts of outer surfaces of vehicles and other equipment by mean of ORO decontaminant. The set is designed to be mounted in the compartment of the vehicle, when ZO-2 set is mounted outside the compartment of the vehicle. Total volume of 1 dm³ of the decontamination mean enables to carry out safe reaching and decontamination of the outer surfaces. Volume of decontamination mean enable decontamination of the surface up to 5 m².

Technical and tactical data of ZO-E

Set weight:

- unfilled 5,4 kg
- filled with decontamination mean 7 kg

Device weight:

- unfilled 0.4–0.5 kg
- filled with decontamination mean 1.5–1.6 kg

Decontamination mean filling unit	1 dm ³
Working pressure in the container	0.35–0.4MPa;
Working pressure workout – by hand pump as an integral part of decontamination device.	
Decontamination device working volume	1 dm ³
Efficiency	0.6 dm ³ /min.
Working temperature range	–20°C–50°C.
Storage temperature range	0°C–20°C.

Composition of a ZO-E set

Spraying device is composed of container equipped with spraying head integrated with safety valve. The container has drum shape finished with threaded inlet on which the head is being screwed. The safety valve ensures exceeding overpressure in the container, by opening when being reached or exceeded.



Figure 8. General view of a ZO-E set (author's photo)

Preparation of the set to work

The device divided into two parts is stored in metal box. The user runs preliminary preparation to carry out decontamination process by taking out the device from the box, unscrewing the container cap, filling with decontamination solution and screwing the spraying head integrated with safety valve. After reaching this form, the device is being transported in crew compartment. Before use one should pump the container with the air to reach overpressure to the level of safety valve cut-off.

Usage

Carrying out the decontamination by using ZO-E set is done by placing decontamination solution on the parts of the surfaces of the vehicle use as evacuation route out of the vehicle and reach ZO-2 set. Then the surfaces of the ZO-2 set is being decontaminated by ZO-E set. During decontamination process lance end ended by nozzle should be in distance ~ 10 cm of contaminated surface.

One should be careful to ensure the density of the decontamination mean on the Surface ~ 200 cm³/m².

3.5.4. PZLS-1 decontamination set

PZLS-1 is designed for decontamination of surfaces of vehicles, armament and equipment. Universal decontaminant UOP and organic decontaminant ORO are used in PZLS-1 set. Elements of set are placed in 13 cases fitted for transport on EUR-pallets. The height of palletized load is 120 cm. PZLS-1 can be transported by any truck. 16 portable decontamination devices is placed in cases numbered 1–4. Each one device contains 8 dm³ of organic decontaminant ORO. Amount of decontaminant in such device is sufficient for decontamination of 40 m² – approximate mean external area of tank. In cases 5 and 6, reserve of decontaminant is placed (120 dm³ in 2 dm³ containers).

Case No 7 contains 16 sets of brushes, spray lances and lengthening pipes for 16 portable decontamination devices placed in cases 1–4. In cases 8–13 is located equipment for decontamination with aqueous solutions of universal decontaminant UOP. Case No 8 contains 2 containers 400 dm³ each and one 1500 dm³ made from rubberized fabric. These containers are used to preparing of UOP decontamination solutions.



Figure 9. General view of cases Nos. 1–4 (author's photo)



Figure 10. General view of the interior of cases Nos. 5 and 6 (author's photo)



Figure 11. General view of the interior of case No. 7 (author's photo)



Figure 12. General view of the interior of case No. 8 (author's photo)



Figure 13. General view of the interior of case No. 10 (author's photo)



Figure 14. General view of the interior of case No 10 (inside lid) (author's photo)

In case No 9 is placed gasoline engine floating pump, canister for gasoline, hose water and packages with surfactant for preparation of solution for deactivation (for removing of radioactive contamination). In case No 10 is located gasoline engine pump, canister for gasoline, hose water and connectors.

On the internal side of lid are mounted simple and triple nozzles, spray lances and lengthening pipes for application of aquatic solutions of UOP onto contaminated surfaces. 60 kg of UOP in total, is placed in cases No 11 and 12. Amount of decontaminant is enough for decontamination of all vehicles in one battalion (average). Decontamination process can be carried out with using UOP solution of 8, 5 or 2%, depending on the tactical conditions and availability of water. Case No 13 contains connectors, hoses, wrenches, flat-top ladders and 4000 dm³ tank made from rubberized fabric.



Figure 15. General view of the interior of case No. 10 (author's photo)



Figure 16. General view of the interior of cases Nos. 11 and 12 (author's photo)



Figure 17. General view of the interior of case No. 13 (author's photo)

PZLS technical and tactical capabilities:

- carry out decontamination process on the surfaces of armoured vehicles and other vehicles composing one team/sub-unit by using decontamination devices containing of 8 dm³ decontamination solution each. Set contains 16 devices;
- refilling of organic decontamination solution in individual decontamination sets for one team/sub-unit. PZLS set contains 120 dm³ of organic decontamination solution ORO-type placed in 60 2-liter containers;
- carrying out decontamination process with using water solution of UOP decontamination mean. The amount of the UOP in PZLS enables to prepare water-based decontamination solution to carry out decontamination of the surfaces of all vehicles in unit of battalion level. Decon process can be done according to availability of water and tactical situation in two ways. First is washing of the equipment with water and then placing decontamination mean with using foaming nozzles. Second technology is based on decontamination process with using brushes;
- carrying out contamination liquidation process by washing of vehicle surfaces with water containing SF-M additives raising effectiveness of the process;
- carry out disinfection process with using UOP solution.



Figure 18. PZLS equipment enabling to prepare water solution of UOP (author's photo)



Figure 19. View of the equipment enabling preparation of organic decontamination solution (author's photo)

3.5.5. Decontamination truck IRS-2C

Decontamination truck IRS-2C is designed for decontamination of:

- vehicles, armament, equipment etc.;
- infrastructure and durable surfaces contaminated by chemical substances or radioactive materials;
- decontamination of personnel (source of warm water).



Figure 20. Decontamination truck IRS-2C, on the left SANIJET C921D washing system (author's photo)

Technical and tactical data

Decontamination truck IRS-2C enables decontamination with the use of cold and hot water with addition of decontaminants under pressure up to 9 MPa. IRS-2C is equipped with 2000 dm³ stirred tank allowing preparation 2% solution of UOP by dissolution of 4 portion packages (10 kg each) of UOP in water. About 2 dm³ of 2% UOP decontamination solution per 1 m² is used.

IRS-2C is equipped with high-pressure decontamination system SANIJET C921D allowing:

- decontamination with the use of cold and hot water (up to 9 MPa);
- decontamination with the use of steam (150–180°C);
- possibility of continuous heating of water (1000 dm³/h);
- possibility of flushing contaminated surfaces or application of decontaminant (as foam) with the same spray lance. Decontamination solution is prepared on line by sucking of powdered decontaminant from container to stream of water.

Decontamination solution is generated dynamically when applying on contaminated surface by sucking powder decontamination mean from container and introducing into water stream. Efficiency of the decontamination generation process is tuned by lance operator.



Figure 21. General view of the process of mass decontamination of an airport using IRS-2C (author's photo)



Figure 22. Decontamination process of an aircraft by SANIJET high-pressure washer as part of IRS-2C (author's photo)

3.5.6. Sorbent-based portable decontamination systems

Sorbents are often used as universal decontaminants. Main advantages of sorbents are:

- high efficiency;
- non-reactivity;
- non-toxicity;
- non-flammability (mineral sorbents);

- non-corrosive;
- readiness to use.

Important: they need precise application for effective decontamination of vertical surfaces.

Usually application of sorbents on such surfaces is complicated, moreover, sorbents often are weakly retained on sloping surfaces. To avoid this – below described TRIBO and CORONA devices are used for application of sorbent on contaminated surfaces. Stream of air with negatively charged sorbent particles is used for decontamination. Charged particles are attracted to surfaces undergoing decontamination and stable layer of sorbent on all surfaces is formed. Contaminated sorbent can be easily removed from decontaminated surfaces (e.g. with vacuum cleaner) and collected as waste.

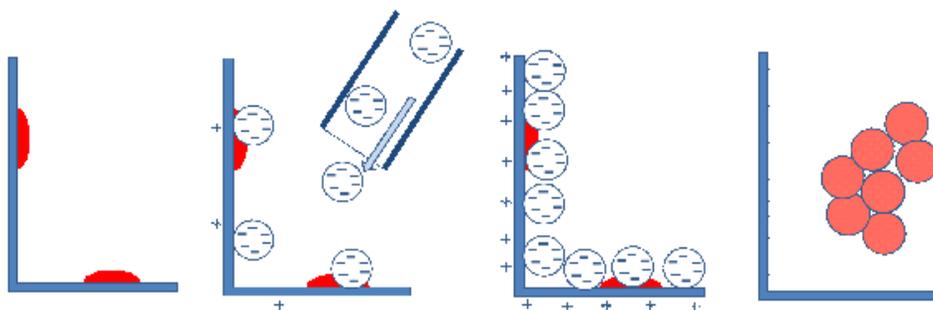


Figure 23. Principle of spraying of sorbents on contaminated surfaces:

- Contaminated surfaces
- Stream of air with negatively charged sorbent particles is directed from nozzle to contaminated surface. Charged sorbent particles are electrostatically attracted to surface and retains on contaminated surfaces.
- Surface covered in sorbent.
- Removing of contaminated sorbent from decontaminated surfaces (vacuum cleaner can be used)

Back-pack sorbent-based portable decontamination system

Technical data:

- Power supply: 12 V;
- Weight: 9.2 kg;
- Load of nano-sorbent container – 400 g;
- Continuous run time (to empty the sorbent container) 20 min.;
- Ability to carry out decontamination process on the surface of up to 10 m².



Figure 24. Back-pack device for application of sorbent based decontaminants (author's photo)

Mobile sorbent-based portable decontamination system



Figure 25. Mobile sorbent based decontamination system – general view (author's photo)

Technical data:

- Power supply: 230 V;
- Weight: 63 kg;
- Load of nano-sorbent container – 2.5 kg;
- Continuous run time (to empty the sorbent container) 30 min.;
- Ability to carry out decontamination process on the surface of up to 50 m².

3.5.7. Mobile decontamination system with using vaporized H₂O₂

Mobile decontamination system using H₂O₂ is designed to carry out chemical decontamination process. Especially it is designed to carry out decontamination process of sensitive equipment (e.g. electronic), cloths, etc. System is placed in container.

Container is divided into two parts: decontamination compartment (Fig. 27) and technical compartment containing installation for generating vaporized H₂O₂ stream and enabling continuous supply and circulation of the decon stream (Fig. 28).



Figure 26. General view of the Mobile decontamination system using H₂O₂ (author's photo)



Figure 27. General view of the decontamination compartment (author's photo)



Figure 28. General view of the technical compartment (author's photo)

3.5.8. Individual decontamination set IPLS-1

Individual decontamination set IPLS-1 is designed for:

- prophylactic security and carry out rapid decontamination uncovered skin surfaces (face, hands, neck) against chemical warfare agents;
- carry out rapid decontamination of surfaces of personal weapons and equipment.



Figure 29. General view of Individual decontamination set IPLS-1 (author's photo)

IPLS-1 set consist of:

- powder decontaminant placed in polyethylene sealed bag;
- glove for organic powder application;
- prophylactic-decontamination cream;
- organic decontamination spray;
- Viscose cloths – 2 pcs.

Tactical and technical data of IPLS-1 set

1) powder decontaminant

Basic container: sealed polyethylene bag. The bag is wrapped by glove designed for application of the decontaminant and the whole is placed in outer polyethylene bag.

Product features:

- homogenized mixture of chemical substances consisting of 82 % weight of magnesium oxide and 18% weight of arsil;
- mixture grade – 0.18 do 0.20 mm;
- decontaminant weight – 100 g;
- decontamination ability – after 30 min. of decontaminant exposure of 400 g/m², on glass surface contaminated with HD 5 g/m², residual contamination of HD < 5·10⁻² g/m².

1) glove for organic powder application

Basic packaging of the glove is sealed polyethylene bag. It is wrapping for powder decontaminant (see above).

Product features:

- outer part of the glove is made of cloth;
- inner part of the glove is made of fibre cloth.

1) prophylactic-decontamination cream

Basic packaging of the prophylactic-decontamination cream is polyamide tubing laminated with polyethylene.

Product features:

- homogenized mixture of chemical substances consisting of 35.6÷37.8 g sodium persulfate, 13.3 g Magnesium stearate, 3.3 g urea i 46.7 g methyl silicone oil 1000;
- mixture grade of dispersed substance (sodium persulfate) < 80 µm;
- cream weight: 85÷95 g;
- protection ability – after 60 min. of decontaminant exposure of 400 g/m², on glass surface contaminated with HD 5 g/m², protection time > 15 min. Residual contamination of HD < 420 mg/m².

1) organic decontamination spray

Basic packaging of the organic decontamination spray is sealed polyethylene bag.

Product features:

- Transparent solution with straw colour and ammonia smell resulting from solution 2.3 weight part metallic sodium in 15.0 weight part of monoethanolamine and 42.7 weight part of anhydrous ethyl alcohol and 40,0 weight part of dimethyl sulfoxide;

- Decontaminant vol. – 200 cm³;

- Decontamination efficiency of steel surfaces covered with epoxy paint ensures residual contamination (c_r) not exceeding:

- $c_r = 0.42 \text{ g/m}^2$ for HD, at initial contamination density $c_0 = 5 \text{ g/m}^2$;
- $c_r = 1.9 \cdot 10^{-2} \text{ g/m}^2$ for GB at initial contamination $c_0 = 5 \text{ g/m}^2$. Vapour concentration of GB after decontamination process not exceed $8 \cdot 10^{-7} \text{ mg/dm}^3$;
- $c_r = 4.5 \cdot 10^{-3} \text{ g/m}^2$ for VX, at initial contamination density $c_0 = 1 \text{ g/m}^2$.

1) viscose clothes

Total composition of IPLS-1 set:

- powder decontaminant:
 - 82% magnesium oxide;
 - 18% arsil (colloidal silicic acid).
- prophylactic-decontamination cream:
 - 46.7% methyl silicone oil (OM-1000);
 - 36.7% sodium persulfate;
 - 13.3% Magnesium stearate;
 - 3.3% urea.
- organic decontamination spray R-18:
 - 42.7% anhydrous ethyl alcohol;
 - 40.0% dimethyl sulfoxide;
 - 15.0% monoethanolamine;
 - 2.3% metallic sodium (in the form of sodium ethylate or ethylamine).

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MODULE V

RADIATION AND NUCLEAR DEFENCE

1. RADIATION AND DEFENSE AGAINST NUCLEAR WEAPONS

Radiation is the sending and transfer of energy at a distance. Energy can be sent in the form of heat, light (as electromagnetic waves or molecules). In the general sense, we are called a radiation electromagnetic waves (EM) propagated in an appropriate medium (vacuum, the earth's atmosphere, water, other example. Soil). In addition to electromagnetic waves, there are other waves, eg acoustic or gravitational waves which in colloquial speech we do not call radiation.

In our environment there are many different sources of radiation in the form of electromagnetic waves – both as sources of natural and artificial.

A typical example of such waves are radio waves and light. Radio waves are of natural origin (star) and artificial (radio and television signals produced by the human radar, mobile telephony, etc.). Also, light can be of natural origin (starlight, some living organisms, fire) and artificial (light sources created by man – lamps, radiators). A particular type of radiation is **ionizing radiation**. They were called because it induced in electrically neutral atoms and molecules of charge change, i.e. ionization.

Man is not equipped by nature with any sense that allows him to feel the presence of ionizing radiation as, for example, our eyes detect and react to light. Therefore, to be able to detect the presence of radiation was necessary to build devices that can we “help” visualize his presence.

Ionizing radiation comes from space, rocks, air and human activity. Ionizing radiation, both natural and artificial, is a part of our environment and is present everywhere. This part of the ionizing radiation that man has created is used in industry, medicine and energy. Therefore, you should be aware of its presence and know the basic principles that apply to its use, taking into account the potential dangers it may bring.

1.1. Properties of radiation

Each electromagnetic wave has three parameters associated with each other, which characterize it unequivocally. These are:

- Length – marks as a Greek letter lambda [λ];
- Frequency – marks as a Greek letter ni [ν];
- Energy – marks as a Latin letter E.

Between these values there is a relationship:

- $E = h \nu$ – where “ h ” is Planck’s constant, or
- $E = h c / \lambda$ – where “ c ” is the speed of light in a vacuum.

The illustration below shows a graphical relationship between these values.

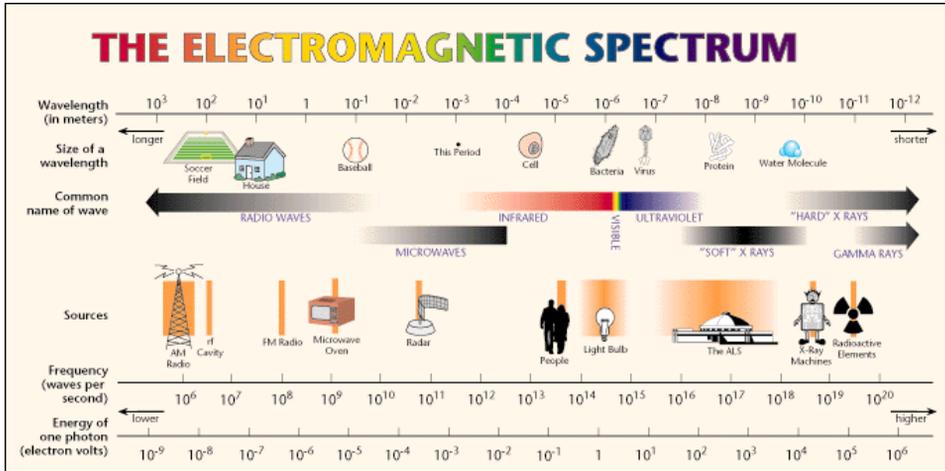


Figure 1. The spectrum of electromagnetic waves (*figure used with permission under Creative Commons license*)

As can be seen from the graph above, there is a fairly strict division into non-ionizing and ionizing radiation. In terms of lower energy, it begins from the ultraviolet with increasing energy is becoming more and more dangerous.

Used to scale the energy unit “electron volt” (termed “eV”) energy is one which is replaced by an electron accelerated in the electric field the potential difference of 1 V

1 eV = 1.6×10^{-19} J, which is a unit of very small. In practice, we use a larger unit, ie. keV and MeV. From the point of view of the SI measurement system, the electronvolt is a system-non-system unit, but allowed for use in nuclear technology.

1.2. Types of radiation (α , β , X-rays, neutrons)

To talk about radiation and radioactivity, the theory of the atomic structure should be brought closer to the listeners. Of course, in the form of a lightweight so that you can later was part of the lecture to refer to the definitions and phenomena listed here.

We will use here the so-called classical model of the atom. Rutherford’s model. Ernst Rutherford with Henri Becquerel and Marie Curie form part of the groups of researchers pioneers in the field of research into the structure and

atom and radioactivity phenomenon at the turn of the century. Our compatriot Maria Skłodowska-Curie was the first victim of radioactivity, because she died as a result of radiation sickness.



Figure 2. Maria Skłodowska-Curie (*figure used with permission under Creative Commons license*)

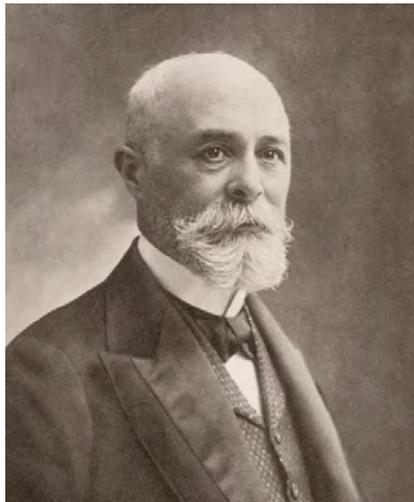


Figure 3. Henri Becquerel (*figure used with permission under Creative Commons license*)



Figure 4. Ernst Rutherford (*figure used with permission under Creative Commons license*)

The model of the Rutherford atom assumed the existence of an atomic nucleus revolve around which electrons elliptical orbits. This model is now a good basis for the lecture on 'atomistics in a nutshell'.

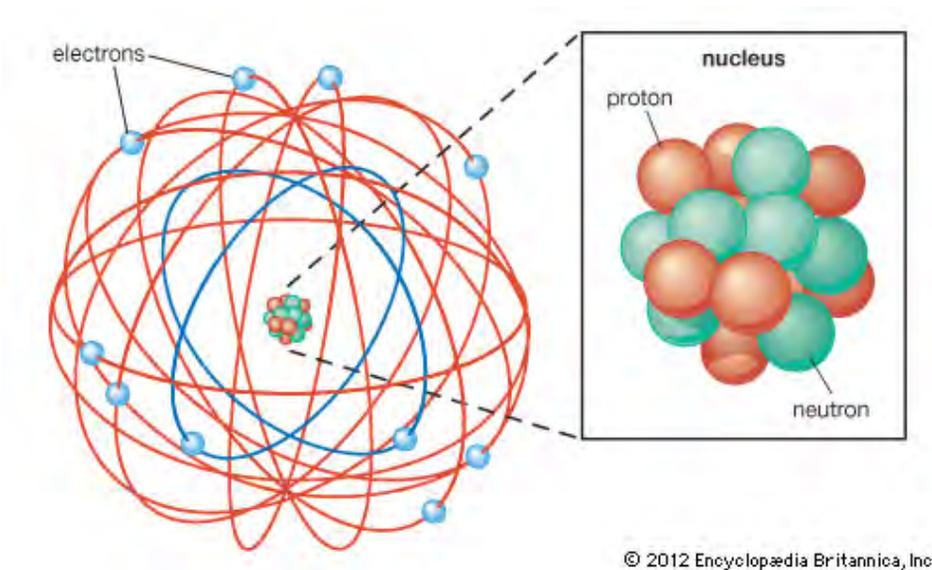


Figure 5. Rutherford atom model. On the left a positive nucleus surrounded by clouds of negative electrons circulating on elliptical orbits. On the right larger core composed of positive protons and electrically neutral neutron (*figure used with permission under Creative Commons license*)

Weight of proton and a neutron in they are approximately equal to each other while the mass of the electron is smaller than the mass of the proton and ne at the throne 1840 times. If you compare the diameter of the nucleus and the diameter of the atom, it turns out that the atom in the middle is “empty”.

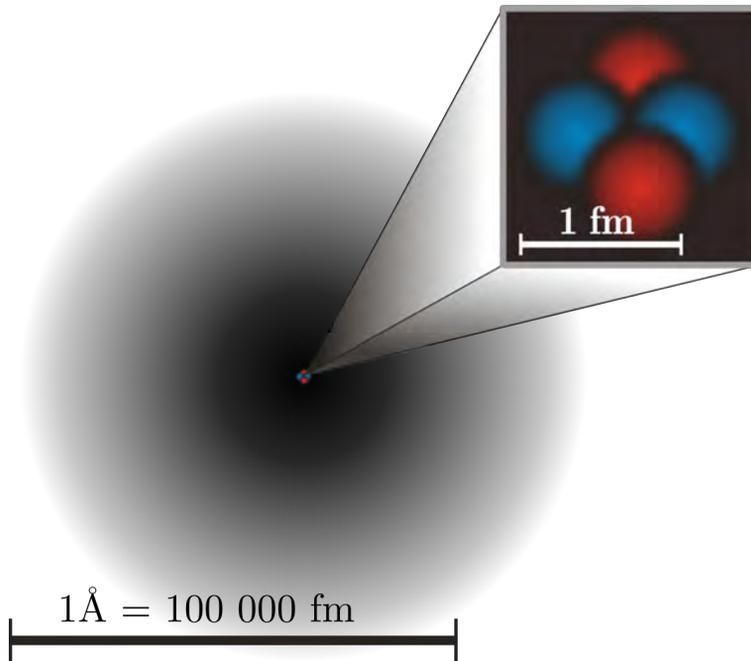


Figure 6. Schematic comparison of the size of the helium atomic nucleus (1fm) and the diameter of the helium atom ($1\text{\AA} = 100,000 \text{ fm}$) (figure used with permission under Creative Commons license)

The electric charge of the proton and electron is the same in magnitude, with the proton having a positive charge and the negative electron. From the standpoint of physics atom of any element by its nature it is electrically neutral.

A cloud of electrons orbiting the nucleus is called the electron shell. If this coating by an external factor is added to or included as one or more electrons to **an atom** receive **ion** with the proviso that if electrons are added to the shell to receive **a negative ion**, and if included is receive **positive ion**.

Each atom is distinguished by the number of protons in the nucleus. Hydrogen has a nucleus of one proton, helium 2 protons, lithium 3 protons and uranium 92 protons.

There are elements of so-called uranium other than uranium. transuranic (prepared artificially, the current has reached the atomic number 118). In addition to protons in the nucleus they are also neutral molecules called neutrons.

Common name of **neutrons** and **protons** in the nucleus of a **nucleons**. The nature of element tells us the number of protons in the nucleus marked with the letter “Z” – this is the **atomic number** in the periodic table (the Periodic Table of the Elements – PTOE). A second parameter characterizing atom is its **atomic mass** representing the number of nucleons in the nucleus marked with letter A. The overall record of the atom of the element:

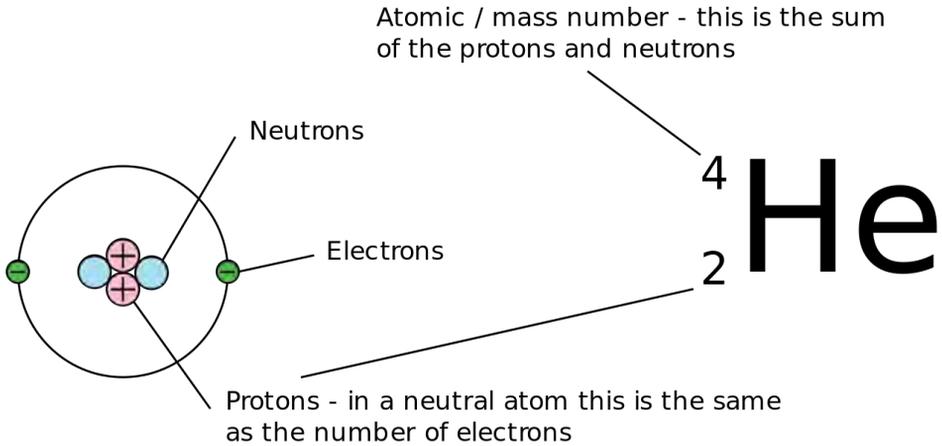


Figure 7. Explanation of mass number and atomic number (figure used with permission under Creative Commons license)

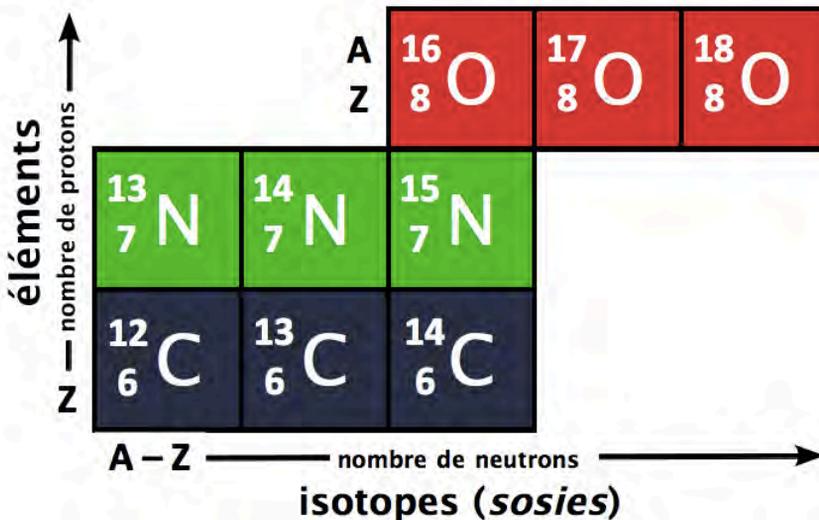


Figure 8. Isotopes of oxygen, nitrogen and carbon (figure used with permission under Creative Commons license)

As indicated, the atoms of a given element must have the same number of protons, but it often happens that they differ in the number of neutrons. Because the PTOE such atoms will be classified in the same place it was called **isotopes** (from the Greek “isos topos” – the same place.) Another name for this **isotope nuclide**. There is also a concept **isobar** ie. Two atoms of different elements having the same mass number.

The simplest example is the hydrogen isotopes:

- Prot – 1 proton in the nucleus;
- Deuterium – 1 proton and 1 neutron in the nucleus;
- Tritium – 1 proton and 2 neutrons in the nucleus.

Prot and deuterium are **stable isotopes** and tritium is a **radioactive isotope**.

PERIODIC TABLE OF THE ELEMENTS

The periodic table is organized into groups (I to 18) and periods (1 to 7). It includes the Lanthanide and Actinide series at the bottom. A key to the chart explains the following:

- Key to Chart:**
 - Atomic Number: 50
 - Symbol: Sn
 - Atomic Weight: 118.710
 - Oxidation States: +2, +4
 - Electron Configuration: 1s-18-4
- Element States:**
 - Metals: Yellow background
 - Non-metallic Solids: Light blue background
 - Liquids: Green background
 - Gases: Pink background

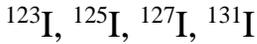
Figure 9. Periodic table of elements (figure used with permission under Creative Commons license)

From the physical point of view, ionization can be caused by particles or electromagnetic waves. The molecular radiation are also talking about the radiation of corpuscular. The particles that cause ionization can be divided into ionizing **directly** or **indirectly**.

In area electromagnetic waves dealing with **X-rays** and **gamma rays** as **ionizing radiation**. In the area of particle radiation, we deal with alpha, beta, neutron and other radiation, e.g. proton or deuteron streams.

ISOTOPES

Atoms having same atomic number but different mass numbers.

**ISOBARS**

Atoms having same number of nucleons but differ in number of protons i.e. have same mass number and different atomic number.

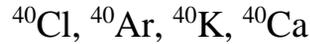


Figure 10. Isotopes vs Isobars

Of alpha, beta, protons or deuterons and other heavier particles are **ionizing** particles **directly**. This means that the neutral electrically atoms and molecules are ionized as a result of electrostatic interactions. From the point of view of physics:

- Alpha particles are helium nuclei;
- Beta particles are simply a stream of electrons;
- Protons are hydrogen nuclei;
- Deuterons are deuterium nuclei (1 proton and 1 neutron).

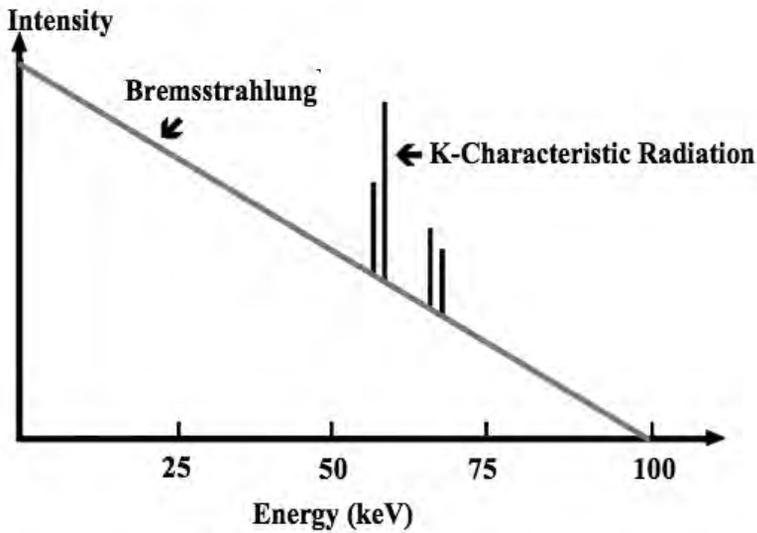
Indirectly ionizing particles are neutrons. Electromagnetic radiation or X-rays and gamma rays include **ionizing radiation indirectly**. Electromagnetic ionizing radiation is divided into:

- X-ray radiation. such that arises due to the interaction of charged particles (electrons) of the electron shell of the atom;
- Gamma radiation, i.e. radiation, which source is the nucleus of the atom.

In turn, the **X-rays** are divided into:

Characteristic Radiation – the impact of the electron beam knock off electrons from the electron shell of atom. Electrons returning to his seat quantum emit radiation, whose energy is closely related to the atomic number “Z” material, which allows you to identify it. This identification method is used in X-ray fluorescence analysis or X-ray spectrometry in electron microscopes.

Bremsstrahlung – it is formed when the electron beam interacts with an electric field an electron shell as a whole. Inhibition of the phenomenon of radiation is used to generate radiation beams “X” for applications in the industrial field (X-ray radiography), Medical X-ray cameras and security. This radiation is also produced as an undesirable by-product, when used the electron beam for welding of metals.



The X-ray spectrum from tungsten

Figure 11. X-rays spectrum with Bremsstrahlung part and characteristic part (figure used with permission under Creative Commons license)

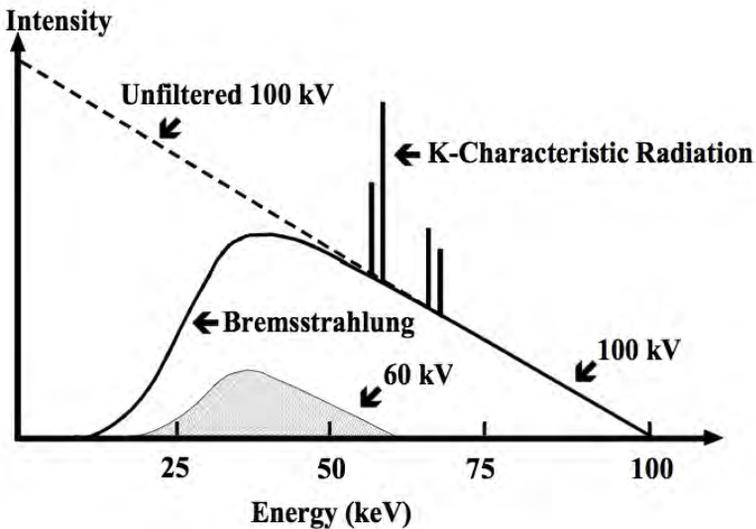


Figure 12. X-ray spectrum for low and high accelerating voltage (figure used with permission under Creative Commons license)

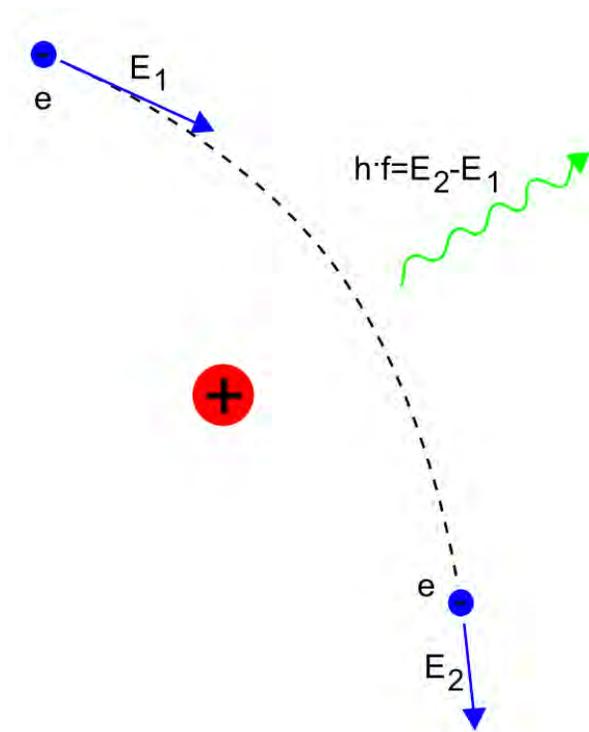


Figure 13. Explanation how Bremsstrahlung radiation is produced

Gamma radiation

Gamma radiation arises as a result of nuclear transformations that take place in the nuclei of radioactive isotopes. The emission of gamma radiation in most cases is composite, i.e. with the accompanying emission of alpha or beta radiation or both at the same time.

Energy ranges

As mentioned before, every EM wave including the waves of ionizing radiation has a parameter that characterizes it, i.e. energy.

Also, they charged particles or uncharged (alpha, beta, protons, neutrons) have an energy, which together with the properties of said particles is characterized by any type of radiation.

Alpha decay – emission of the He 4 nucleus stream. Discrete spectrum. Tick s energy of particles emitted from a few to several MeV.

Beta decay – electron beam emission. Continuous spectrum. Energy range E_{\max} to a few MeV.

The transformation of gamma – quantum emission stream. Discrete spectrum. The scope of energy to a few MeV.

1.3. Radiation properties

1.3.1. Radioactive decay: scheme and parameters

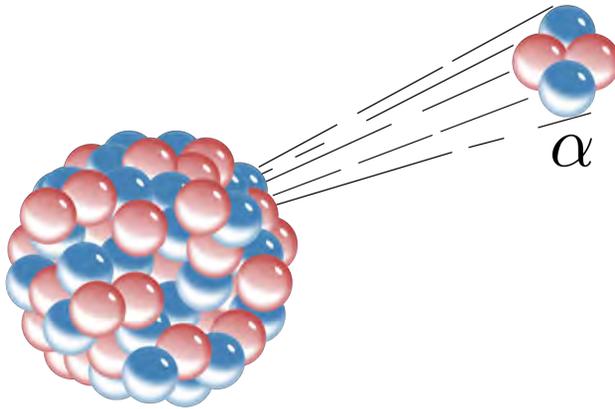


Figure 14. Graphical representation of the alpha decay. The kernel drops an alpha molecule (figure used with permission under Creative Commons license)

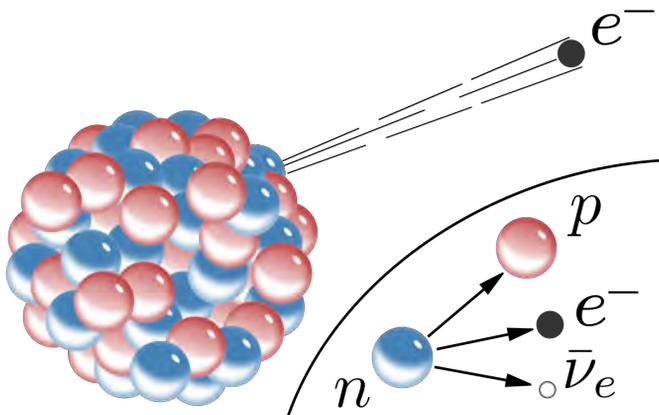


Figure 15. Chart of beta decay. The neutron in the nucleus decays into a proton and an electron (figure used with permission under Creative Commons license)

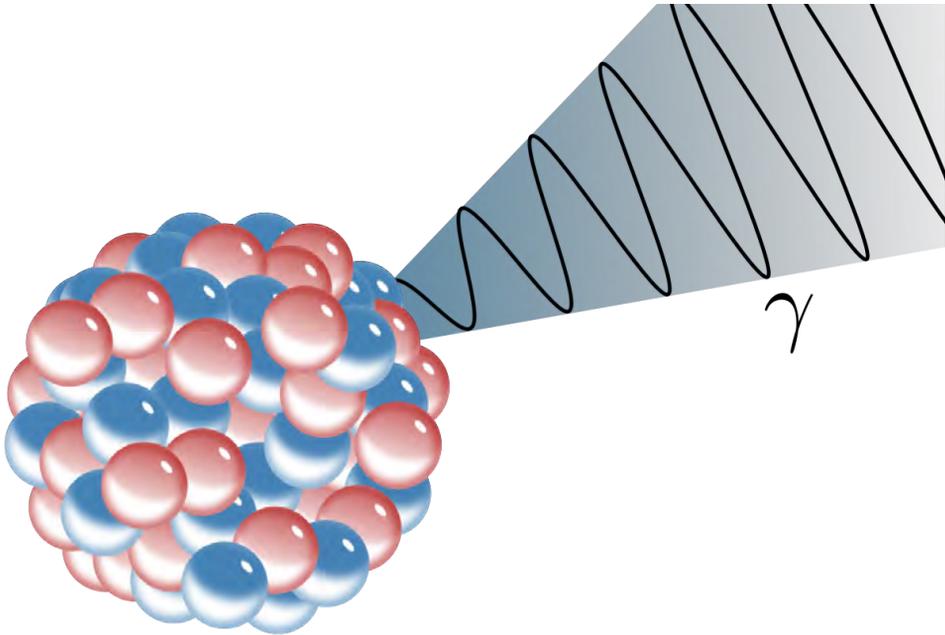


Figure 16. Chart of the conversion gamma (*figure used with permission under Creative Commons license*)

The pictures above illustrate graphically the alpha and beta decay and gamma transformation. Phenomena most often occur together, i.e. the alpha or beta decay is accompanied by the emission of gamma quanta. There are nuclides that are pure alpha or beta emitters.

In ancient world atom (from Greek “a-tomos” – no split able) was found as smallest part of environment. Now in XXI century we know that not only atom can divided in parts (nuclear reaction like fission or spallation) but even kernel is a complex structure in real not only consist of protons and neutrons.

Of course it is some simplification to say – we have only protons and neutrons. It is very useful to explain some nuclear phenomena but reality is more complicated.

Only for example you will find on image below how current science describe whole atom and its nucleus on Helium element.

A helium atom

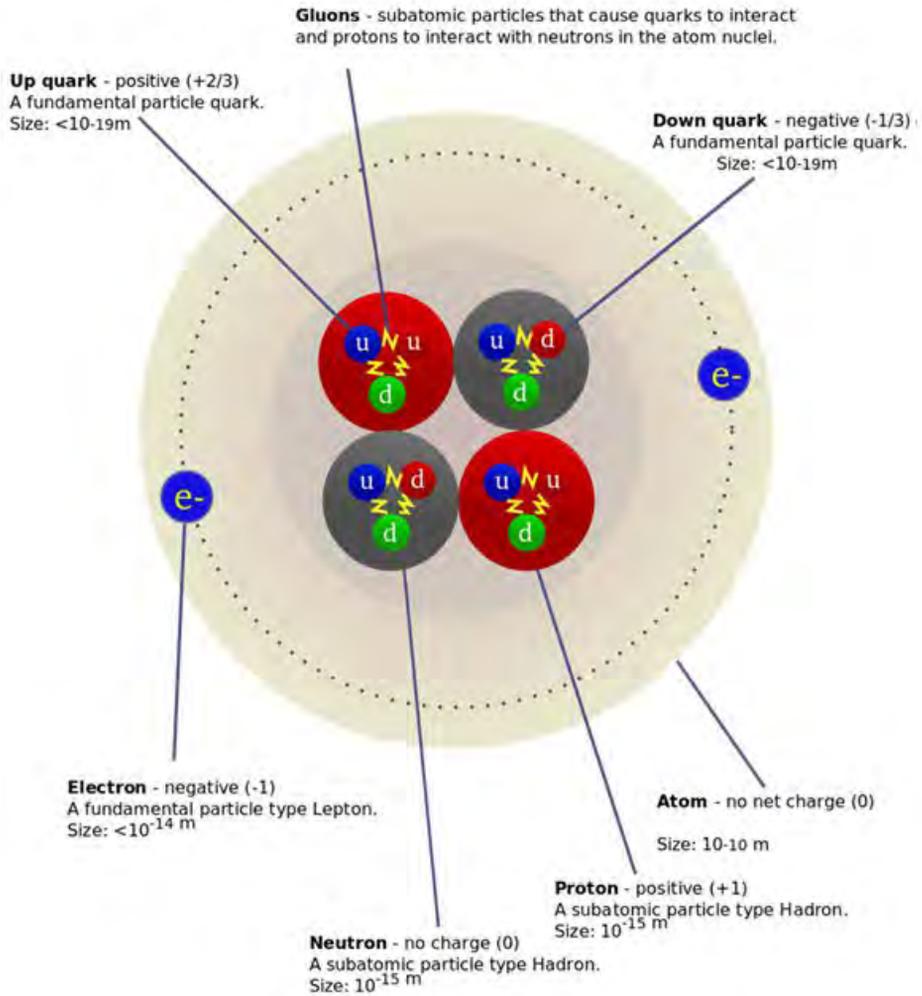


Figure 17. Detailed description of helium atom including nucleus structure (*figure used with permission under Creative Commons license*)

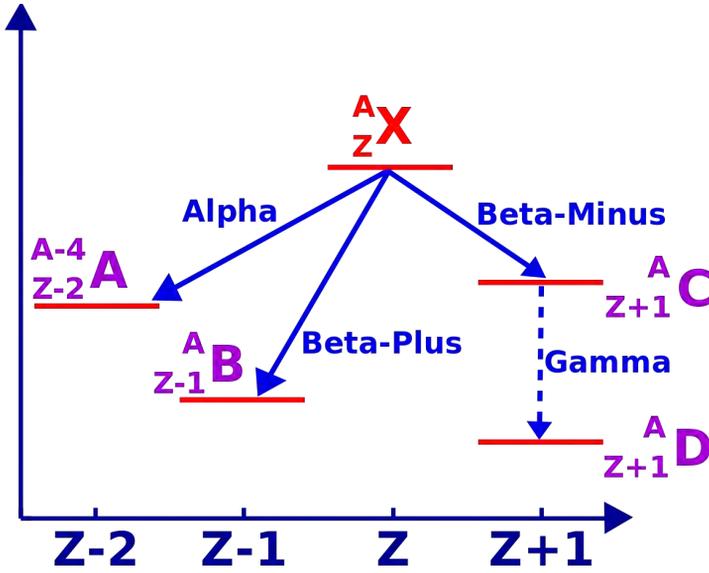


Figure 18. Nuclear decay parameters, Vertical axis – Energy, Horizontal axis – Atomic number

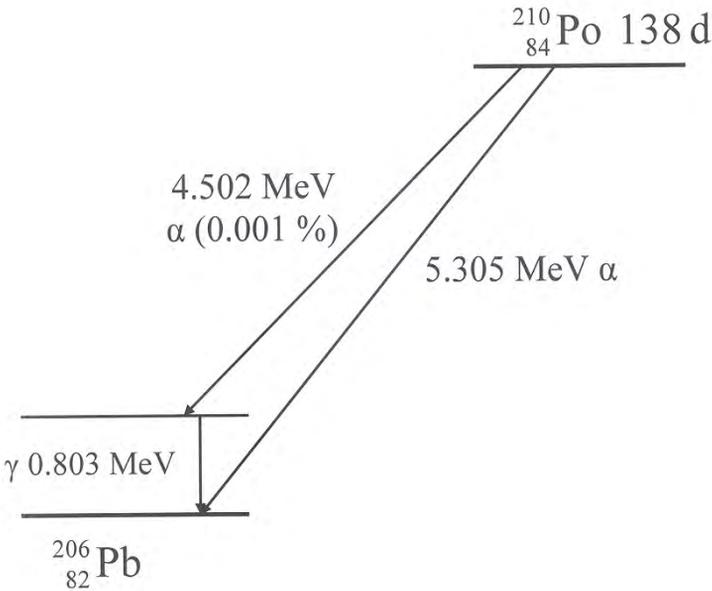


Figure 19. Scheme decay of polonium-210. Visible alpha decays and the accompanying transition and gamma (figure used with permission under Creative Commons license)

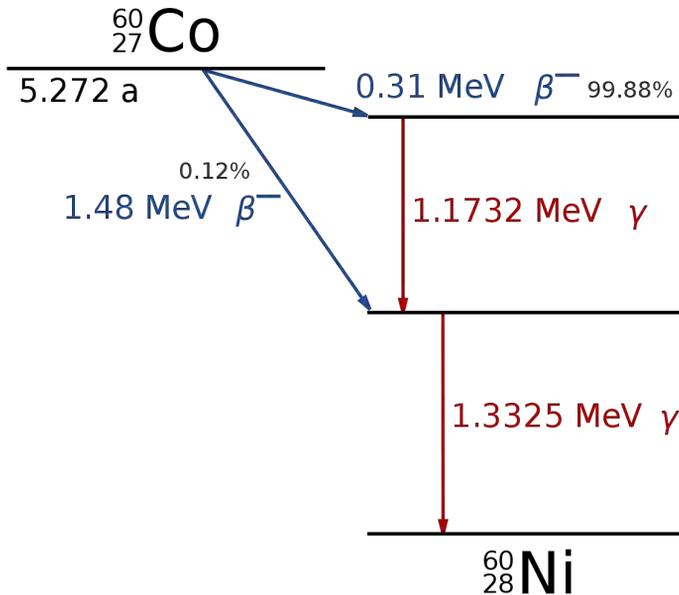


Figure 20. Schematic diagram of Co-60 cobalt disintegration. See two 1.33 and 1.17 MeV gamma transitions (*figure used with permission under Creative Commons license*)

1.4. Radiometry – basic parameters

The discovery of the phenomenon of radioactivity occurred in principle quite accidentally. Becquerel during the study of uranium ore kept it wrapped in black paper and photographic film. After developing the film, it turned out that it was exposed. The scholars began to wonder what could have caused the film to be exposed, and so began in the history of the human era of the atom.

At the beginning, they were not aware that research on radioactivity is associated with some danger. However, in an effort to define a certain size were introduced certain concepts. They gave rise to science called radiometry and its derivative dosimetry.

Concepts used in radiometry:

- Law of atomic decay. When studying nuclear decays and transformations, a certain regularity was observed when measuring radiation. This resulted in the formulation of **the law of atomic decay**:

- This law determines how the processes of radioactive decay take place. For each nuclide, there is a unique set the speed at which it disintegrates.

– **The rate of decay** is exponential nature of the evanescent i.e. that the rate of decay of the radiation is a function of the general type $\exp(-x)$ where “x” is the unit of time.

– And defined the concept of **activity** is given in units which will be measured. These units evolved over time to be consistent with the international system of SI measures.

– To be able to speak about the effects of physical activity introduces the concept of radiation **dose radiation**.

– To be able to talk about the biological effects of the concept of **equivalent doses** in relation to man and his organs.

1.5. Types of radiation doses and units used in radiological protection

In radiation science, the concept of radiation doses was introduced and thus defined:

- Absorbed dose – $D = dE/dm$ – Unit J/kg (Gy);
- Equivalent dose – $H_T = D * W_R$ – Unit J/kg (Sv);
- Effective dose – $E = H_T * W_T$ – Unit J / kg (Sv).

The **absorbed** dose is the amount of energy dissipated in the ionization mass. There is a physical unit and describes the energy effects. **Equivalent** dose is the absorbed dose, taking into account the coefficient W_R that takes into account the biological effects associated with different types of radiation being absorbed. **Effective Dose (RMS)** introduces a factor W_T ie the weight factor of tissues taking into account the sensitivity of individual organs and tissues to radiation.

Due to distinguish between different types of dose, although each of them has a physical dimension [J/kg] is a unit of absorbed dose in **gray (Gy)** and the other two are located in the unit **sivert (Sv)**. Sivert is a unit of all doses related to the exposure of a living organism. In the case of exposure to various types of radiation and exposure of various organs, we use the sum of exposure from different types of radiation and various organs.

$$H = \sum_i D_i * W_{Ri}$$

$$E = \sum_i H_i * W_{Ti}$$

Below the image presents the compares doses absorbed in some real circumstances with dose limits on US NRC regulatory.

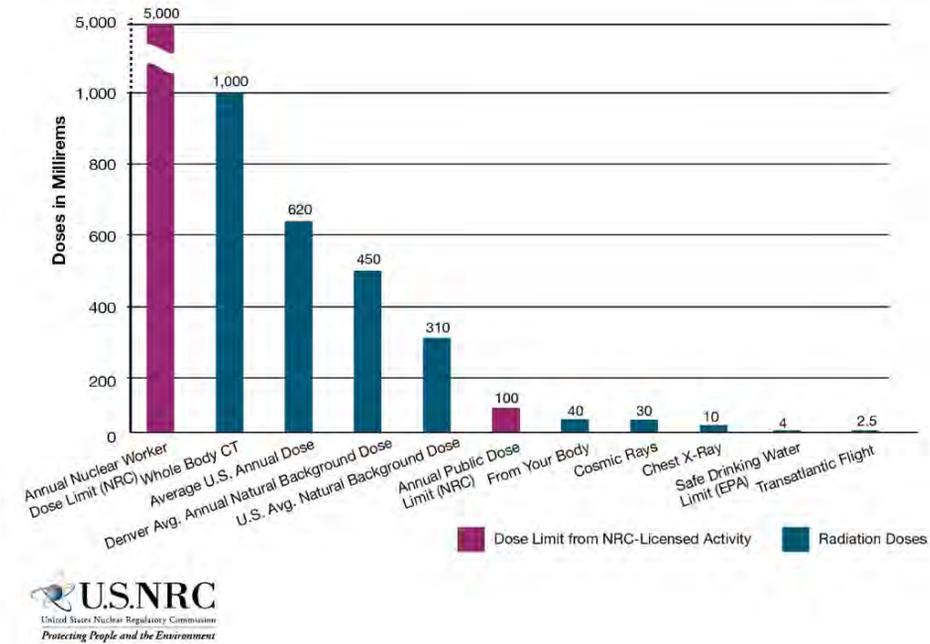


Figure 21. Radiation doses and Regulatory Limits by US NRC. In USA still old units are in use. Conversion from mrem to mSv is 1 mrem ⇔ 0.01 mSv (figure used with permission under Creative Commons license)

1.6. Radioactive isotopes: the concept of half-life and activity

The law of radioactive decay gives the way in which the number of atoms of a given nuclide decreases.

$$N(t) = N_0 e^{-\lambda t}$$

Where:

N_0 – the number radioactive atoms at the beginning of life of source,

$N(t)$ – number of radioactive atoms at time “t”,

λ – constant of radioactive decay.

The radioactive decay constant determines the rate at which radioactive atoms “disappear”. At the moment, we are introducing the concept of the half-past period.

$T_{1/2}$ => Is the period which is **half of the radioactive atoms**.

There is relation between half time period and constant of radioactive decay

$$\lambda * T_{1/2} = \ln 2$$

The half-life period is also referred to as the half-life time.

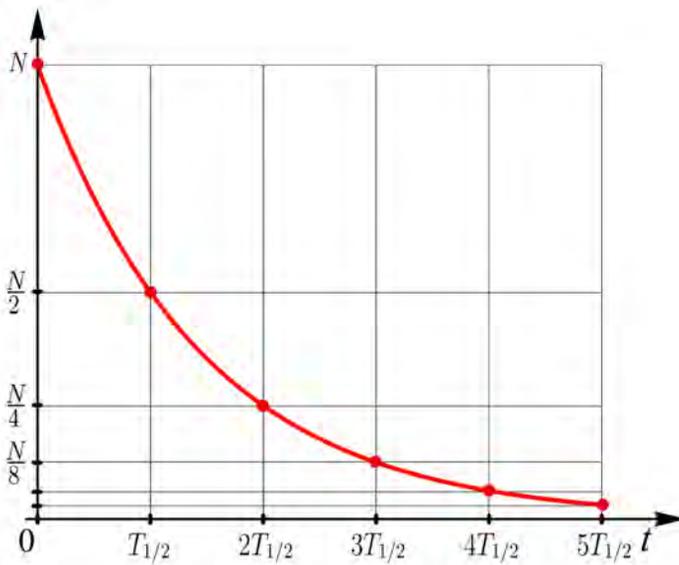


Figure 22. Graphic illustration of the law of radioactive decay. After each half-life, the number of radioactive atoms decreases twice (*figure used with permission under Creative Commons license*)

Radioactive activity is simply the number of radioactive atoms that break down in a unit of time.

$$A = -dN/dt$$

After transformation we obtain a pattern corresponding to the pattern of the number of atoms disintegrating:

$$A(t) = A_0 e^{-\lambda t}$$

A_0 – is the source activity at the time of production,

$A(t)$ – Is the source activity after time t ,

Value A_0 is a parameter which is given for radioactive sources while source is manufactured. Value of A_0 is written in source certificate.

We measure our activity in Becquerels (a unit named after H. Becquerel). 1 Bq is 1 disintegration per second (s^{-1}), so it is very small unit. Practically we use a larger unit kBq, MBq GBq and TBq.

The historical unit is a kiuir named after Maria Skłodowska-Curie. It has a practical basis because the activity of 1 Ci had 1g Ra-226 produced by Maria

and donated to Poland. In turn, Ci unit was very high and practically used his aliquots ie. mCi, uCi, nCi. The relationship between these units is **1 Ci = 37 GBq**.

Sometimes, some literature position and companies still Ci is using to determine the activity of sources. For example Smiths Detection in GID-3 manual has Ni-63 sources in mCi described.

1.7. Sources of radiation

1.7.1. Natural radioactivity

As mentioned earlier, radioactivity occurs naturally in nature. Radioactive elements that occur in air, soil and water together with cosmic radiation make up the radioactive background that surrounds us.

Radioactive elements found in nature

The elements that occur naturally in nature are:

- Coal C-14;
- Potassium K-40;
- Three elements of the so-called. radioactive ranks;
- Others listed in the figure below.

Element	Half-life (years)
²³⁵ U	7×10^8
²³⁸ U	4.47×10^9
²³² Th	1.4×10^{10}
¹⁷⁶ Lu	3.6×10^{10}
¹⁸⁷ Re	4.3×10^{10}
⁸⁷ Rb	4.9×10^{10}
¹³⁸ La	1×10^{11}
¹⁴⁷ Sm	1.05×10^{11}
¹⁹⁰ Pt	6.9×10^{11}

Figure 23. Naturally radioactive isotopes. The first three marked in red are the origins of three natural radioactive ranks

1.8. Radioactive series

There are 4 radioactive series. Below are shown graphical representation of them. Due to fact that emitting alpha particle mass number of daughter element decreases by 4 from its parent it can be **only** four series.

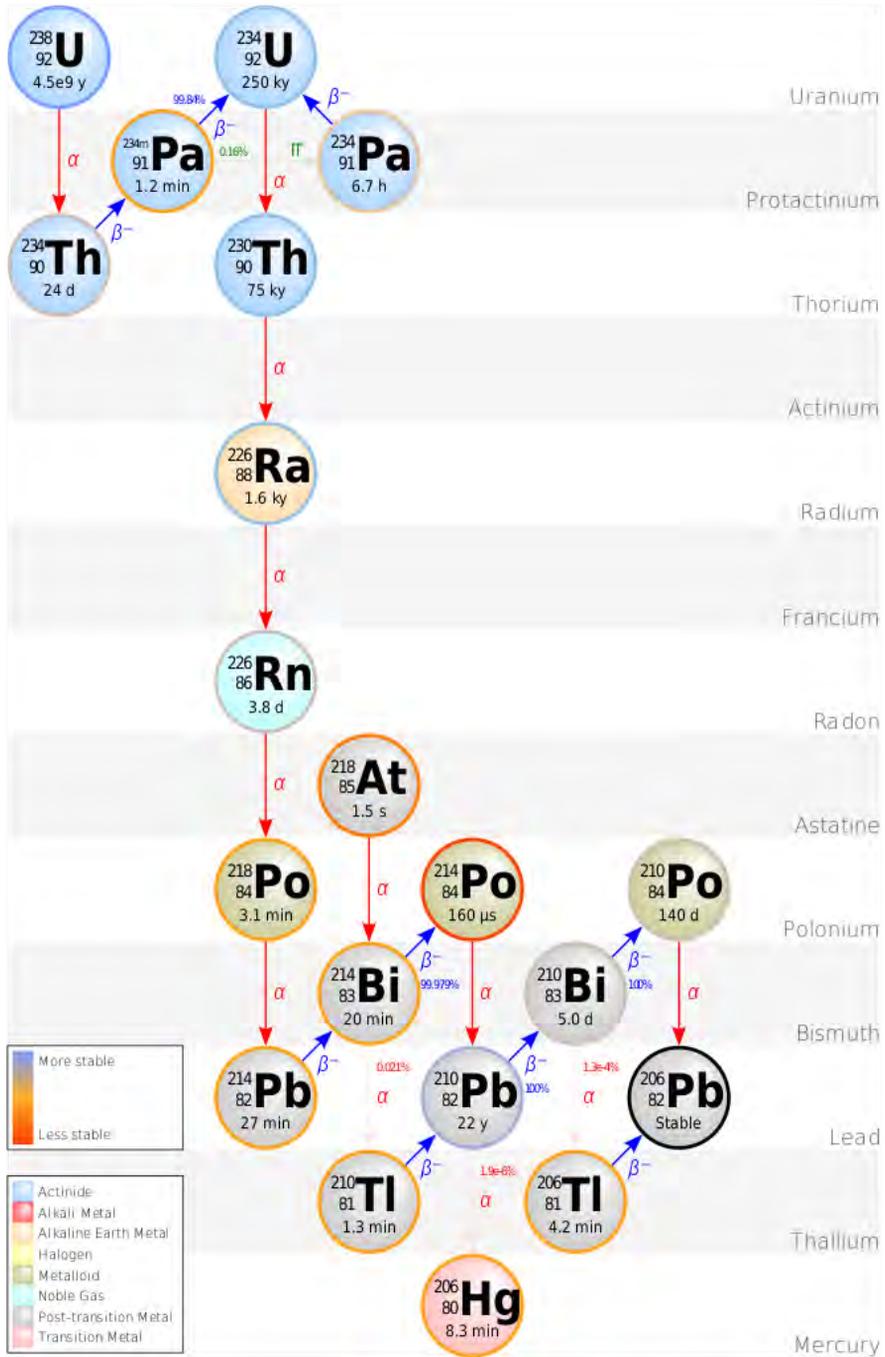


Figure 24. Decay scheme of uranium series (figure used with permission under Creative Commons license)

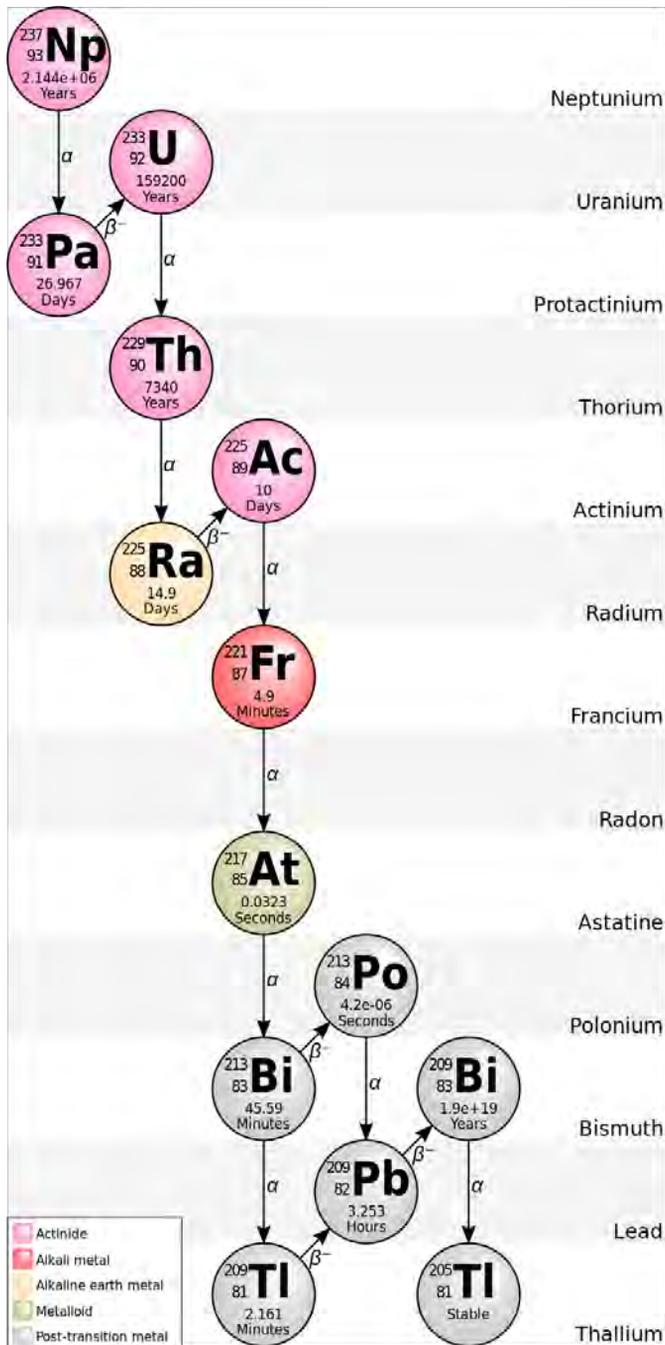


Figure 25. Decay scheme of neptunium series (figure used with permission under Creative Commons license)

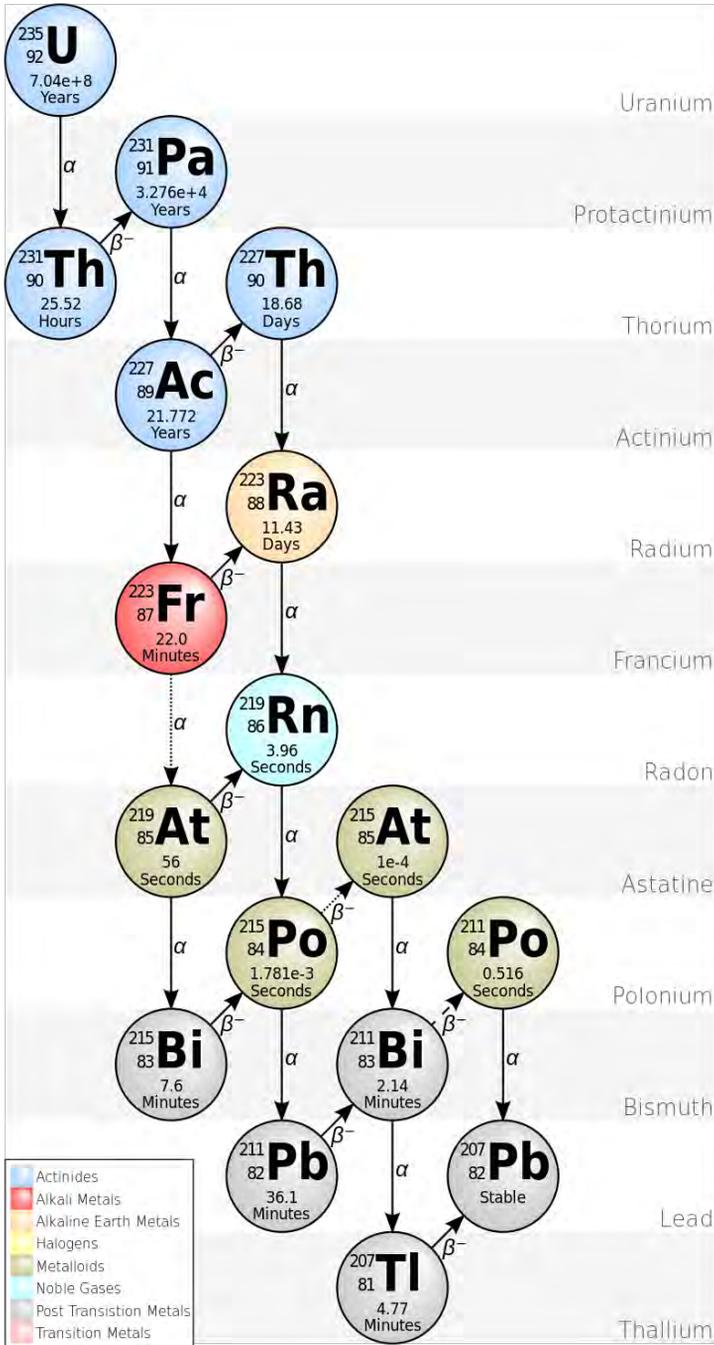


Figure 26. Decay scheme of actinium series (figure used with permission under Creative Commons license)

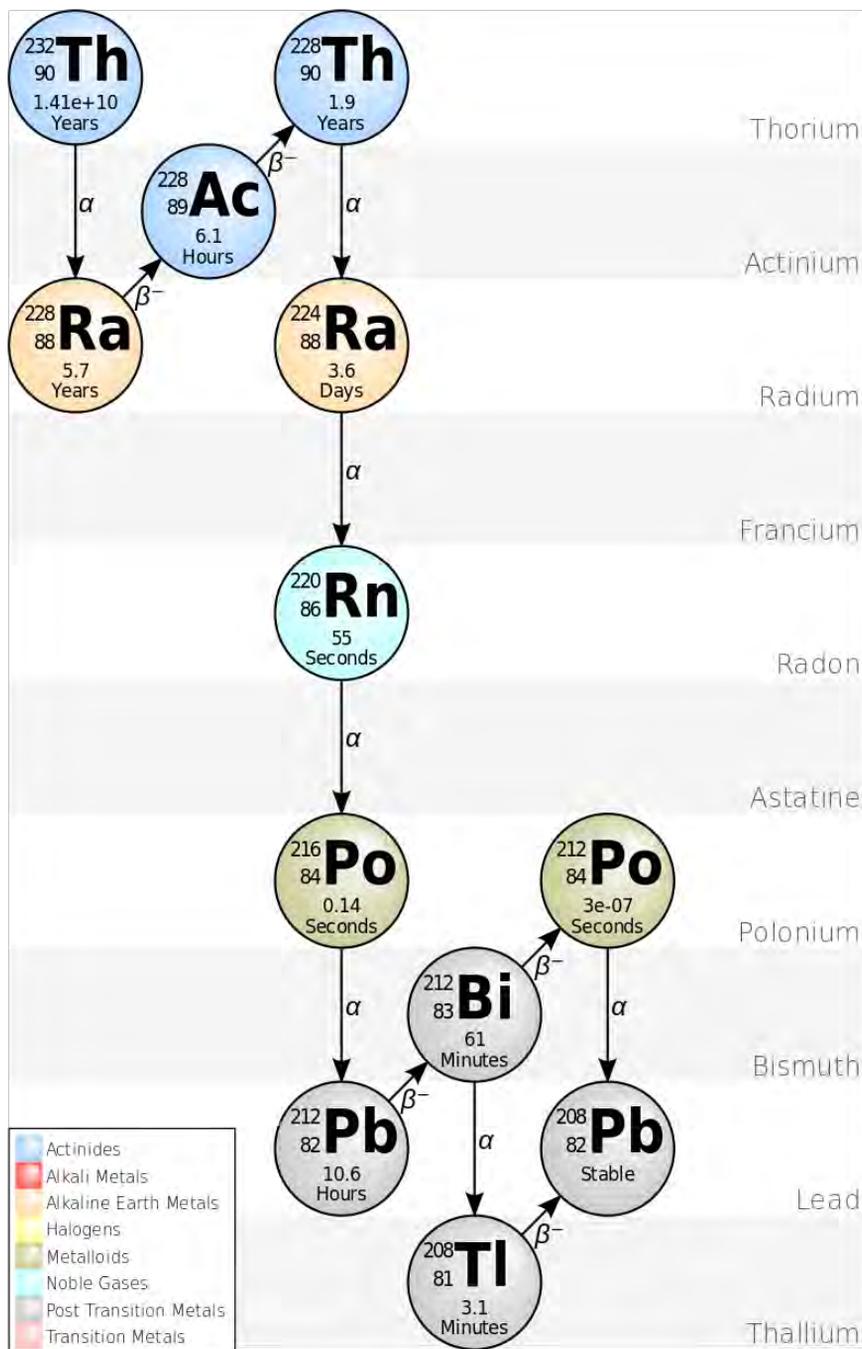


Figure 27. Decay scheme of thorium series (figure used with permission under Creative Commons license)

1.9. Radioactive elements produced artificially (by man)

In the national economy, ionizing radiation is widely used:

- In industry;
- In medicine;
- In security-related departments.

Most isotopes are produced in nuclear reactors and accelerators. They are mostly gamma and beta-radioactive.

1.9.1. Isotopes used in the medicine

When used in Medicine, radioisotopes fall into one of two groups:

- **Diagnostic Radionuclides**
- **Therapeutic Radionuclides**

A representative list of medical radioisotopes is shown in the table.

Sodium-24 (^{24}Na)	15 Hours	Study of general biological processes
Iron - 59 (^{59}Fe)	46.3 Days	Diagnosis of Blood Disease
Technetium -99m ($^{99\text{m}}\text{Tc}$)	6 Hours	Diagnosis of various diseases
Cobalt - 60 (^{60}Co)	5.3 Years	Treatment of Cancer
Strontium - 90 (^{90}Sr)	27.7 Years	Treatment of Tumors
Iodine-131 (^{131}I)	2.6 Minutes	

1.9.2. Radioisotopes used in the industry

Table 1. List of radioisotopes use in industry

Radioisotope	Industrial Applications
Americium-241	For uniform thickness when rolling steel and paper, determine location of oil wells
Sodium-24	Oil well studies and to locate leaks in pipe lines
Iridium-192	Test integrity of boilers and aircraft parts
Uranium-235	Nuclear power plant and naval propulsion systems fuel, production of fluorescent glassware and colored wall tiles
Califomium-252	Determine moisture content of soil – important for road construction and building industries

1.10. Nuclear fuel

The following table lists of the nuclear reactor core including the fuel type.

Reactor Type	Coolant	Moderator	Fuel	Comment
Pressurised water reactors (PWR, WER)	Light water	Light water	Enriched uranium	Steam generated in secondary loop
Boiling water reactors (BWR)	Light water	Light water	Enriched uranium	Steam from boiling water fed to turbine
Pressurised heavy water reactor (PHWR)	Heavy water	Heavy water	Natural uranium	–
Gas-cooled reactors (Magnox, AGR, UNGG)	CO ₂	Graphite	Natural or enriched uranium	–
Light water graphite reactors (RBMK)	Pressurised boiling water	Graphite	Enriched uranium	Soviet design

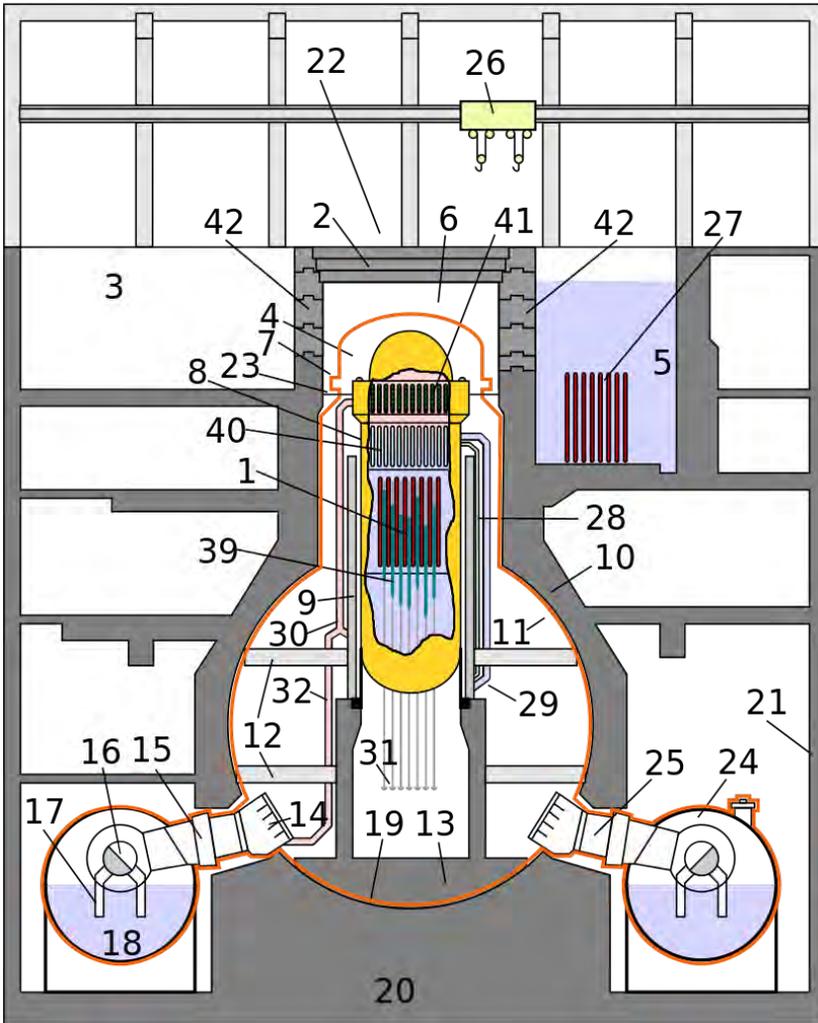


Figure 28. Scheme of BWR nuclear reactor Mark 1 (figure used with permission under Creative Commons license)

Description to figure:

– orange: primary containment vessel:

1 – Core with fuel rods;

2 – Concrete shield plug;

3 – Equipment pool;

4 – Drywell head;

5 – Fuel storage pool; spent fuel area;

6 – Refueling cavity;

7 – Drywell flange;

8 – Reactor pressure vessel;

9 – Biological shield;

10 – Secondary concrete shield wall;

11 – Free standing steel drywell;

12 – Radial beam;

- 13 – Concrete embedment;
- 14 – Jet deflector;
- 15 – Expansion bellows;
- 16 – Vent header;
- 17 – Downcomer pipe;
- 18 – Water (wetwell);
- 19 – Embedded shell region;
- 20 – Basement;
- 21 – Reactor building;
- 22 – Refueling platform;
- 23 – Refueling Bulkhead;
- 24 – Pressure suppression chamber (runs in a torus around the reactor);
- 25 – Vent (81 inch diameter);
- 26 – Crane;
- 27 – Spent Fuel;
- 28 – Coolant pipe;
- 29 – Cold water pipe (from generator);
- 30 – Steam pipe (to generator);
- 31 – Control rod drives;
- 32 – Blow down line;
- 39 – Control rods;
- 40 – Steam separators (water normally goes to this level);
- 41 – Steam dryer;
- 42 – Gates (removed during refuelling).

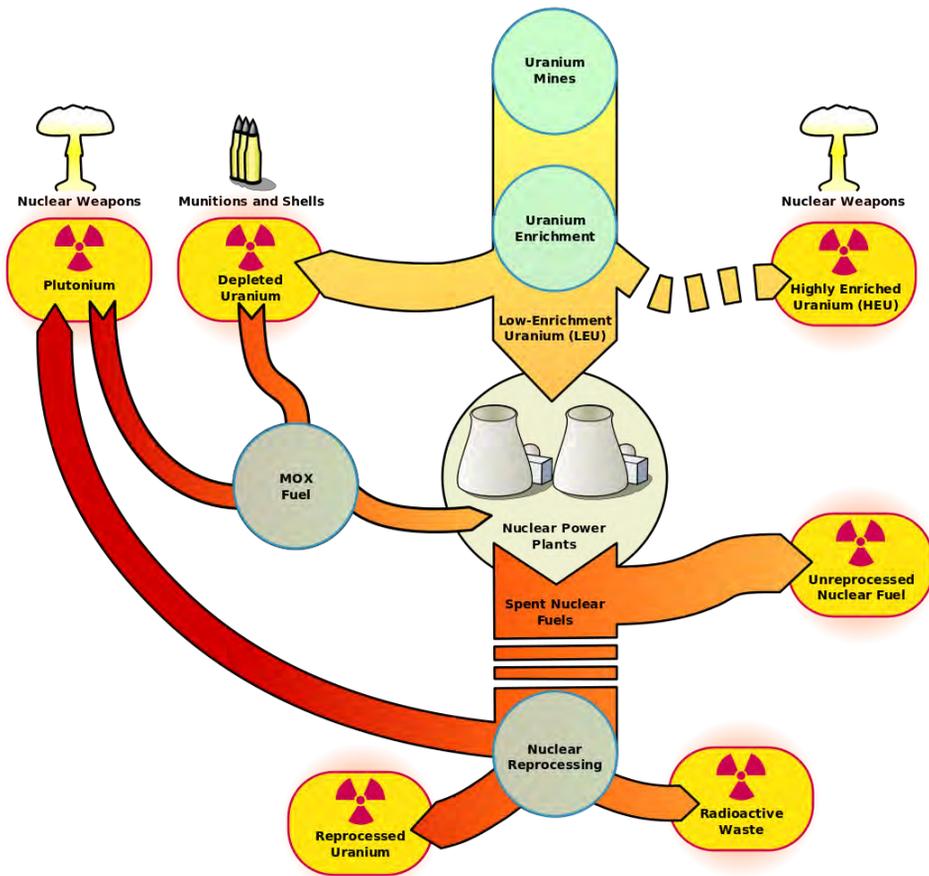


Figure 29. Nuclear fuel process (figure used with permission under Creative Commons license)

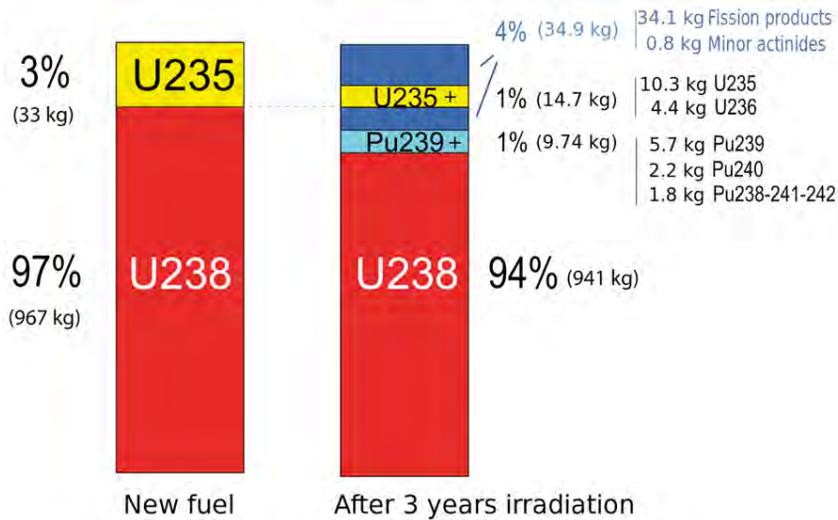


Figure 30. Nuclear fuel composition (figure used with permission under Creative Commons license)



Figure 31. Nuclear fuel pellets (figure used with permission under Creative Commons license)

1.11. Radioactive waste

Polish “Atomic Law Act” has a number of articles devoted to radioactive waste. Generally, the waste can be divided into:

- Solid and liquid;
- Low- medium and high-intensity;
- Short and long-lived.

The law also defines the rights and duties of the Department of Radioactive Waste Management (DRWM) and functioning KSOP (National Radioactive Waste Repository).

The DRWM operates on the premises and in the structure of the NCBJ (National Center for Nuclear Research). On the website of the department you can find out about the conditions of waste collection also the current price list is included.

During the operation of the energy reactor, the so-called fuel cycle. It consists in the fact that during operation some of the rods is “burned” in the exchange requires fresh.

The fired rods are material to be processed in spent fuel processing plants. Part of the fuel is designed for reuse. The rest is considered as radioactive waste and requires appropriate treatment.

Because Poland belongs to IAEA (International Atomic Energy Agency) we are obliged to fulfill all requirements about treatment of radioactive waste which Agency recommend its members. Below you will find how radioactive waste are classified by IAEA.

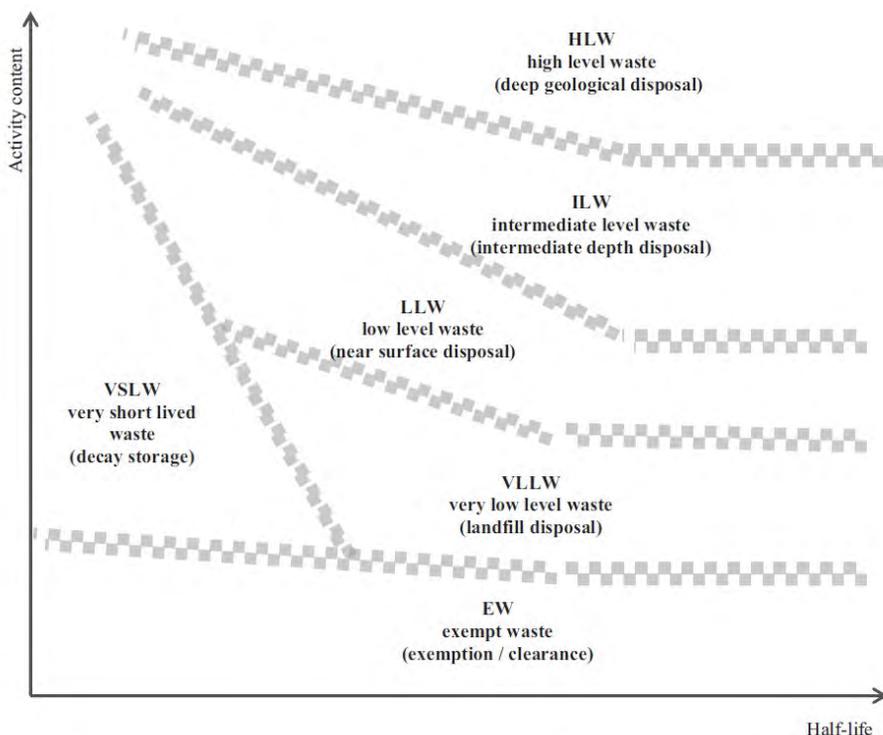


Figure 32. Nuclear waste classification (figure used with permission under Creative Commons license)

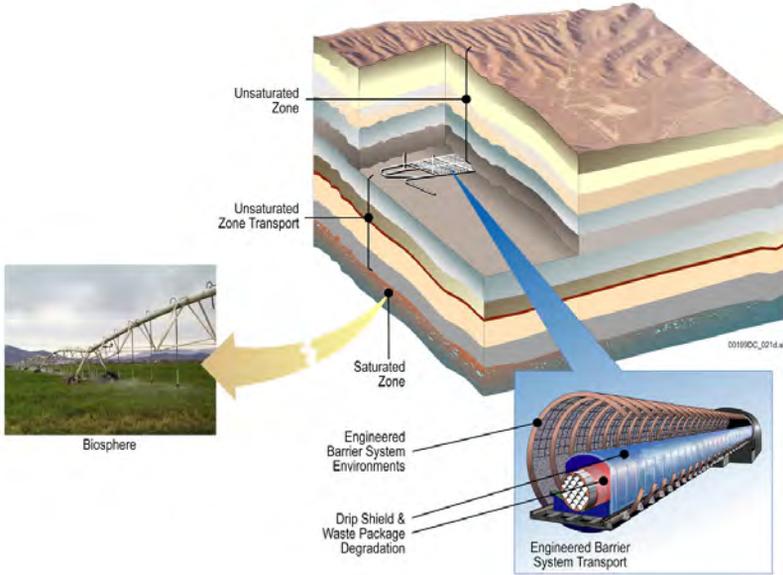


Figure 33. Yucca Mountain nuclear waste repository cross-section (figure used with permission under Creative Commons license)

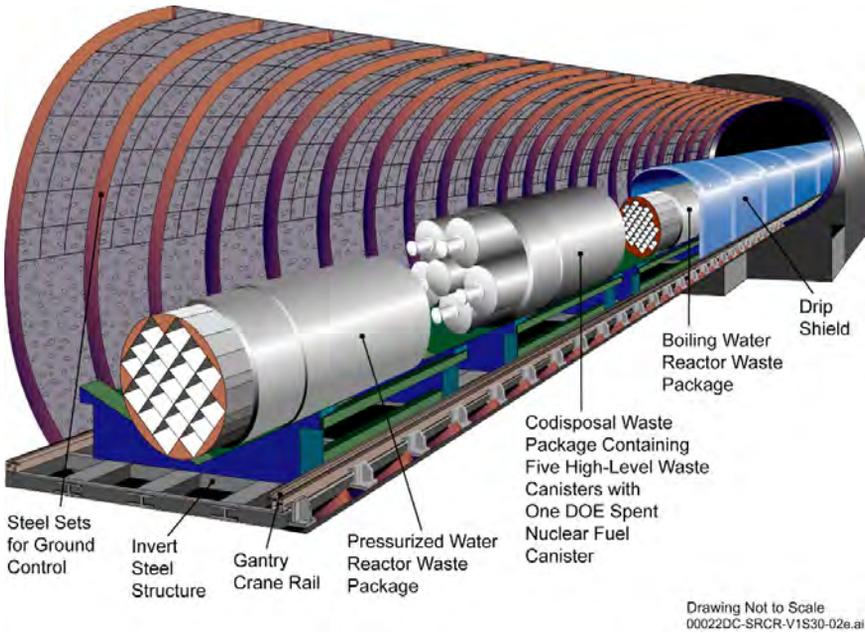


Figure 34. Yucca Mountain waste packages (figure used with permission under Creative Commons license)

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2. RADIATION DETECTION AND PROTECTION PROCEDURES

2.1. Dosimetric equipment to measure the dose and dose rate/ equivalents of doses and equivalents dose rate of radiation

Dose rate is the dose absorbed in unit time and indicates the amount of radioactive dose received by a person within a certain period of time. The dose rate is often given in thousandths of sieverts per hour. Depending on the calibration procedures used by the manufacturer, detectors provide information about the strength of the radioactive source.

2.1.1. Operational dose quantities

It is not possible to directly, and physically measure the amount of energy deposited (i.e. doses) in organs or people. Therefore alternative operational dose quantities are used by international convention to provide best and accepted estimates. It is Good Practice to measure doses to personnel with a personal dosimeter, but an alternative method is to estimate the effective dose with a hand held monitoring instrument.

The convention in radiation protection for area monitoring is to use:

Ambient dose equivalent - for control of effective dose to people, e.g. area **monitoring**. Ambient dose equivalent $H^*(d)$ is the normal monitoring quantity for X, gamma and neutron radiation where d is the depth at which the dose rate applies. International convention in radiation protection is to use the ambient dose equivalent at 10 mm depth i.e. $H^*(10)$. The ambient dose gives a conservative estimate of the effective dose a person would receive when staying at the point of the monitoring instrument.

Directional dose equivalent – for control of doses to skin and lens of eye. Directional dose equivalent $H'(d)$ is intended for use with less penetrating radiation. Its main use is for skin dose at a depth of 0.07 mm, but it is also used for dose to the eye lens at 3 mm. Operational quantities that should be measured using portable instruments during dose rate surveys are therefore either:

- ambient dose equivalent at 10 mm depth, $H^*(10)$ – SI unit: sievert (Sv);
- ambient dose equivalent rate, $H^*(10) \text{ h}^{-1}$ – SI unit: sievert per hour (Sv h^{-1});

- directional dose equivalent at 0.07 mm depth, $H'(0.07)$ – SI unit: sievert (Sv);
- directional dose equivalent rate, $H'(0.07) \text{ h}^{-1}$ – SI unit: sievert per hour (Sv h^{-1}).

Some instruments may use different quantities such as fluence rate, air kerma rate, absorbed dose rate in air. Estimation of dose is a specialist task when using instruments not scaled in dose equivalent or dose equivalent rate.

2.1.2. Measurement of contamination

The units most commonly found on instruments used for contamination monitoring are:

- counts per second (cs^{-1} , cps, ps^{-1} , and s^{-1});
- disintegrations per minute (dpm);
- becquerel (Bq);
- becquerels per square centimetre (Bq cm^{-2}).

If the instrument indicates ‘becquerel’ or ‘becquerel per square centimetre’, a calibration factor will have been stored in the instrument. Conversion of counts per second to becquerels or becquerels per square centimetre can be complicated. This task requires a comprehensive knowledge of decay schemes, instrument performance and some estimation of how the local conditions (e.g. surface construction) might effect the observed count rate.

2.1.3. Examples of dosimetric equipment to measure the dose and dose rate



Figure 1. DPO radiometer

DPO radiometer is designed for measurement of X and gamma radiation ambient dose equivalent rate and ambient dose equivalent. The device enables also detection and measurement of surface contamination with alpha, beta and gamma radiation emitters. The appliance can be installed not only in stationary military defensive and protective objects or command points but also can be treated as a primary dosimetry unit, used on ships and other buoyant objects of navy. It is also possible to use it as a handheld radiometer in the field research.



Figure 2. RK-100-2 radiometer

Portable radiometer RK-100-2 is dedicated for measurement of ambient dose equivalent rate, ambient dose equivalent, absorbed dose rate in air, absorbed dose in air of X, gamma radiation and surface contamination (alpha, beta and gamma emitters). It is easy to use radiometer with a durable case and light weight.

The **SVG 2** is a newly developed, hand-held, hardened microprocessor controlled radiation detector based on state-of-the-art semiconductor technology. The SVG 2 consists of a base instrument with integrated energy-compensated MOSFET and PiN-diode sensors for neutron radiation detection, an external personal dosimeter which records the assimilated dose of the respective carrier, and an external probe. Additional probes, like a scintillation probe and a Geiger-Muller-Counting Tube can be connected. The base instrument is easy to handle and is particularly distinguished by its low weight and power consumption. Not only the current dose rate but also the integrated dose over a certain time can be displayed. Additionally, the initial and assimilated dose of radiation is measured by an internal dosimeter, even if the instrument is shut off! Both modes of total and differential dose display are available. Alarm thresholds for all the modes are adjustable. The alarm is given either optically by a red flashlight and acoustically by a horn (> 90 dB A) or only by the flashlight. An earphone can alternatively be used.

2.1.4. Spectrometric equipment for identification of the radioactive sources

The devices listed in this section are able to identify radioactive isotopes by analyzing the characteristic gamma emission spectra. The devices are often multipurpose/multitask instruments able to detect different forms of radiation and perform a variety of functions; used to search, detect and identify radioactive materials.

Hand-held Radioisotope Identification Devices (RIIDs) take this another step further and are designed to identify the isotopic composition of radioactive sources. A RIID is typically a small, handheld device that is generally easy to use and deploy. The devices are often multipurpose/multitask instruments able to detect different forms of radiation and perform a variety of functions; used to search, detect and identify radioactive materials. Radioisotope Identification Devices (RIIDs) are instruments that are designed to determine the identity of radioactive materials by measuring the energy of the emitted gamma rays. Law enforcement, customs, and other personnel are being equipped with RIIDs as part of a national strategy to interdict illicit movement of radioactive material. When radiation sources are detected by screening devices such as radiation portal monitors or radiation pagers, RIIDs are used to determine whether the source of radioactivity constitutes a high level threat. Radiological emergency personnel, firefighters and other response personnel also use RIIDs for situational assessment during radiological emergencies.

Most radioisotopes emit gamma rays with characteristic energies. Gamma rays emitted by a radioactive source strike a detector within the RIID and are converted into a signal that indicates the energy of the incident gamma ray. The number of gamma rays at each energy are counted and plotted versus energy in an energy spectrum which reveals characteristic energy peaks. Identification is based on matching the peaks in the spectrum to the known peaks and peak ratios of gamma emitters. This matching process is done using proprietary isotope identification software which is a critical component of these instruments. Two key RIID features are energy resolution and sensitivity.

Resolution is a measure of how close two energy peaks can be and still be differentiated; the lower the percent resolution, the better the detectors' ability to distinguish two or more closely spaced peaks.

Sensitivity is a measure of how efficiently incoming gamma rays are detected; this determines the counting time needed to obtain a spectrum

Two different types of detectors are used in commercially available RIIDs.

1. Scintillator detectors are transparent materials (crystals) that, when struck by gamma rays, produce light pulses with intensities that are proportional to the gamma ray energies. These light pulses are converted to electrical pulses by a photomultiplier tube (PMT) and then processed by a multi-channel

spectrometer to produce characteristic gamma ray spectra. RIIDs using scintillation detectors are lighter and less expensive than other technologies, but have poorer resolution. The most common scintillator material used in RIIDs is thallium doped sodium iodide (NaI(Tl)) crystals. A relatively new scintillator material, cerium doped lanthanum bromide (LaBr₃:Ce) has twice the resolution of NaI(Tl), and also has a higher light output, but RIIDs using this material could cost about twice as much.

2. Semiconductor detectors are made from specially processed crystalline material. Gamma rays striking the detector create free positive and negative charges that are detected as a current; the higher the energy of the gamma ray, the higher the current that is generated. The most common semiconductor material used in RIIDs is high purity germanium (HPGe) crystals (2 inches dia.x 1.2inches high), these must be cooled to liquid nitrogen temperatures (-321°F) with a built-in refrigeration system in order to operate. HPGe RIIDs are much larger and heavier, and cost about fivetimes as much as NaI(Tl) RIIDs. While scintillator RIIDs are ready to operate in a few minutes, an HPGe based RIID that is at room temperature is inoperable until it is fully cooled, which takes approximately two hours.

Handheld RIIDs are battery powered, have built- in software for spectral analysis and are capable of identifying the radioisotopes most commonly encountered by emergency responders. Radioactive isotopes are divided into four groups:

- special nuclear material (SNM), plutonium, highly enriched uranium;
- medical isotopes (used in radiotherapy and medical imaging);
- industrial isotopes (used in weld inspection devices, civil engineering equipment, food irradiators);
- naturally occurring radioactive material (NORM), commercial products such as ceramics and fertilizers containing radioactive elements such as potassium, uranium, thorium and radium.

Limitations

Radioactive sources can be shielded so that gamma or neutron radiation is below the RIID detection limits or the spectrum is greatly distorted. It is particularly easy to shield SNM isotopes that emit only low energy gamma rays. For example, about 1 inch of lead will reduce the emission from plutonium by about a factor of 1000, and 0.25 inch of lead will reduce the emission from HEU by the same factor. The energy calibration of some RIIDs can be affected by temperature changes such as moving between indoors and outdoors, which may result in misidentifications. Some devices contain a built in check source for recalibration. Isotope identification software currently used in RIIDs can misidentify radioactive sources even when the data was collected with a properly

calibrated RIID. For this reason, the DHS Domestic Nuclear Detection Office established the Joint Analysis Center (JAC) Regional Reachback Program. Highly trained gamma spectrometry specialists are available on a 24/7 basis through this program to provide analysis of RIID data to first responders.

Example of spectrometric equipment for identification of the radioactive sources

The **identiFINDER R500 (radHUNTER)** is an extremely sensitive and accurate digital hand-held gamma radionuclide identification device (RID). It is the culmination of over eight years of development of micro-miniature, digital signal processing electronics; operating power conservation; and advancements in the scintillation detector, radionuclide template matching identification algorithm. The identiFINDER R500 development was supported in part by the U.S. Department of Homeland Security (DHS) / Domestic Nuclear Detection Office (DNDO), Human Portable Radiation Detection System (HPRDS) program.

Available in two configurations (NaI and LaBr), the identiFINDER R500 is able to quickly detect, rapidly locate, accurately measure and precisely identify sources of contamination from their gamma radiation signature. The identiFINDER R500 NGH ultra 3n uses a 0.75" thick by 4" diameter NaI(Tl) detector while the identiFINDER R500 LGH ultra 3n uses a 1.5" by 1.5" LaBr₃:(Ce) detector. Both instruments come with a GM tube for high gamma dose rate measurements and a sealed He₃ detector with moderator for neutron detection. Each instrument is supplied in a carrying case with recharging unit, communication cables, synchronization and analysis software. Variations in normal operating conditions can affect the performance of RID with scintillation detectors such as NaI(Tl) and LaBr₃:(Ce). Dramatic temperature variations can affect the scintillation detector and/or the PMT and can also cause instabilities and non-linearities in the electronics.

The InSpector™ 1000 is a high-performance, hand-held NaI spectrometer for use primarily in first responder, customs, homeland security, and health-physics applications. One-click simplicity masks the sophisticated spectral processing facilities that lie within the instrument - providing a level of performance previously available only in more complicated laboratory systems. The InSpector 1000 is an instrument that provides answers not just data! With the InSpector 1000, users from law enforcement officers to Health Physics technicians can obtain the results they desire with an easy to use, intuitive interface. InSpector 1000 has been designed for easy operation. The high-resolution color LCD display is clearly visible from bright sunlight to night conditions. The unit can be held and operated in one hand (even if wearing gloves). The convenient hand strap leaves the hand positioned to easily reach the controls with the thumb. This leaves the

second hand free for other operations - holding a ladder, sorting through suit cases or packages, etc. In addition, for applications requiring flexible positioning of the detector relative to the object being inspected, the detector can be detached from the instrument body and placed in any position or narrow gap. This greatly increases the probability of detecting small amounts of radiation and precisely locating sources by reducing the measurement distance.

InSpector 1000 was designed to be used in all types of environmental conditions. Its ruggedized, light weight packaging meets the IP 54 specifications and is easily decontaminated. It also has a wide operating range for both temperature and humidity. The InSpector 1000 instrument has been designed to survive a one meter drop onto concrete.

InSpector 1000 is an easy-to-use digital multichannel analyzer, ideally suited for homeland security applications, custom and border protection, healthphysics, treaty and non-proliferation compliance, monitoring of nuclear transportation, and in situ measurement of objects, surfaces etc. The user interface provides the ultimate flexibility in field operations. The InSpector 1000 is readily usable by less sophisticated users without the need of extensive training and also offers high-level spectrometry analysis capabilities for expert users. It can be used in any field measurement application requiring nuclide identification, activity measurements, dose/count rate measurements, or spectrum acquisition and analysis. Although the instrument has a powerful suite of spectroscopy functions, the InSpector 1000's simple user interface isolates the user from complicated spectrum analysis while still providing this data to more advanced users.

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MODULE VI

**THE BASIS OF
SAPPER-PYROTECHNIC
RECOGNITION**

1. PYROTECHNIC RECOGNITION

Explosion is a rapid, physical, chemical or nuclear transformation of a system accompanied by a transformation of the potential energy into mechanical work – destruction of the environment (e.g. bursting, fracturing, splitting, splintering, spraying or relocation of objects in the surroundings). The phenomenon is accompanied by an acoustic effect such as a sound and a visual effect such as a flash.

1.1. Types of explosions

Physical explosion

It is a very rapid transformation of the matter while releasing a considerable volume of energy in the form of heavily compressed starting substances or gaseous products produced during a transformation e.g. under the influence of a strong electrical current (a lightning), a rupture of a steam boiler or a compressed gas cylinder.

Nuclear explosion

Rapid generation of nuclear energy occurring as a result of an uncontrolled chain reaction of heavy element fission (nuclear weapons). In the so-called thermonuclear weapons, the force of nuclear explosion is multiplied by causing a nuclear synthesis of nuclei of light elements.

Chemical Explosion

Rapid (millisecond-lasting), exothermic chemical processes occurring in solid and liquid explosives and explosive gaseous mixtures.

Chemical explosion properties:

– **velocity of reaction** e.g. 1 kg of TNT detonates during approximately 0.00001s,

– **high velocity of transformation** e.g. in crush explosives from 1,000 do 8,500m/s,

- **exothermic** e.g. for crush explosives, the explosion temperature is approximately 3,000 degrees C.,
- **production of large quantities of gaseous products and related overpressure** (chiefly steam, carbon oxide or dioxide, nitrogen and oxygen)
- e.g. from 1 kg of explosives approximately 1,000 litres of post-explosion gases are produced. Overpressure is dependent on the distance from the blast epicenter.

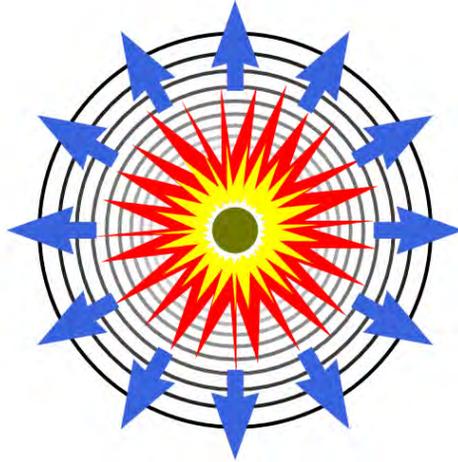


Figure 1. Overpressure model of explosion

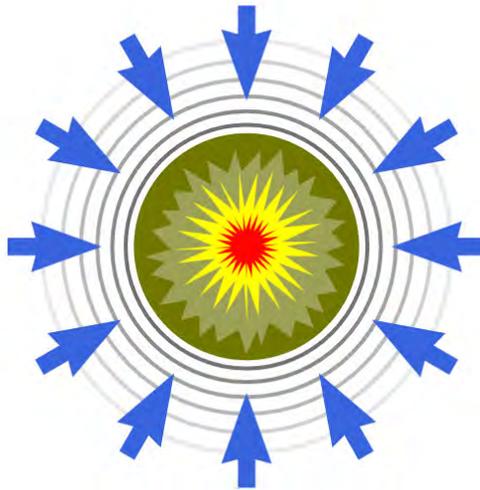


Figure 2. Underpressure model of explosion

1.2. Types of transformations

Detonation

Detonation is an blast transformation occurring in an explosive at constant and highest velocity in the form of a detonation wave which travels faster than sound.

Explosion

Explosion is a temporary form of a blast transformation of undermined velocity. Depending on the conditions, it may turn into detonation or deflagration.

Deflagration

Deflagration is otherwise known as explosive combustion, a type of blast transformation where thermal energy transmission from the reaction zone to the zone of an explosive material occurs through conductivity and radiation. Linear velocity of the dissipation process is significantly lower than sonic velocity in the same material (>340 m/s) and is external pressure-dependent.

1.3. Explosives

Explosives are solid or liquid chemical substances or mixtures of substances, which are chemically reactive and produce gas of such temperature and pressure and such speed that they may cause damages in their environment, as well as products filled with explosives.

Sensitivity of explosives is the capability of such explosive to be initiated by a specific external impulse. It is the smallest amount of external energy which should be supplied to cause an explosive reaction. This property is an initiating impulse. The smallest the initiating impulse required for causing an explosive reaction is, the more sensitive explosives are.

Factors defining sensitivity of explosives:

- **physical state** – explosives in a liquid state tend to be more sensitive than explosives in a solid state; case explosives demonstrate lower propensity for detonation than the pressed ones do;
- **temperature** – sensitivity increases with its growth;
- **density** – higher density causes (usually) lower sensitivity;
- **admixture** – additives harder than explosives, the so-called *sensitisers* e.g. metal fillings, crushed glass, sand – increase, while those of lower hardness, the so-called *flegmatizing agents* e.g. oils, petrolatum, paraffin – reduce sensitivity of explosives.

1.3.1. Division of explosives

Explosives are categories in terms of their:

I. **Chemical constitution**, including:

- 1) **chemical individual** (individual explosives) such as:
 - nitro-compounds (TNT, picric acid, hexyl);
 - nitrogen acid esters (nitroglycerine, nitroglycol, cellulose nitrate, penthrite);
 - nitroamines (hexogen, octogen);
 - chloric and perchloric acid salts (ammonium chlorate, ammonium perchlorate);
 - hydrazonic acid derivatives (lead(II) azide, copper azide);
 - fulminic acid derivatives (mercury(II) fulminate);
 - acetilne derivatives (acedilides – mainly silver and copper acetilides);
 - other explosive compounds (tetrazene, organic peroxides).
- 2) **explosive mixtures**, in two main groups:
 - explosive mixtures where at least component is an explosive e.g. a mixture of ammonium perchlorate and paraffin. An explosive mixture may also contain more explosive compounds. Such mixtures are mining explosive mixtures;
 - mixtures, which do not contain a single explosive component. The group includes black powder and similar mixtures and a large group of pyrotechnic mixtures.

II. **Application (purpose) presented in Figure 3:**

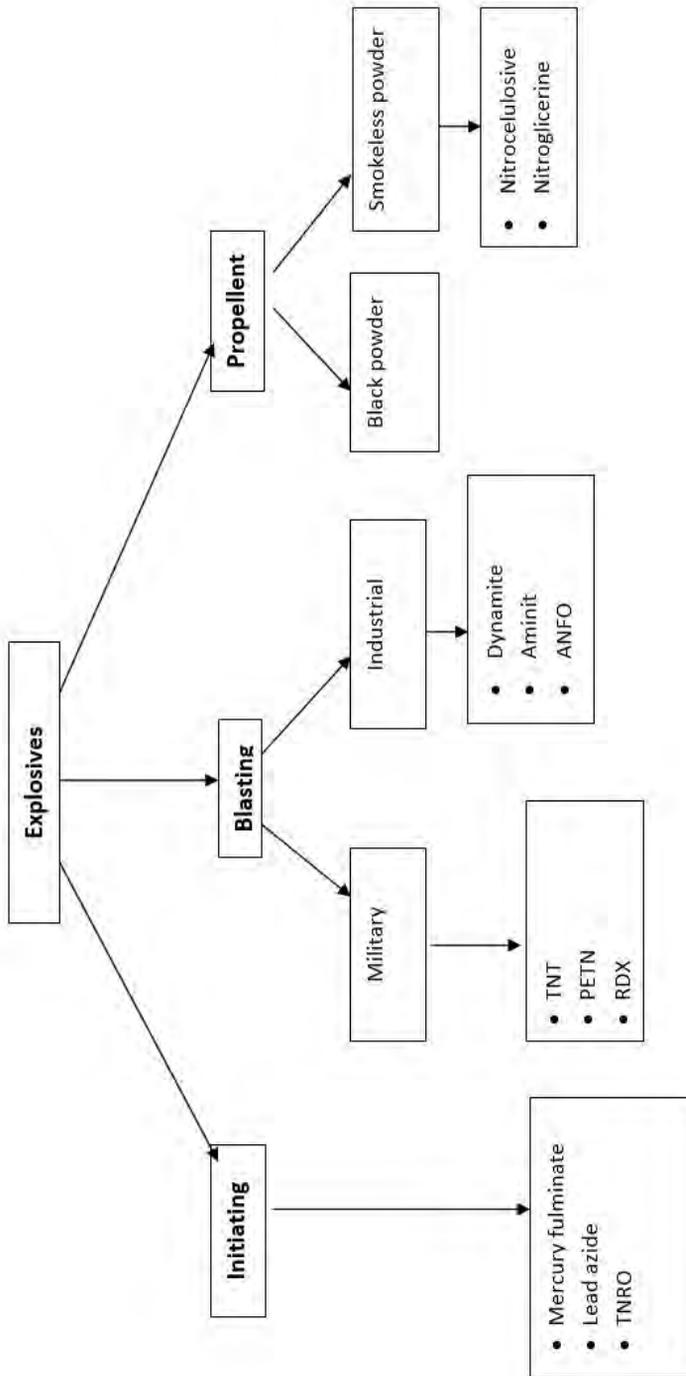


Figure 3. Application of explosives

- 1) **initiating explosives** i.e. chemical compounds or mixtures of chemical compounds who are capable of explosive detonation when impacted by an external stimulus e.g. a flame, blow, friction, used in initiating agents to initiate an explosive transformation (detonation) in other explosives, such as:
- mercury fulminate;
It is a white or grey crystalline body with sweet metallic taste, poisonous properties, poisonous properties causing irritation of the mucous membrane of nose, eyes and larynx and, when moist – eczema-resembling skin irritation.
 - lead azide;
A white, fine-crystalline substance. It is not hygroscopic and it is insoluble in water. It is characterised by a very short passage from ignition to detonation and that is why it detonates when impacted by any type of external stimuli, even if in very small quantities.
 - TNRO – lead trinitroresocynian;
It is a fine-crystalline, dark yellow solid, easily ignitable from a flame or an electric spark. It is a low hygroscopic solid, practically it does not dissolve in water and in organic solvents.
 - tetrazene;
A light yellow, non-hygroscopic and insoluble crystalline substance of sensitivity similar to mercury fulminate. Due to its relatively high sensitivity, predominantly used mixed with other initiators (2–3%) to enhance their sensitivity (sensitiser).
 - organic peroxides;
White chemical compounds of crystalline structure, produced mainly in home laboratories, such as:
 - HMTD – Hexamethylene triperoxide diamine (urotropine peroxide);
 - TATP/TCAP/CTATP – triacetone triperoxide (acetone peroxide).
- 2) **blasting explosives**, i.e. materials whose main explosive transformation is detonation. This group of explosives is used to fill different munition (missiles, bombs, grenades, mines) and rock shooting works in the mining industry, including:
- military, of normal potency, such as:
 - trinitrotoluene – TNT
Hard, light yellow or light brown crystalline substance with a bitter taste, insoluble in water, soluble in alcohol and acetone. Ignited, it burns with a heavily smoking flame but does not explode. Shot through with a rifle missile does not explode and does not ignite. Its forms: powdered, pressed and cast (Fig. 4).

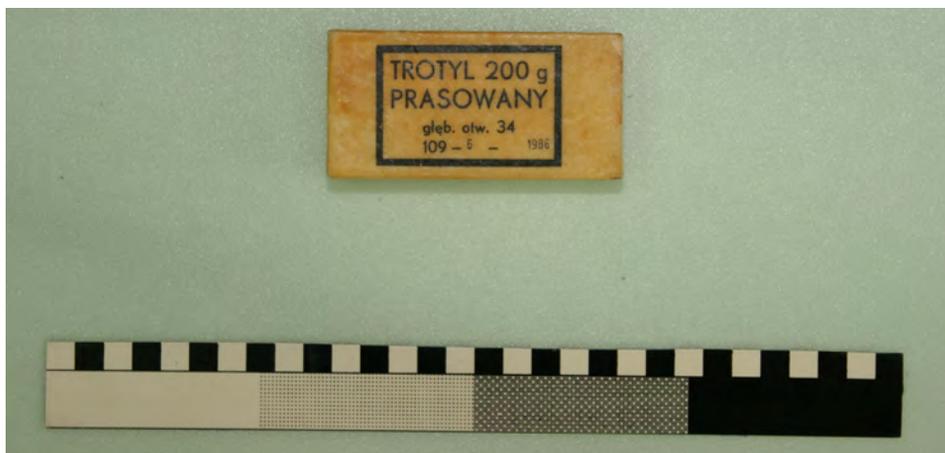


Figure 4. TNT cube (author's photo)

- melinite (picric acid) – TNF
A hard, light yellow, very bitter crystalline substance, weakly soluble in cold water, its solubility in warm water slightly improved. Solution dyes skin and animal tissue yellow. It is 1.5 times more sensitive to mechanical stimuli than trotyl. May explode when shot through.
- tetril
Tetril is a white-yellow powder material. It is slightly salty-tasting powder. It is non hygroscopic. It dyes skin red. It is very sensitive to friction and shock and detonates easier than trotyl and melinite.
- military, of increased potency, such as:
 - nitroglycerin (NG)
Chemically pure is a colourless, oily substance. Technical product is usually light yellow. Of all explosives used in practice, nitroglycerin is the least durable substance. It is very sensitive to shock and friction and, in this respect, its properties are similar to initiating explosives. Liquid nitroglycerine is more sensitive to shock while crystalline glycerine is less sensitive.
 - penthrite – PETN
A white, crystalline substance. It is toxic – it causes headache when touched with hands. Weakly soluble, in moisture does not lose its properties. It explodes when shot through with a rifle missile. It is difficult to ignite.
 - hexogen – RDX

A white, tasteless and odourless crystalline substance, which is non-hygroscopic and insoluble in water. Its sensitivity to mechanical stimuli and detonation is higher than sensitivity of trotyl and tetril. Does not explode when shot through with a rifle missile.

- octogen – HMX
 - Its chemical and explosive properties resemble those of hexogen. It is more durable than hexogen. Its high production cost prevents its wide application.
- used in the mining (industry) sector, such as:
 - dynamite
 - Material invented by Alfred Nobel and patented in 1867. Initially, it contained 75% of nitroglycerine and 25% of diatomaceous earth. Diatomaceous earth saturated with nitroglycerine is doughy and can be rolled in cartridges without the risk of a premature explosion. Modern dynamite contains approximately 10% of nitroglycerine and other components used in its production may be: diatomaceous earth, magnesium carbonate, wood flour, nitrocellulosis and ammonium nitrate (Fig. 5).



Figure 5. Dynamite (author's photo)

- Aminit
 - An explosive mixture with the ammonium nitrate as its main component. Another component is typically a substance with explosive properties e.g. trotyl or with flammable properties e.g. wood flour or aluminium dust. Aminit is weakly sensitive to thermal and mechanical stimuli. Depending on the additional substance mixed with ammonium nitrate, aminit is referred to as:

- amatol – a mixture with trotyl;
 - amonal – a mixture with aluminium dust;
 - saletrol “ANFO” – a mixture with liquid fuel e.g. engine oil.
- 3) **Propellant explosives** i.e. such with their basic form of explosive transformation is explosive burning (deflagration) such as:

- gunpowder (black powder)

Is a mechanical mixture of potassium saltpetre, charcoal and sulphur. Explodes from fire, from a spark, a thunderbolt, shooting a rifle mission and and also from rapid warming. Is hygroscopic, on contact with moisture it emits sulphur and becomes useless when wet. It is used for many purposes, including:

- production of flammable cords;
- filling gunpowder paths of time fuses;
- for production of ballast, ensuring loads of smokeless powder.

- smokeless powder

Is a type of powder, which burns in parallel layers without smoke (Fig. 6) Divides into:

- gunpowder on volatile solvent, known as nitrocellulosive,
- powder on a hardly volatile solvent, including: nitroglycerine and nitroglycol powder.

Nitrocellulose powder is grey and green and nitroglycerine powder is brown, they both demonstrate low sensitivity to friction and shock. They do not ignite when shot through with a rifle missile. Used for production of propellants for small arms and artillery.



Figure 6. Smokeless powder (author's photo)

- 4) **Pyrotechnic masses and mixtures** i.e. flammable mixtures, which give thermal, luminary, smoke, sound and incendiary effects used both in the military and civilian technology. In particular, the igniting and illuminating chemicals have been developed. Most pyrotechnic masses usually contain oxidants and flammable substances in the form of mixtures. In pyrotechnic termite and flame mixtures, magnesium and aluminium (their alloys and mixtures) are used while organic compounds are typically used in smoke mixtures. Nitrates and perchlorates are the main oxides in flame pyrotechnic masses while metal oxides are the main oxides in smoke mixtures. Furthermore, additions are introduced to the composition of pyrotechnic mixtures, e.g. flame-colouring salts, binder improving pressing and giving the required mechanical properties to the mass as well as stabilisers and phlegmatising agents to ensure securing in production and storage.

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MODULE VII

COUNTER-TERRORISM

1. FORMAL AND LEGAL BASIS FOR COMBATING TERRORISM

The threat of the use of CBRN-E in terrorist attacks means that law enforcement agencies must always be prepared to respond to such threats. This is just one part of the fight against terrorism. Efficiency in under such conditions requires interdisciplinary knowledge of:

- The formal and legal basis for combating terrorism;
- Decision-making under emergency conditions (e.g. in terrorist attacks);
- Terrorism as a threat to public order;
- Anti-terrorist actions involving CBRN materials.

Knowledge of these areas will help incident responders to identify the threats posed by terrorism to public safety, to understand the functioning of the various anti-terrorist systems, and to prepare them for decision-making in response to terrorist incidents – particularly CBRN-E.

Ensuring public security is one of the basic functions of the modern state. Terrorism is one of the greatest threats to the world and forces us to take action in the fight against it, to prevent and react to attacks. Like any state activity, the fight against terrorism must be based on legally binding acts and organisational arrangements. These must be influenced by both the domestic and international environments. Escalation of the potential for terrorist threats makes it necessary to continually improve anti-terrorism systems. Understanding the formal and legal bases for combating terrorism, and knowing what the legal and public security procedures are at the site of a terrorist attack, are all necessary preparations in the fight against terrorism. Security specialists should:

1. Have a basic knowledge of the structures and institutions of the state, including in the field of Counter-terrorism (CT), and the relationships between them.

2. Have an elementary knowledge of the international security structures, institutions and organisations combating terrorism in the EU, and the relationships between them.

3. Be able to utilise basic theoretical knowledge to analyse specific processes and social phenomena, and characterise the national security environment, including its dynamics and essential elements of the fight against terrorism.

4. Be able to analyse the causes and course of specific processes and phenomena in the field of international security. This provides the specialist with

a knowledge and understanding of the mechanisms of the various institutions, especially those engaged in combating terrorism.

5. Be able to participate in the preparation of various CT activities in national and international security, taking into account the legal and political aspects.

1.1. Basic concepts of counter-terrorism

State reactions to terrorist threats should consist in a complete, coherent system that provides for the most effective operations. The most common model includes activities in two areas:

1. Combating terrorism through society as a whole (defensive actions).
2. Combating terrorists directly (offensive actions).

This division takes into account NATO's military concept of defence against terrorism, which defines the fight against terrorism as the preventive, defensive and offensive measures taken to reduce the vulnerability of forces, individuals and property to terrorist threats and acts of terror. In the context of the NATO Comprehensive Approach, this can be combined with or followed by measures enabling recovery from terrorist acts. This position indicates that, despite NATO's military character, it takes a broader view of security into account. NATO activities in this area focus on:

- Compliance with International Law;
- Support for Allies;
- Non-Duplication and Complementarity.

NATO's international CT work is achieved through, amongst others, close contact and ongoing communication with various international organisations, including the United Nations, the European Union, the Organization for Security and Co-operation in Europe (OSCE), and the Global Counterterrorism Forum (GCTF). An example of its engagement in Europe is the creation on February the 16th, 2017 of its Regional Hub for the South, based at NATO's Joint Force Command in Naples. The Hub is a focal point for enhancing both the Alliance's understanding of the challenges of the region and its ability to respond to them.

Proponents of the security sciences propose a number of different models for combating terrorism, analysis of which should provide a comprehensive approach to the issue. Some of these go beyond the scope of defensive and offensive activities.

Prevention of terrorism includes deterrents designed to discourage individuals or groups from engaging in terrorist activities, as well as the social policy of the state on people considered vulnerable to indoctrination by extremist groups. Combating terrorism has the following phases:

- Establish: recognize and identify opponents;
- Neutralise: arrest or eliminate threats;

- Punish;
- Impede: prevent opponents from returning to terrorist activity.

These phases include forecasting the identification of potential targets, risk analysis, and risk assessment of the likelihood of attacks. Responses include measures to minimise the effects of terrorist attacks. Protection should be provided for both people and infrastructure likely to be threatened by terrorists.

The most common model of combatting terrorism, however, is a model that consists of two phases:

1. Anti-terrorism.
2. Counter-terrorism.

1.1.1. Anti-terrorism

Anti-terrorism is the combating of terrorism. K. Jałoszyński defines anti-terrorism as the use of protective measures reducing the vulnerability of people, forces and property to terrorism. It is realised in the following ways:

- Diplomatic;
- Economic;
- Administrative;
- Legal;
- Police and paramilitary;
- Intelligence;
- Social Policy.

Diplomatic activities involved in anti-terrorism are all forms of international policy aimed at creating a unified front against terrorism. The most visible manifestation of this is the formation of international anti-terrorist coalitions, which includes work between militaries or police forces. Poland is consistently present in the global anti-terrorist coalition.

Economic activities are focused on creating the capacity to control the flow of certain funds, to halt terrorist financing. For example, the US Patriot Act gave federal agencies the opportunity to request access to information from American financial institutions. This privilege has been strengthened with a tool for enforcing these demands. Refusal now results in an order to cease cooperation with any suspect entities. Within the Council of Europe, work in this area is primarily carried out by the Committee of Experts on the Evaluation of Anti-Money Laundering Measures and the Financing of Terrorism – MONEYVAL. This is a permanent monitoring body of the Council of Europe, entrusted with assessing compliance with principal international standards countering money laundering and the financing of terrorism, and monitoring the effectiveness of their implementation. It also has the task of making recommendations to national authorities on necessary improvements to their own systems. Through a dynamic process of mutual evaluation, peer review and regular follow-ups on

its reports, MONEYVAL aims to improve the capacities of national authorities to fight money laundering and the financing of terrorism more effectively. In Poland, the Chief Inspector of Financial Information is responsible for obtaining, collecting, processing and analysing information and taking action to counter money laundering and terrorist financing.

Administrative activities are aimed at the creation and improvement of government crisis response systems, which define the responsibilities of responders in taking concrete action to prepare for, react to, and recover from terrorist activities. One examples of this is the uniform system of alarm levels in the event of terrorist threats. Declaring an alarm imposes the obligation to perform the specific actions assigned to the given alarm level. This makes it possible to co-ordinate readiness to respond to terrorist events. These actions also include the issuing of decisions by the authorities, such as the prohibition of assembly in an area where there is a threat of a terrorist attack.

Legal activities consist in the creation of national and international law enabling prosecution and counter terrorism. Poland has ratified a number of international conventions and international agreements on the fight against terrorism, including those created by, for example:

- The United Nations;
- The Council of Europe.

These agreements, conventions, and decisions affect the international environment to varying degrees, and have different effects. One of the effects of international law is the cataloguing of crimes that cannot be considered political offenses that are nevertheless involved in a political offense, or committed for political reasons. The consequences of defining such a catalogue of crimes is to set aside the possibility of refusing to release the perpetrator of a terrorist offense to a state applying for extradition (the European Convention on the Suppression of Terrorism, Strasbourg, 27 January 1977). Another legal area in which anti-terrorist functions are being implemented is criminal law. Criminalization of terrorist behaviour is an essential element in the fight against terrorism. The Polish Penal Code defines terrorist offenses in Art. 115 Par. 20, making them a prohibited act punishable by imprisonment of at least 5 years. This includes the act of, or threatening to:

1. Seriously intimidate many people.
2. Force the authorities of the Republic of Poland or another state or organ of an international organization to take or discontinue certain activities.
3. Cause serious disturbances in the political system or economy of the Republic of Poland, other states or international organization.

Apart from terrorist crimes in Poland, the following are also related, punishable offenses:

- Leadership or participation in a group for the purpose of committing terrorist offenses;

- Financing of terrorism;
- Provision of knowledge and information for the purposes of committing a terrorist offense;
- Participation in training that would allow the commissioning of a terrorist offense;
- Crossing the border of the Republic of Poland to commit terrorist offenses in other countries;
- Making public calls for terrorist offenses;
- Persuading others to commit terrorist offenses (recruitment);
- Not sharing knowledge of planned terrorist offenses with the authorities.

Police and paramilitary activities are aimed at increasing police powers to stop people, check luggage and search residences; increasing security measures in public places that may be subject to terrorist attacks, and checking and strengthening critical infrastructure security. In these activities, the armed forces are often used as support for preventive and patrol activities in public spaces. An example of such a systemic approach is the VIGIPIRATE program, in which street patrols are made by units of the French Armed Forces.

Intelligence consists in gathering information and data basing it to enable the identification of suspects and the fight against terrorism. For these purposes, methods include the work of human agents, technical information gathering (eavesdropping, surveillance, satellite intelligence, etc), trawling the Internet, and so on. These activities are dealt with by special services with legal and technical capacities in these fields.

Social policy consists in running social programs discouraging terrorist activity, by counteracting radicalization, xenophobia and social exclusion. Successfully educating the public and residents of a country can limit the impact of terrorism on its society, and prevent it from developing in the first place.

1.1.2. Counter-terrorism

Counter-terrorism activities are carried out by special units that directly address the threat posed by terrorists using both firearms and non-lethal weapons. Counter-terrorism activities can be separated into:

- Counter-terrorist liquidation operations – conducted in response to terrorist incidents (combat rescue operations);
- Pre-emptive counter-terrorist combat actions – to prevent terrorist offenses by eliminating or stopping the perpetrators before they can act.

Counter-terrorist liquidation operations (combat rescue operations) are carried out by police or military units specialising in CT operations, with the aim of:

- Saving the lives of people threatened by terrorists;
- Neutralizing terrorists.

Characteristics of counter-terrorist liquidation operations include:

- Strict time limits;
- Immediate response;
- Highly dynamic action;
- Very high risk;
- Persistent threat to the lives and health of victims and CT operators.

Examples of counter-terrorist liquidation operations include the GIGN action against the Kouachi brothers in Dammartin-en-Goële, after the attack on Charlie Hebdo; the RAID action against Amedy Coulibaly at a kosher supermarket in Paris on the 10th of January 2015, and the BRI and RAID response in Paris, on the 13th of November 2015, in response to the attack at the Bataclan theatre.

Pre-emptive counter-terrorist combat actions are carried out by police or military units specialising in CT operations against terrorists who have been found to be still in the planning and preparation phase of their attacks, in order to:

- Stop or neutralise the terrorists;
- Prevent planned attacks from occurring.

Characteristics of pre-emptive counter-terrorist combat actions include:

- Time and place of the operation determined by the authorities/CT units;
- Detailed planning and preparation process;
- Highly dynamic implementation;
- Operations conducted so as to minimise the risk to civilians;
- Direct threat to the lives and health of the CT operators.

Examples of pre-emptive counter-terrorist combat actions include the January 15th 2015 arrest in Verviers, Belgium, of three Islamic terrorists at their place of residence (two were killed, one was wounded). The operation was carried out by the Belgian police's counter-terrorism unit (DSU), in a joint operation with the GIGN. In the same operation, 13 other terrorists were arrested.

Counter-terrorism tactics evolve with the methods and tactics of the terrorists. CT operators modify their tactics depending on the situation, as well as their equipment loadouts. All of their options are analysed to try to reach the ideal solution. In most countries, there are special rules for CT operators' use of firearms against terrorists (e.g. 'kill shots' and 'Shoot on sight/Shoot to kill'). This ground-level approach to fighting terrorism is particularly important to the safety and security of civilians.

1.2. Example: Combating terrorism in the Polish Republic

Poland's anti-terrorist system is composed of services and institutions designed to identify and neutralise terrorist threats. It has appropriate powers and competences, is equipped with the means of providing a physical response,

and integrates communications systems, information exchange and direct action under the same command, all while keeping the public informed about real and potential threats. The Polish anti-terrorist system operates on three levels:

- Strategic;
- Operational;
- Tactical.

At the **strategic** level of the Polish anti-terrorist system, the legislation, anti-terrorism policy and organisational solutions needed to legally conduct CT operations are all devised. Systemic solutions are created at this level, defining the state's reactions in the event of terrorist incidents. Entities in the executive branch are supported by collegiate bodies, which provide substantive support in the implementation of the country's anti-terrorism operations.

The Prime Minister instigates activities by issuing regulations and guidelines for the implementation of statutory instruments in anti-terrorism policy. He also works directly, overseeing the work of the Government Crisis Management Team.

The Council of Ministers is then responsible for establishing and approving counter-terrorism strategy, subsequently creating state policy around it. It also makes decisions on other applicable regulations. The Council can also ask the president to declare a state of emergency, and pass emergency laws. To assist the Council of Ministers in its work, the Interdepartmental Task Force on Terrorist Threats was established, which is chaired by the Minister of Internal Affairs.

The Minister of Internal Affairs is responsible for the protection of public security and public order, and for crisis management. In emergency situations, it can work independently. The Minister is also responsible for the preparation, response and re-supply of the resources used to combat terrorism. The Minister of Internal Affairs supervises the services working in the anti-terrorist system, including the Police, Border Guard, Government Protection Bureau, and Fire Department.

The Interdepartmental Task Force on Terrorist Threats is an auxiliary body of the Council of Ministers, and is appointed by the Prime Minister. Its meetings are held on a regular basis, as and when needed. The Task Force consists of:

- Chairman – Minister of Internal Affairs;
- Deputy – Minister in charge of public finance, Minister of National Defence, Minister of foreign affairs, Minister of Justice, and Members of the Council of Ministers, Coordinator of Special Services;
- Secretary – a person appointed by the chairman of the team from the staff of the office, to serve the Minister of Internal Affairs;
- Secretary of State at the Ministry of Interior;
- The Secretary of the Special Services Board, or their substitute;
- Chief of Civil Defence of the country or their deputy;
- The Head of the Internal Security Agency or their deputy;

- The Head of the Foreign Intelligence Agency or their deputy;
- The Head of the Government Protection Bureau or their deputy;
- Commander in Chief of the Police or their deputy;
- Commander in Chief of Border Guards or their deputy;
- Commander in Chief of the State Fire Service or their deputy;
- The Chief of General Staff of the Polish Army or their deputy;
- Operation Commander of the Armed Forces or their deputy;
- The Head of the Military Intelligence Service or their deputy;
- The Head of the Military Counterintelligence Service or their deputy;
- Commander in Chief of the Military Police or their deputy;
- General Inspector of Revenue Control or its replacement;
- General Inspector of Financial Information or their replacement;
- Head of the Customs Service or their deputy.

The Interdepartmental Task Force on Terrorist Threats' responsibilities include:

- Monitoring, analysis and evaluation of terrorist threats;
- Presentation of opinions and proposals to the Council of Ministers;
- Development of draft anti-terrorism standards and procedures, in particular standards for assessing and evaluating the presence of a threat;
- Initiation, coordination and monitoring of activities undertaken by the competent governmental administration, particularly regarding the use of information and the identification, counteracting and combating of terrorism;
- Requesting the appropriate ministers to take legislative action to improve the means and forms of combating terrorism;
- Arranging work with other countries in the fight against terrorism and coordination of the exchange of information and joint operations;
- Initiating training sessions and conferences on counter-terrorism.

Finally, the Government Crisis Management Team is the body responsible for initiating and coordinating crisis management activities, including those related to terrorist threats. It is the task of the Team to prepare proposals for the use of forces and resources needed to deal with emergencies, and to advise on the coordination of the activities of governmental authorities, including in the case of terrorist attacks.

The **operational** level of Poland's anti-terrorist system handles the coordination of information exchange and work between anti-terrorist system operators, including the ongoing analysis of terrorist threats. The Internal Security Agency's Anti-Terrorism Centre is responsible for coordinating analytical and information activities conducted by all anti-terrorist organizations.

The essence of the Centre's activity is the rapid and efficient transmission of information on terrorist threats, specifically:

- Terrorist incidents occurring outside the Republic of Poland that affect the security of Poland and its citizens;

- Terrorist incidents occurring inside the Republic of Poland that affect the security of Poland and its citizens;
- Information on potential threats to Poland and abroad;
- Information on money laundering or transfers of funds that may indicate the financing of terrorist activity.

The Anti-Terrorism Centre is active all the time, and is staffed by officers and soldiers who provide all of its services as part of the country's AT system.

The Government Security Centre/Government Crisis Management Team fulfils the role of a national crisis management centre. It provides full-time, on-call duty for crisis management issues, permanent on-call defence forces, and exchange of information on international and national issues, including implementation of the NATO Crisis Response System.

The **tactical** level of the anti-terrorist system encompasses ground-level combat forces and emergency responders involved in the protection of Poland. These include:

- Internal Security Agency;
- Foreign Intelligence Agency;
- Military Counterintelligence Service;
- Military Intelligence Service;
- Central Anti-Corruption Bureau;
- General Inspector of Financial Information;
- Police;
- Border Guard;
- Government Protection Bureau;
- Fire Brigade;
- Customs Service;
- Military Police;
- Ministry of National Defence;
- Ministry of Foreign Affairs.

All of these entities carry out the tasks resulting from the legal provisions directly connected with prevention, preparation for reacting, reacting and rebuilding of resources after activities conducted within the anti-terrorist system of the Republic of Poland.

1.3. The legal basis for combating terrorism

Over the last few years, the legal and organisational framework for combating terrorism in Poland has changed significantly:

- Until 2014, there were no systemic regulations;
- In 2015–2016, attempts were made to streamline operations at the political and administrative levels;

– 2016 saw the introduction of the Act of 10 June 2016 on Counter-terrorism Activities. This comprehensive law regulates the fight against terrorism at all levels.

Until as recently as 2014, the fight against terrorism in Poland was based on a variety of scattered legal grounds, with many areas not regulated at all. Recognition, detection and prevention of terrorist offenses, and the prosecution of their perpetrators, were the domain of the Internal Security Agency. In this work, the ISA was the only one with any clearly indicated responsibility. Furthermore, there was no one service assigned the task of directly combating terrorism and responding to terrorist attacks. The most developed CT system at the time existed with the Police, as part of their overall remit to protect public safety. Eventually, the lack of fully-fledged anti-terrorist law was seen to be very dangerous when it appeared that terrorist attacks could actually happen in Poland. Concerns were so great that in 2015 an administrative agreement was signed between the heads of the major emergency services, designating responsibility for terrorist incidents. The co-signees were the:

- Head of the Internal Security Agency;
- Commander in Chief of the Police;
- Commander in Chief of the Border Guard;
- Commander in Chief of the Military Police;
- Commander in Chief of the State Fire Service.

The agreement states that, in the event of terrorist incidents or suspicion of such incidents, incident management shall order the services' work together through appropriate utilisation of personal, material and financial resources. As a general rule, it was assumed that responding officers would be managed by a Police officer and a Military Police officer.

The next step taken in building responsibility for Poland's anti-terrorism response came in 2014, with the enactment of the 2015–2019 National Anti-terrorist Program. In the core document of the program, a general interpretation of the responsibilities of the various entities in Poland's anti-terrorist system was made, in the context of the legal framework. The National Anti-terrorist Program was not a source of legislation itself, and although it didn't alter the extent of the duties of the individual services involved, it did clarify them. It also crystallised the importance of the need for co-ordination of the activity of the participating services, and defined the range of duties of the heads of the services as at January 2015. The police are responsible for coordinating threat response in civilian areas, with the Military Police (with the consent of the subordinate parts of the Ministry of National Defence).

But the breakthrough moment for the gradually coalescing anti-terrorist system in the Republic of Poland came in 2016, when the Act of 10 June 2016 on Anti-terrorist Activities (Journal of Laws of 2016, item 904) was passed. It was originally created to ensure safety at the NATO summit in Warsaw and the

World Youth Day in Krakow, both in July of that year. The Act comprehensively regulates the fight against terrorism in Poland, and specifies which entities have which duties. Some of its stipulations sanction the existing organisational arrangements, and also legally oblige the various services to perform certain tasks. The Act also introduced special rules on the use of weapons in counter-terrorist operations.

1.4. Example: The 2015–2019 National Anti-Terrorism Program

Adopted by the Council of Ministers in 2014, the main objective of the 2015–2019 National Anti-terrorist Program was to legally reinforce and clarify Poland's anti-terrorist system. With this in mind, the Program sets specific objectives:

- Improve the nation's capacity to prevent terrorist attacks;
- Strengthen the preparedness of services and institutions for the possibility of terrorist incidents;
 - Increase responsiveness in the event of a terrorist incident;
 - Improve the efficiency of the forces and resources utilised;
 - Improve existing procedures for dealing with terrorist threats.

These objectives are met in order to better respond to, and deal with, the following events, which are classified as being terrorist incidents:

- Detonation of explosive devices;
- Assault with firearms;
- Abduction of people, including taking them abroad;
- Occupation of means of transport, including the holding of hostages;
- Abduction of means of transport:
 - land transport,
 - water transport,
 - air transport.
- Assault with aircraft having RENEGADE status;
- Assault with a vessel or a floating object for which it was granted the status of MARITIME RENEGADE;
- Assault on the health, life or freedom of protected persons;
- Assault with the use of:
 - Biological agents,
 - Chemicals,
 - Radioactive material.
- Cyber terrorist attacks;
- Attacks on critical infrastructure;
- Other types of terrorist attack;
- False notification of a terrorist threat.

For each of the five specific objectives listed above, areas of responsibility were identified and priorities assigned in the various phases of anti-terrorism prevention and response in Poland, as well as the lead entity in charge of each phase. These can be broken down as follows:

Prevention

1. Recognising terrorist threats – Internal Security Agency (international terrorism is handled by the Foreign Intelligence Agency).
2. Analysing, forecasting and assessing terrorist threats, including conducting analytical and information-gathering activities – Internal Security Agency (international terrorism is handled by the Foreign Intelligence Agency).
3. Coordinating the exchange of information on terrorist threats – Internal Security Agency.
4. Monitoring the media for information with which to prevent terrorist incidents – Internal Security Agency.
5. International cooperation on the prevention of terrorist threats – Internal Security Agency.
6. Informing the public of terrorist threats – Internal Security Agency.
7. Counteracting the financing of terrorism – General Inspector of Financial Information.
8. Public information and education policy – Ministry of the Interior.
9. Migration and asylum policy – Ministry of the Interior.
10. Protection of the state border – Border Guard.
11. Protection of Polish airspace – Polish Armed Forces.
12. Participation in stabilization, peacekeeping and international missions, and anti-terrorist coalitions – Ministry of National Defence.

Preparedness

Although the 2015–2019 National Anti-terrorist Program does not designate responsibilities in the preparedness phase, the following areas of action are identified:

1. Development of plans and increasing of resources.
2. Evaluation of potential targets of terrorist attack.
3. Critical Infrastructure Protection.
4. Protection of transport systems.
5. Protection of population centres.
6. Work with the private sector.
7. Participation in exercises and training.

Response

In responding to a terrorist incident, the following areas of action are specified:

1. Oversight of crisis management on the territory of Poland – The Council of Ministers (in urgent situations – the Minister of Internal Affairs).
2. Oversight of the Government Crisis Management Team – The Prime Minister (in urgent situations – the Minister of Internal Affairs or the Minister of National Defence).
3. Provision of crisis management services to the Government Security Centre.
4. Coordination of information exchange and the information policy of the public administration during a crisis – Government Security Centre.
5. Preparation for launching emergency management procedures (in the event of an incident) – Government Security Centre.
6. Actions against floating facilities used in terrorist attacks – Border Guard, followed by the Polish Armed Forces.
7. Actions against aircraft used in terrorist attacks – the Polish Armed Forces.
8. Recovering personnel lost as a result of abduction by terrorist organizations or organized crime groups – Ministry of Foreign Affairs (for soldiers – Ministry of Defence).
9. Recommendations for handling crises as a result of terrorist incidents, that threaten critical infrastructure, the life or health of people, property of considerable size or national heritage, etc, or the environment – Internal Security Agency.
10. Creation, conduct and coordination of information policy – Ministry of Interior.
11. Response to chemical contamination incidents, including in the context of terrorist attacks – the State Fire Service.
12. Responding to the use of biological agents in terrorist attacks – Ministry of Interior, with the support of the Ministry of Health, the Ministry of the Environment and the Ministry of National Defence.
13. Oversight of the elimination of nuclear threats to the nation, including in the context of terrorist attacks – Minister of Internal Affairs, with the help of the President of the National Atomic Energy Agency.
14. Decontamination procedures – Voivode or competent territorial self-government unit.

At an incident scene

1. Medical emergency operations at the scene of a terrorist incident – State Medical Rescue and National Rescue and Firefighting System (at sea – Marine Search and Rescue Service).

2. Physical combat against terrorists/counter-terrorism – Police (on military territory – Military Police).
3. Securing incident scenes – Police (on military territory – Military Police).
4. Mining-pyrotechnic activities at an incident scene – Police (on military territory – Military Police).
5. Coordination the work of the emergency services at the scene of a terrorist incident in a pre-accession stage investigation – Police (on military territory – Military Police).
6. Investigation of terrorist incidents – Public Prosecutor’s Office.
7. Detection of terrorist offenses and prosecution of their perpetrators – Internal Security Agency.
8. Preliminary decontamination activities – State Fire Brigade.

Reconstruction

At this stage, all of the entities involved in an incident response are required to re-supply the resources they need to respond to future incidents. The Minister for Internal Affairs is responsible for overseeing this task.

An Action Plan is also part of the 2015–2019 National Anti-terrorist Program, which specifies the projects to be implemented in all phases. Implementation of these tasks is overseen by the Interdepartmental Task Force on Terrorist Hazards, on the basis of a timetable for the tasks’ completion by the various services, developed by the heads of the individual services.

Competences of CT entities in the Republic of Poland

Under the 2016 Act on Anti-terrorist Activities, the key entities in Poland’s anti-terrorist system, their areas of responsibility and powers are all clearly defined, as follows:

Head of the Internal Security Agency

Area of responsibility:

- Prevention of terrorist incidents.

Powers:

- Collecting information on terrorist threats;
- Coordination of analytical and informational activities undertaken by other services in the field of terrorist threats;
- Issuing information and instructions to entities threatened by terrorist incidents, to prevent, remove or minimise the threat;
- Maintaining a database on people involved in terrorist activities;
- Immediately informing the President of the Republic of Poland, the

Prime Minister, the minister responsible for Internal Affairs, the Minister for National Defence, the Minister for Foreign Affairs, the Minister for the Special Services Coordinator of any information relevant to preventing terrorist incidents;

- Coordination of intelligence and reconnaissance activities in terrorist incidents;

- Issuing recommendations to special services and supporting entities on eliminating or minimising the threat of terrorism;

- Implicit management of non-RP citizens using operational techniques (correspondence control, eavesdropping);

- Access to state registers;

- Issuing Police recommendations on protection of specific facilities, in the face of terrorist incidents;

- Appointing coordinating staff in the event of a terrorist alert or CRP (cyber terrorism) alert.

The Minister of Internal Affairs (Minister of Internal Affairs and Administration)

Area of responsibility:

- Managing terrorist incident response through planned actions, responding to incidents as they occur, and maintaining needed resources.

Powers:

- In urgent situations, the introduction, changing and cancellation of alerts;

- Applying to the Minister of National Defence for use of the Armed Forces of the Republic of Poland in assisting the Police in the event of third- or fourth-level alerts (acc. to the 4-level alert system used in Poland);

- Supervising the Police, Border Guard, State Fire Service and Government Protection Office in their tasks under the Anti-Terrorism Act.

The Minister of Foreign Affairs (with the Minister of Special Services Coordinator)

Area of responsibility:

- Coordination of the activities of special services and entities responding to terrorist incidents outside the Republic of Poland, involving the citizens or property of the Republic of Poland.

Powers:

- Requesting appropriate departments and services to provide information, reports and options for proposed solutions;

- Requesting consultation on activities undertaken by special services and entities.

The Minister of National Defence (with the Minister of Foreign Affairs)

Area of responsibility:

– Coordination of the activities of special services and entities responding to terrorist incidents outside the Republic of Poland, involving the personnel or property of the Armed Forces of the Republic of Poland.

Powers:

– Requesting appropriate departments and services to provide information, reports and options for proposed solutions;
 – Requesting consultation on activities undertaken by special services and entities.

Police/Military Police (In military areas)

Area of responsibility:

– Conducting counter-terrorist activities.

Powers:

– Directing anti-terrorist activities at the scene of a terrorist incident;
 – Command of counter-terrorist forces.

1.5. System of police anti-terrorist units

In February 1976, the first Polish counter-terrorist unit was established – the Security Department of the Capital of the Civic Militia, and was one of the first of its kind in Europe. The Germans had established their GSG-9 the year before, in 1974, while Austria formed its COBRA unit in 1977, the Portuguese their GOE in 1979, and France founded its RAID CT team in 1985.

Until 1990 in Poland, special militia forces were stationed in the largest regional towns, and these also had the task of combating terrorism. In 1990, specific Anti-Terrorism Units were then established in nine provincial capitals (Łódź, Gdańsk, Białystok, Rzeszów, Kraków, Katowice, Wrocław, Poznań, Szczecin), along with the Warsaw Police Department's own Anti-terrorist Department. In 2000, the name 'Anti-Terrorism Units' was changed to 'Independent Police Anti-terrorist Subdivisions'. At the same time, sub-units were created, being the Anti-Terrorist Sections of the Provincial Police Headquarters (in Opole, Gorzów Wielkopolski, Bydgoszcz, Olsztyn, Radom, Lublin and Kielce).

On March the 6th, 2003, the greatest tragedy in the history of Polish anti-terrorism occurred. The Warsaw Unit, which was at that time part of the Central Bureau of Investigation of the National Police Headquarters, stormed a house in which two dangerous criminals were hiding. As a result of the detonation of an explosive device, two policemen were killed and 17 wounded. During an exchange of gunfire, both the criminals were killed.

Over the next almost 15 years, until May 2017, the organizational structure of all Police Counterterrorism units was changed to make 18 independent anti-terrorist subdivisions stationed in all regional cities, with the Anti-terrorist Operations Bureau of the National Police Headquarters in Warsaw as the central, coordinating unit. At present, the Police have a system of counter-terrorist units prepared at all times to conduct combat activities throughout Poland, as well as to participate in joint operations in the EU.

1.5.1. The Anti-terrorist Operations Bureau at the National Police Headquarters

The Anti-terrorist Operations Bureau at the National Police Headquarters [BOA KGP] is subordinate to the Deputy Commander of the Office of the National Chief of Police. The office is made up of combat units with specific specialisations, including a combat training division and a support division.

The BOA KGP's Tasks include combating terrorism and organising, coordinating and supervising the activities of the Police in this regard (counter-terrorist activities, including physical combat). In the execution of its tasks, it is legally considered a 'special intervention unit', as referred to in Council Decision 2008/617/JHA of 23 June 2008 on improving the cooperation between special intervention units of the Member States of the European Union in crisis situations.

The Commander in Chief of the Police, or the authorized Deputy Commander in Chief of the Police, direct the activities of the BOA KGP's officers within Poland. In the capital city of Warsaw and its surrounding Mazowieckie province, the Director of the BOA KGP directs the officers' activities.

1.5.2. The Independent Anti-Terrorist Sub-units

These are subject to the Deputy Commissar of the Voivodship Police of the Department of Prevention, and consist of combat teams, a combat-training team, and a support team. Their tasks include:

- Preparation for and conduct of counter-terrorist activities, including physical combat;
- Preparation for and conduct of rescue operations in the event of direct threats to human life and health;
- Preparation for and conduct of combat operations requiring the use of specialized forces and means, or the application of special tactics;
- Implementation of tasks in the protection and support of protected persons;
- Conduct of pyrotechnic activities;
- Supporting rescue operations.

Conducting training and professional development of the police officers of the Police Counterterrorism Unit and the organisational unit of the Police Headquarters, competent in anti-terrorist matters, in terms of their acquisition of skills and competences and maintaining the high level of physical fitness necessary for the execution of their tasks. Their work is directed by the Voivodship Police Commander or an authorised deputy Voivodship Police Commander.

1.5.3. The Central Rear Guard Anti-Terrorist Unit of the Chief of Police

The Police anti-terrorist units and the organizational unit of the Police Headquarters, which are competent in anti-terrorist matters, as well as mining and pyrotechnical units, constitute the Central Rear Guard Anti-Terrorist Unit of the Chief of Police (COAT KGP). Its tasks include:

- Actions requiring the use of the forces and resources of more than one anti-terrorism sub-unit of the Police, or its police mining and pyrotechnical units, carried out as part of police operations,
- Announcement of second-level emergency alerts (as described in the Decree of the Prime Minister on the list of projects and procedures of the crisis management system),
- Announcement of third-level emergency alerts (as described in the Decision of the Minister of Internal Affairs on establishing the manner and mode of introducing, changing or cancelling alert levels).

As soon as COAT KGP is activated, all Police AT units are subordinated to the COAT KGP commander. The COAT KGP commander could be the director or deputy director of BOA KGP, and is appointed each time by the Chief Police Officer. The COAT KGP commander has the authority to:

- Activate police anti-terrorist sub-units and police mining and pyrotechnical cells,
- Coordinate the use of the forces and resources of the anti-terrorist sub-units, and the police mining and pyrotechnical units,
- Recommend commanders for police operations to the commanders of counter-terrorism operations.

The Central Rear Guard Anti-Terrorist Unit of the Chief of Police is a driving force in the work of CT units throughout the country.

1.6. The ATLAS European Special Police Units network

After the attacks in New York City on September 11, 2001, it seemed that nowhere in the world was secure, and that the threat of terrorism could take on an unprecedented scale. In the European Union, consideration was given

to strengthening its capacity to respond to terrorist threats. On October 15, 2001, in Brussels, at a meeting of 15 European police counter-terrorist units, the willingness to cooperate in the preparation of counter-terrorist operations was expressed. A slogan expressing this mutual desire was picked, *All Together To Protect You*. However, initial work on this mutual system of cooperation was undertaken without the support of the EU. In 2005, the Council of Europe adopted an anti-terrorism strategy. Then, in 2007, Javier Solana, the EU representative for Common Foreign and Security Policy, appointed Gilles de Kerchove as Counter-Terrorism Coordinator. The CT Coordinator was given the task of:

- Coordinating the work of the Council in the fight against terrorism;
- Presenting policy recommendations to the Council and propose action priorities, on the basis of threat analyses and reports prepared by the EU Intelligence Analysis Centre and Europol;
- Closely monitoring the implementation of EU Counter-Terrorism Strategy;
- Following all the instruments at the disposal of the European Union, regularly reporting to the Council and effectively implementing its decisions;
- Coordinating activities with the relevant preparatory bodies of the Council, the Commission and the EEAS, and sharing information about their work;
- Overseeing EU activity in the fight against terrorism;
- Improving relevant communications between the EU and other countries.

After this, in 2008 the legal basis for the operation and financing of the European ATLAS Collaboration Platform was established. This was made possible by the introduction of a number of Council decisions on cross-border cooperation and improved cooperation between the special intervention units of the Member States of the European Union. Chief among these was Council Decision 2008/617/JHA of 23 June 2008 on improving cooperation between the special intervention units of the Member States of the European Union in crisis situations (EU L 210, item 73). From the 1st of January 2009, it was then possible for special intervention units from one country to provide assistance to another European country, if requested, for assistance. This legal decision assumes that no one Member State has all of the means, resources and technical know-how needed to respond effectively to a crisis or crisis situation, requiring special intervention on a large scale. It was therefore considered essential that special intervention units meet on a regular basis and organise joint training sessions to share their experience. The records of these first meetings provided the opportunity for the ATLAS Group to act formally, and to obtain a budget from the Council of Europe. According to the ATLAS Convention, the aim of the group is to strive to create states in which every member of an anti-terrorist unit is at the same – highest possible – level. ATLAS achieves this by creating a network of mutual cooperation and assistance, thus achieving equal opportunities in the

fight against crime and terrorism (ATLAS Convention – not published). ATLAS members are national counter-terrorist units, as reported by their state authorities (limited to 2 units per member state). Units from non-EU countries can also be invited to participate as non-voting observers, after a majority vote by all voting members. These units are represented by their commander or a designated officer. ATLAS is managed by a president, elected for two-year terms (the current president is from COBRA, Austria), and this function is non-profit. Decisions are made jointly, at the ATLAS Commanders Forum twice a year. Organisational support is provided by the Executive Office, financed by the European Council, which gives ATLAS a budget of 1.5 million Euros. At present, ATLAS conducts training, information exchange, research projects and joint operations. Due to the situation in Europe, the cooperation of ATLAS units has become more important over the last two years, and today there are joint anti-terrorism operations all throughout the EU.

Since 2005, Poland's ATLAS member has been the Anti-Terrorist Operations Bureau of the National Police Headquarters. Units from Norway and Switzerland are included in ATLAS as observers, and in the event of a continued UK presence within the EU, SCO19 will also receive observer status. Altogether, ATLAS brings together 35 police CT units from 27 EU states:

1. ACVILA – Grupul Special de Protectie si Interventie 'Acvila' (The Eagle Unit), Romania.
2. AKS – Politiets Aktionsstyrke (The Special Intervention Unit), Denmark.
3. ARAS – Lietuvos policijos antiteroristinių operacijų rinktinė ARAS (Lithuanian Police Anti-terrorist Operations Unit ARAS), Lithuania.
4. ATJ Lučko – Antiteroristička jedinica Lučko (Anti-Terrorist Unit Lučko) Croatia.
5. ATU – Anti-Terrorism Unit, Switzerland.
6. BOA – Biuro Operacji Antyterrorystycznych Komendy Głównej Policji, Poland.
7. DELTA – Beredskapstroppen 'Delta' (The 'Delta' Emergency Response Unit), Norway.
8. DSI – Department of Special Interventions, Nederland.
9. DSU – The Directorate of special units (French: Direction des unités spéciales), Belgium.
10. EAO – Ειδικός Αντιτρομοκρατικός Ουλαμός (Special Anti-terrorist Squad), Cyprus.
11. EKAM – The Special Counter-Terrorist Unit (Greek: E.K.A.M. – Ειδική Κατασταλτική Αντιτρομοκρατική Μονάδα, Eidiki Katastaltiki Antitromokratiki Monada), Greece.
12. EKO Cobra – Einsatzkommando Cobra, Austria.
13. ERU – The Emergency Response Unit (Irish: Aonad Práinnfhreagartha), Ireland.

14. GEO – The Grupo Especial de Operaciones (Special Operations Group), Spain.
15. GIGN – Groupe d'intervention de la Gendarmerie nationale (National Gendarmerie Intervention Group), France.
16. GIS – Gruppo di Intervento Speciale (Special Intervention Group), Italy.
17. GOE – The Grupo de Operações Especiais (Portuguese for Special Operations Group), Portugal.
18. GSG 9 – Grenzschutzgruppe 9 der Bundespolizei (Border Protection Group 9 of the Federal Police), Germany.
19. KARHU – Karhu-ryhmä, The Police Rapid Response Unit (Finnish: Poliisin valmiusyksikkö), Finland.
20. K-Commando – K-Komando, Estonia.
21. LYNX – Útvar osobitného určenia Lynx Commando, Slovakia.
22. NI – Nationella insatsstyrkan, znana jako National Task Force of the Swedish Civilian Police (Swedish: Ordningspolisens nationella insatsstyrka), Sweden.
23. NOCS – The Nucleo Operativo Centrale di Sicurezza (Central Security Operations Service), Italy.
24. OMEGA – Pretterorisma Vieniba 'Omega' ('Omega' Counter-terrorism special police unit), Latvia.
25. RAID – Recherche, Assistance, Intervention, Dissuasion (Search, Assistance, Intervention, Deterrence), France.
26. RED PANTHER – Specialna Enota Policije 'Red Panthers' ('Red Panthers' Special Police Unit), Slovenia.
27. SCO19 – Specialist Crime and Operations Specialist Firearms Command, UK.
28. SEK BWL – Spezialeinsatzkommandos Baden-Württemberg (Special Deployment Commandos of the State of Baden-Württemberg), Germany.
29. SAG – Lo Special Assignments Group (Special Assignments Group), Malta.
30. SIAS – Serviciul Independent pentru Intervenții și Acțiuni Speciale (Independent Special Actions and Intervention Service, SIAS), Romania.
31. SOBT – Специализиран отряд за борба с тероризма – СОБТ (Special Unit for Combating Terrorism), Bulgaria.
32. TEK – Terrorelhárítási Központ (Counter Terrorism Centre), Hungary.
33. UEI – Unidad Especial de Intervención (Special Intervention Unit), Spain.
34. URNA – Útvar rychlého nasazení – ÚRN (Rapid reaction unit), Czech Republic.
35. USP – Unité Spéciale de la Police (Special Unit of the Police), Luxembourg.

1.7. Grounds and procedures for working with foreign officers in anti-terrorist operations on Polish soil

The European legal basis for joint responses to threats was created in 2008, with Council Decision 2008/615/JHA of 23 June 2008 on stepping up cross-border cooperation, particularly in the fight against terrorism and cross-border crime (Official Journal of the European Union 210 of 6 August 2008). This EC decision regulates the following areas:

- Conditions for and means of automatic transmission of DNA and fingerprint data;
- Conditions and procedures for transmitting other data on relevant international events;
- Conditions and procedures for providing information to prevent terrorist offenses;
- Conditions and procedures for intensifying cross-border police cooperation, including joint operations.

Among other things, it created the possibility of granting the powers of the host Member State officials under the direction and in the presence of a host Member State (including the use of firearms). The obligation to establish the details of a proposed joint operation was also established. Based on the EU law, Poland then passed the Act of 07 February 2014 on the participation of foreign officers or employees in joint operations or joint rescue operations in the territory of the Republic of Poland. This law regulates the mode and rules of participation of foreign officers in joint operations occurring on the territory of the Republic of Poland.

From Poland's perspective, joint operations are defined as actions conducted on its territory that involve foreign officers or employees of the Member States of the European Union, or other countries applying the Schengen acquis. These could be, for example:

- Joint patrols or other joint actions to protect public order, and for safety and crime prevention, carried out by officers or employees of the Police, Border Guard or Government Protection Bureau;
- In the case of assemblies, mass events or similar events, natural disasters and serious accidents, in order to protect public order and for safety and crime prevention, carried out by officers or employees of the Police, Border Guard or Government Protection Bureau;
- Within the framework of the assistance provided by the Special Intervention Unit referred to in Council Decision 2008/617/ HA of 23 June 2008 on improving the cooperation between the special intervention units of the Member States of the European Union in crisis situations, by police officers or employees of the Police, Border Guard or Government Protection Bureau.

To initiate a joint operation, the ‘inviting’ State must apply to a ‘sending’ State. Prerequisites for such invitations include:

- Explanation of the circumstances justifying a joint operation or joint rescue operation;
- An explanation of why, due to the nature of the incident justifying a joint operation or joint rescue operation, the use of only national forces and resources is impossible or insufficient;
- If a joint operation is agreed, in particular at a mass event or similar, a substantial number of nationals of the invited country are envisaged;
- By virtue of its nature, the participation of nationals of other States in an event justifying a joint operation, in particular a mass event or similar, can pose a serious threat to public security and order.

The persons qualified to apply for joint operations are:

- The Commander in Chief of the Police, Commander in Chief of the Border Guard, or Commander in Chief of the State Fire Service – with the prior consent of the Minister of Internal Affairs;
- The Minister of Internal Affairs – *ex officio* or at the request of the Commander in Chief of the Police, Commander in Chief of the Border Guard, Commander in Chief of the State Fire Service or the Head of the Government Protection Bureau;
- The Prime Minister, at the request of the Minister of Internal Affairs.

Applications should include:

- The purpose and type of joint operation or joint rescue operation;
- The type of assistance that the ‘sending’ State is to provide to the Republic of Poland;
- The area in which the joint operation or joint rescue operation is to be conducted;
- The anticipated duration of the stay of foreign officers or employees on the territory of the Republic of Poland;
- The type of military or emergency service requested to participate in the joint operation or joint rescue operation;
- The anticipated costs of the joint operation or joint rescue operation and the means of its financing.

Upon acceptance, a detailed list shall be drawn up specifying numerous details, including:

- The purpose and type of activities;
- The nature of the ‘sending’ state’s role;
- A list of foreign officers to be involved;
- The duration of the operation;
- The date the ‘sending’ state’s personnel would cross the border;
- The data of the ‘sending’ state’s participating commanding commanders;
- The number and types of firearms and other necessary equipment to be used;

- The language to be used;
- The costs of the joint operation or joint rescue operation.

In Poland, joint operations can be coordinated by: a regional police chief; a commander of a Border Guard unit; a person designated by the Head of the Government Protection Bureau, or a person designated by the Commander in Chief of the Police, Border Guard or State Fire Service. The choice largely depends on the main force that the officers and employees participating in the operation originate from. If more than one is involved in the operation, it is supervised by the appropriate Commanders in Chief, or by the Minister of Internal Affairs.

Importantly, officers and foreign employees taking part in a joint operation on Polish soil benefit from the same legal protection as Polish officers. They have the right, amongst other things, to:

- Wear a uniform;
- Carry firearms, ammunition and means of direct coercion;
- Use firearms specified in the Anti-Terrorism Act;
- Use the same methods of direct coercion prescribed for Polish Police officers;
- Formally identify people;
- Stop people;
- Collect fingerprints;
- Search people and rooms;
- Perform body searches and searches of luggage and bags;
- Observe and record public events using technical means;
- Request assistance;
- Drive their vehicles as privileged vehicles;
- Use free medical care.

Foreign officers can also use firearms:

- To repel a direct and unlawful attack on the life, health or freedom of foreign officers or employees or other persons;
- In other situations, at the discretion of the commanding officer.

Foreign officers participating in a joint operation enjoy the privileges conferred by Polish law, under the command of, and in the presence of, a Polish officer.

Prior to the commencement of the joint operation, training for foreign officials on the rules of participation in the joint operation must be given, in particular the rules and conditions for the use of firearms and direct coercion.

1.8. Organization of activities at the scene of a terrorist incident

Part 4 of the Anti-Terrorism Act regulates the organisation of anti-terrorist activities at the scene of terrorist incidents. The definition of a terrorist incident scene covers two areas:

- Open or closed spaces in which a terrorist event has occurred or is expected to occur;

- Any space in which a terrorist threat is occurring.

It further defines a ‘terrorist incident’ as a situation in which there is a suspicion of terrorist activity arising from previous terrorist offenses, or a threat to commit such an offense.

Various activities are conducted at the scene of a terrorist incident, and counter-terrorist activities constitute one element of these. Counter-terrorist activities are actions taken against perpetrators or persons preparing or assisting in a terrorist offense, to eliminate the immediate threat to the life, health or freedom of persons or property using specialized forces, tactics and resources.

Counter-terrorist actions have several elements:

- Operational situation – an immediate threat to the life, health or freedom of persons or property by terrorists;

- Target entities – perpetrators, persons preparing or assisting in a terrorist threat or incident;

- Expected result – elimination of the threat to the life, health or freedom of persons or property by terrorists;

- Execution – intervention using specialized forces, tactics and resources.

Polish anti-terrorism law specifies the persons responsible for managing anti-terrorist activities at the site of a terrorist incident in Poland, in order to unambiguously define the responsibilities of all entities involved, not just individual units. For the purposes of these definitions, anti-terrorist actions are defined as one of two kinds:

- Those conducted in and around civilian areas (buildings, streets etc.);

- Those conducted in and around military areas or areas belonging to entities supervised by the Ministry of Defence.

In civilian areas, the responsibilities of responding units are assigned by the Commander in Chief of the Police, or the relevant officer at the local Police Headquarters. In military areas, they are assigned by the Minister of Defence, by the Commander in Chief of the Military Police, or by a Military Police officer.

Commanding officers at terrorist incident scenes have the right to:

- Order the evacuation of persons or property from the area of a terrorist incident to a designated place, building or area;

- Stop or restrict pedestrian traffic and prohibit unauthorized persons from staying at or near a terrorist incident scene;

- Stop or restrict the movement of vehicles at the scene of a terrorist incident or its surroundings, as well as to re-route public transportation vehicles in consultation with the authorities, or re-direct them to a designated location;

- Request the railway authorities to suspend or limit rail traffic at or near a terrorist incident scene, or stop a railway vehicle or re-route it to a designated location;

- Request the port authorities to suspend or limit the water traffic at or near a terrorist incident scene, or to detain a vessel or re-route it to a designated location;
- Request the air traffic authorities to suspend or limit air traffic at airports being the scene of a terrorist incident;
- Demand cost-free use of property or movable property, including means of transport, as well as objects and equipment necessary for carrying out anti-terrorist activities;
- Request assistance from an institution, organization, entrepreneur or natural person, or advise others on how to help minimise or eliminate the effects of a terrorist incident, prevent further terrorist incidents, or detect and identify those responsible for the terrorist incidents.

All persons and entities to whom these orders and demands are directed are legally obliged to comply with them. In the process, the State Treasury bears civil (financial) liability for any damage caused by the anti-terrorist authorities.

1.9. Rules of engagement in anti-terrorist operations

One very important power of the executive is the power to authorise the use of firearms in counter-terrorist activities. In Poland there are two legal acts underlying the use of force by authorized entities:

- Journal of Laws of 2013, item 628 – a general act;
- Journal of Laws of 2016, item 904 – the specific legislation regulating the use of firearms.

The general principle that firearms officers must at all times follow is that of minimising injury, i.e. that weapons should be used in the least detrimental way. Officers must take the necessary precautions, while taking into account the threat to the life and health of their colleagues. Generally speaking, officers cannot be ordered to fire their weapons. Each officer must decide for themselves to fire their weapon and assumes full responsibility for doing so. There are, however, prerequisite conditions for discharging a firearm (including a clear call for cessation of illegal activities, a clear warning that the officer is armed, warning shots etc.). These can be waived only in exceptional cases. After discharging their firearm, the officer must prepare a detailed written report about their use of the firearm during the incident.

Although the two legal acts cited above do not clearly define the situations in which a life can be specifically taken by a firearms officer, the Anti-Terrorism Act does contain a special ‘use of weapons’ formula, according to which officers may legally shoot to kill. This is still an exceptional solution and is, as mentioned in the previous paragraph, constrained by precisely defined restrictions, including:

- Counter-terrorism activities must be conducted in accordance with Anti-Terrorism law;

- Special use of arms should be given to prevent the direct, unlawful, violent assassination of a person's life or health, or to release a hostage;
- The use of weapons on general principles, even in the least detrimental way, is insufficient reasoning to take a kill shot;
- Use of firearms is permitted when counteracting an attack or releasing a hostage in any other way is impossible;
- All of the circumstances of the terrorist incident and the options for countering it must be taken into account;
- Use of firearms is permitted only against persons carrying out an attack or taking or holding hostages.

'Special use' of firearms is carried out under order, after a formal procedure, and only in the context of counter-terrorism. In these situations, there is no obligation to warn about the use of firearms, to give warning shots or to request compliance.

The procedure for allowing special weapons use is:

- Decision to classify the situation as a terrorist event and designate a counter-terrorist authority to take action, made by the competent services or entities as part of their statutory tasks at the scene of a terrorist incident;
- Decision on the admissibility of special use of weapons by the commanding officer at the scene, based on an analysis of the operational situation;
- Decision by the commander of the responding counter-terrorist unit;
- Notification of the decision on the admissibility of the special use of weapons by the relevant authority;
- Issuing of the order by the CT group commander to the officers, on the special use of weapons. The procedure for and purpose of the special use of weapons must be specified in the order.

After the operation, a detailed report on the special use of weapons is made by the commander of the CT forces. The special use of weapons is authorized in the context of counter-terrorist activities only for:

- Police officers;
- Border Guard officers;
- officers of the Internal Security Agency;
- soldiers of the Armed Forces of the Republic of Poland;
- Military Police.

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2. DECISION-MAKING IN EMERGENCY RESPONSES TO TERRORIST ATTACKS

2.1. The crisis and the crisis situation

In the social sciences, the concepts of crisis and crisis situations are defined in various ways. Legally, they are defined in the Act of April 26, 2007 on crisis management. It describes a crisis situation as a situation that negatively effects the level of safety of persons, property or the environment.

2.1.1. Crisis recognition

One necessary condition of a crisis is that the authorities are unable to cope with the situation normally, using standard procedures. Other conditions determining an event as a 'crisis' are:

- Surprise;
- Time and information deficits;
- Delayed response to development of the situation;
- Escalation of events;
- Periodic loss of control over the development of events;
- Threat to life, vital interests or priorities;
- Wide media attention;
- Collapse of the normal decision-making process.

Crisis is a word of Greek origin meaning 'solstice', 'breakthrough', 'decisiveness', and 'turning point'. This concept appears in all areas of human life and society. The social sciences speak about political crises, Government crises, crises of the State, economic crises, psychological crises and international crises, amongst others. The literature on the subject most often focuses on the following types of crisis:

- Developmental crisis – moments of transition from one stage to the next;
- Situational crisis – when a person encounters something unusual and is not able to cope with it or control it (violence, job loss, accidents);
- Chronic crisis – coping mechanisms that the human brain deploys to deal with the immediate aftermath of a crisis;

– Existential crisis – internal conflict and fears. Often combined with the belief that we have wasted our lives, that we have made the wrong decisions. Existential crises often occur at certain moments of human life, for example a mid-life crisis (at about 40 years in men, or the so-called balance sheet crisis appearing near the end of life).

The concept of a crisis can also be defined as an unpredictable event that has potentially negative effects, significantly limiting production, services, employment, financial health and reputation.

2.1.2. A comparison of the statutory definition of selected definitions of psychology, management and command

A crisis is a set of external and internal circumstances affecting a system in such a way that they have lasting changes. The result of these changes can be a qualitatively new layout or a new structure and function in an existing status, or growing destabilization, insecurity and social tensions. From this it can be concluded that crises are inevitable, accompanying people throughout their life and not having only a negative aspect, as is commonly believed, because it is apparent from the definition (and above all else, experience and history), that the consequences of a crisis can also be progress, development and new values. From the beginning of its existence, humanity has had to deal with crisis situations. These are considered to be accidents, personal failures and successes. The life of every person is marked by continuous changes in the wake of critical events that erode its homeostasis. Human behaviour cannot be fully controlled and predictable. The forces of nature cannot be fully anticipated. Therefore, each unit – family or community – sooner or later finds itself involuntarily or accidentally in a crisis situation. The situations that cause crises can be limited and their effects minimised. However, they cannot be eliminated completely.

2.1.3. Attributes of a crisis

A crisis is made up of three elements: time pressure, threat, and surprise. Every crisis is different, although most are, to some extent, characterized by the following features:

- Surprise and time pressure;
- Insufficient and uncertain information;
- Loss of or inability to take control of the situation;
- Panic;
- Outdated emergency response systems;
- Policy makers focused on short-term plan of action;
- Interruption of the normal decision-making process.

2.2. Decisions and the decision-making processes

It would be difficult to create a single universal definition of a decision that fully satisfies all those dealing with the issue. Decision-making is a conscious process, with at least two possibilities desirable from the point of view of the public interest. The term order determines all further proceedings, not only of the decision-maker, but also of the entities affected by the decision. A similar definition of the word 'decision' is that a decision is not random, but is supported by analysis of a number of variant solutions possible at the time. Most often a decision is a choice between two modes of action, neither of which are more verifiable than the other.

2.2.1. Attributes of a decision

- Selection of possibilities;
- Choice of action;
- Choice of objectives;
- Choice of activities leading to implementation of the objectives;
- Decision based on preferences and comparisons;
- Troubleshooting the chosen solution;
- Statement of intent;
- Implementation.

The most important attribute of a decision is that it is a freely-made choice.

2.2.2. Decision-making processes

The decision-making process is a unique thought process each time. A decision made in another, identical situation, even by the same person, could be the product of an entirely different thought processes. The steps in such a process include:

- Identification of the situation;
- Identification and design of variant decisions;
- Assessment of these variants and choice of one particular one;
- Creation of the conditions for execution of the chosen decision;
- Monitoring of the effects of the decision.

Each of these steps needs to meet specific information needs, and the continuous flow of relevant information can only be accessed by a smoothly functioning information system.

2.2.3. Leadership decisions and the decision-making processes

A command decision is an act of will, formalised and passed on for implementation. The decision-making process at the command level is the result of a series of actions, events and information enabling command-level personnel to make decisions in a crisis situation.

2.3. The formal/classical model of decision-making

Formal models of decision-making are used to determine the an optimal solution to a problem. The aim of rational thinking is to arrive at the optimal decision, i.e. the one that has the greatest benefit, or minimises losses as far as possible.

2.3.1. Presentation of the basic elements of the classic model

Formal models of decision-making follow a specific sequence of thought, which can be illustrated as follows:

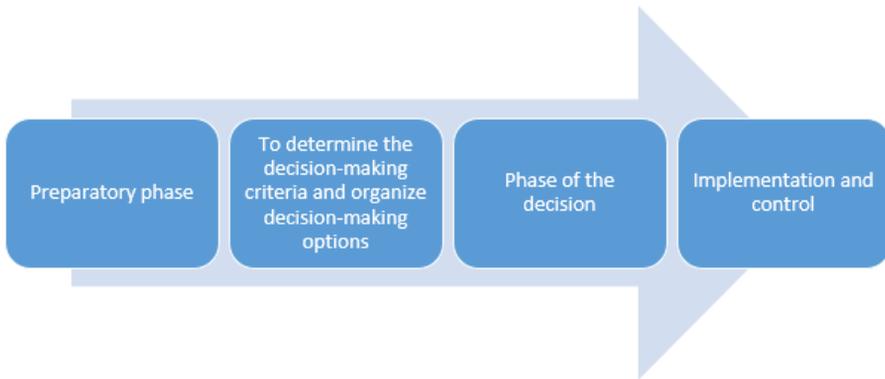


Figure 1. Phases in the formal model of the decision-making process

Preparatory – Determination of the causes of the problem. The problem might be identified by asking the following questions:

- Has the problem happened before?;
- What are the time and location concerns?;
- Is it a recurring problem, or a one-off event?;
- Who is responsible?

Determining decision-making criteria – This phase has three steps:

- Formulation of different variants of a solution;
- Definition of the solution assessment criteria;
- Evaluating the chosen solution.

Decision – Comparison of possible solutions to the problem and choosing the one that best meets the previously adopted criteria.

Implementation and control – Putting the chosen solution into practise and monitoring its progress and results.

2.3.2. The rules of the decision-maker operating in a rational model

Regardless of the adopted solution, the decision-maker must adhere to certain rules of rational thought:

- They must have access to the full range of information uniquely identifying the situation requiring a decision and be able to process them calmly and intelligently;
- They must be able to arrive at a full set of possible variant solutions;
- They must know and understand the consequences of each variant solution;
- They must be able to identify and prioritise all objectives;
- They must always choose the option most advantageous from the point of view of the problem assessment criteria;
- They must have a consistent value system that would allow them to make the same decision again, under the same conditions.

2.4. Models of decision-making under conditions of bounded rationality

The bounded rationality model of decision-making assumes that the decision-maker has incomplete information. This does not always mean a shortage of information, but can also mean their inability to separate the relevant information from the irrelevant. If they do not have the appropriate information, they cannot not create all possible solution variants. This limitation most often arises from the limitations of human knowledge and reasoning.

In terms of having only limited or incomplete information, the decision-making process is as follows:

- Formulation of the problem-identification problems visible, reflecting the interests and prior experiences of decision-makers;
- Identification of assessment criteria;
- Assignment of weights to each criterion;
- Determination of variant solutions;
- Analysis of variant solutions;
- Implementation of the chosen solution;
- Implementation and monitoring of the chosen solution and its effects.

2.4.1. The practical model of decision-making – characteristics and critical analysis of its use in emergency situations

The practical model defines a simplified decision-making process. This simplification is imposed by a conscious adjustment by the decision-maker to the realities of the situation in hand, such as time constraints and limitations on

information gathering. As a result of this approach, the decision-making process is itself simplified.

2.4.2. The Janis & Mann rational model – characteristics and critical analysis of its possible use in emergency situations

Rational decision-making models come down to application of the appropriate course of action. The free effort of the decision-maker is replaced by use of an algorithm designed to achieve optimal results – from the identification of the problem through the collection of data to the creation of variant solutions and selection of the most advantageous variant. In following the rational model, the decision-maker should:

- Have access to the full range of information about the problem;
- Have access to the full set of possible solution variants;
- Know and understand the consequences of each variant solution;
- Be able to identify all objectives;
- Always choose the option most advantageous from the point of view of the problem assessment criteria;
- Have a consistent value system that would allow them to make the same decision again, under the same conditions.

2.4.3. The advantages of the rational model

- Allows for repetitive decisions;
- Is measurable;
- Newcomers can determine what it is they do not know;
- Is systematic, prevents omission of any items;
- Is of a general nature and can be used in a wide range of situations.

2.5. Decision-making based on recognition

This model assumes the use of a variety of methods of making a decision, depending on the conditions under which the decision-maker is operating. An important part of this process is that the decision-maker must determine whether the problem is a 'decision problem'. Decision problems have the following characteristics:

- The number of possible solutions is large or unknown;
- The final solution variants may differ from the initial variants;
- Not all the possible solutions are equally desirable or feasible;
- The choice of variant of the best solution is a difficult, complex one, that requires the collection of additional information, as well as many more calculations and measurements.

2.5.1. Pre-requisites for optimizing a decision

- The problem is understandable;
- There are clearly defined criterion for the optimal solution;
- The number of options from which to select is small and they are understandable;
- No cost limits and ample time to make a decision.

2.5.2. The importance of expert competence in decision-making

- Experts have much greater knowledge and utility;
- They can use pattern recognition in their decision-making;
- They openly seek feedback;
- They can make improved assessment and identification of problems;
- They possess specialised memory;
- They automatically proceed in small steps;
- They are able to naturally monitor the decision-making process.

2.5.3. Decision-making by experienced individuals

- Experienced individuals can easily evaluate situations on the basis of experience and practise, rather than through formal analysis or comparison;
 - Their first-considered possibility is usually acceptable, but not necessary to future solutions;
 - They generate and evaluate options one by one, without comparing their advantages and disadvantages;
 - In considering the possibilities, they test each one in their imagination, so that the decision-maker can then detect weak points in it and find a workaround;
 - Their main emphasis is on action, not on assessing all possible solutions.

2.5.4. Expert perception of crises – characteristics

- They check for regularity;
- They look for anomalies – events for which there is no explanation, or some other non-compliant factor. Beginners do not know what should happen, so when something does happen, they can't recognize it as important. In comparison, experts can identify it immediately. Anomalous events can be called 'negative guidance'. Experience plays a very important role in the formulation of predictions and making use of them. Experience plays a very important role in the formulation of predictions and their use – only by being able to anticipate things are we able to see when something has happened;

- They have a better overall picture ('tall orientation') – experts have a general sense of what is happening in a given situation, which allows them to assess their typicality;
- Mechanisms – experts have behind-the-scenes knowledge of how to perform specific tasks, and so can better coordinate the work of the team;
- Opportunities and occasions to improvise – experts have the ability to develop explanations and predictions that conflict with available data;
- Events that have already taken place or have only occurred once – experts consider every situation not only from the current perspective, but see it as part of a pattern from past to present to the future;
- Experts can see details that are too small for newcomers to notice;
- Experts are sensitive to the limitations of their own knowledge and memory, which helps them take specific steps to avoid problems. Experts are able to look critically at themselves, which makes it easier for them to see that in a given situation they might make a mistake. They then often submit their decisions and plans for critical assessment.

2.6. Limitations of decision-making models under operating conditions

2.6.1. Characteristics of the RPD (Recognition Primed Decision) model

This is a decision-making model based on the concept of naturalistic decision-making, (Naturalistic Decision-making), in which the decision-making process often takes place under conditions creating the maximum number of factors affecting the decision-maker and the decision-making process. These factors include:

- Time pressure, high-stakes games;
- The experience of the decision-makers;
- A lack of information;
- Inadequately defined objectives;
- Dynamically changing conditions.

The RPD model is based on two processes:

1. Assessment of the situation and development of the best solution by the decision-maker.
2. Evaluation of the efficacy of individual actions by imagining their symptoms.

2.6.2. Terms of the RPD model

The simplest situation in which a decision must be made occurs when a person recognizes a situation as being typical, known to them, and so takes appropriate action. They are able to clearly define the objectives, to recognize the essential factors

and predict developments. From this, they can formulate a solution. The combination of these four elements (objective, guidance, prediction of developments, definition of a typical course of action), allows for a decision to be made. In the RPD model, the decision will be a simple match with previous experience. Information obtained by the decision-maker may not match the information in a typical case, or might indicate more than one possibility. In this case, the decision-maker is forced to extend the decision-making process, and must collect more information that hopefully matches the characteristics of known cases, or clarifies the discrepancies.

Another obstacle that can meet decision-makers is a lack of elements in a situation that are common to known cases.

An important feature of decision-making by means of diagnosing a situation is assessment of a selected solution without comparative analysis. The RPD model specifies that the decision-maker does not propose parallel, alternative solutions to resolve a situation, but that they imagine implementation of the decision and its consequences in their mind. If it appears to be a good solution but one that may have faults, they modify it accordingly. If it appears to be not a very good solution, they will develop another one, which will be subject to the same mental assessment and analysis.

Can you learn to make decisions in a crisis?

The RPD model does not rely on knowledge of the model itself, but on acquisition of experience of decision-making situations, as well as actively searching for opportunities to acquire practical experience. Each opportunity must have a specific purpose and criteria for evaluation. Accumulation of experience in this way is considered the richest resource of knowledge and experience. Obtaining feedback is also highly valuable, enriching one's own knowledge by analysing past experience – experts learn from mistakes made, and formulate new proposals using that knowledge.

2.7. Psychological aspects of decision-making

2.7.1. The psychological conditions of decision-making – general characteristics

When making a decision, a decision-maker must consider three important factors:

- Legal certainty – the precise, quantifiable and reliable information on the legal effects of each of the solutions under consideration that are known to the decision-maker;

- Risk – the probability of obtaining the desired result from different solutions;
- Uncertainty – unpredictable external circumstances or lack of information necessary to determine the likelihood of specific effects of a solution.

Decision-making situations should be assessed on a scale of certainty (using the full capabilities of prediction), by risk, and also uncertainty (based on the minimum possibility of predicting events and their consequences).

2.7.2. Factors distorting the decision-making process

- Information;
- Resources;
- Social;
- Bureaucratic;
- Organizational;
- Conformity;
- Group thinking syndrome;
- Emotions;
- Personality.

2.7.3. Personality features influencing the decision-making processes

- Locus of control;
- The need for achievement;
- Authoritarianism;
- Machiavellianism;
- Willingness to take risks;
- Type of motivation;
- Stress resistance.

2.7.4. Features of a professional commander – general characteristics

- Professionalism and respect for subordinates;
- Unconventionality;
- The need for achievement, the pursuit of a healthy rivalry;
- The ability to resist superiors when they are wrong;
- The ability to provide adequate assessment of subordinates;
- An aversion to unnecessary use of subordinates;
- An interest in the lives and problems of their subordinates;
- Openness;
- An appreciation of the enemy;
- The ability to adapt to new situations and make decisions in changing conditions;

- Empathy;
- An adequate rating in terms of their setbacks and successes;
- Activity, vitality;
- Serene temperament.

2.7.5. The role of the commander in minimizing stress

Decision-making is a process that does not end at the time the decision is issued, but continues until effects of the decision are made completely clear. The decision-maker gains experience from the current course of events that can be used in planning for future events. Decision-making in a crisis is often accompanied by severe stress, not only for the commanding officers, but also their subordinates. But the commanding officer can have an impact on minimising the stress on their subordinates at every stage of the action. This begins before the decision-making process even begins, at the start of the crisis, through the individual phases of the decision-making, planning, preparation and implementation. COs can relieve pressure on their subordinates by:

- Issuing clear and unambiguous orders;
- Giving a clear and unambiguous briefing on the terrain, objectives, and entities involved;
- Allocating tasks according to experience and competence, taking into account social relations;
- Considering the needs of subordinates;
- Carefully planning to use appropriate means and forces to achieve the objectives.

Commanding officers must be aware of their position's power and responsibility, and how it can affect their subordinates. Their authority can be built up over time in a variety of ways, including by being firm but consistent, allowing for their subordinates to clearly understand what to expect of their CO in any given situation. COs are responsible for the safety and well-being of their people, should back them up in difficult situations, and offer them advice and support when needed.

After an event, when the authorities have stood down and returned to normal duties, the Commander should properly debrief their subordinates, assessing together the course of their responses, and collecting opinions and information from them. This particularly applies to any mistakes and perceived inefficiencies, in order to eliminate them in the future, as well as highlighting good decisions and well-implemented actions. A good Commander knows their subordinates and can easily see changes in their thinking and actions, and respond appropriately. For example, by providing comfort and necessary counselling, especially after tough, demanding events.

2.8. Group decisions

2.8.1. The concept of synergy

In a synergistic team, the outcome of a decision or action is always greater than the sum of the results obtained by the individual team members. Synergistic actions made in close cooperation and synchronization with each other have bigger, better effects. Another benefit of a truly well-coordinated team is that each person can offer extra support to other people, with other tasks. A good example of synergy is two people working together to move a heavy wardrobe – individually, they could move it no more than a few metres.

2.8.2. The advantages and disadvantages of making group decisions

Make important and risky decision as a team has its advantages. First of all, a group can analyse the situation from multiple different points of view. This can yield multiple good solutions and open up the pool of information available, which helps reduce uncertainty. Decision-making in a group confers a shared responsibility for implementation of the chosen solution, which increases the focus and feeling of responsibility of the decision-making team.

On the other hand, there are disadvantages too. A team making a group decision can be prone to committing various errors. In analyses of various group decisions the most common errors are the so-called groupthink effect, which is the increased likelihood of bad decisions made simply to ensure harmony and mutual consent. The other problem is polarization of opinions, which is divisive and unhelpful. Other problems with group decision-making are when preference is given to just one kind of decision-making model, and when a team becomes over-confident and develops a resistance to criticism. Various other pitfalls include:

- Groups working in isolation;
- Having an over-strong leader;
- Severe stress, most commonly from time pressure or high risk;
- Lack of a decision-making strategy – the group members do not apply any techniques for decision-making making under pressure.

It is difficult to talk about universal advantages and disadvantages of group decision-making. There are many variables that can have an impact on the decision-makers and their choice of solution. These include the type of situation, the personality, qualifications and experience of the decision-makers, and time pressure. The advantages and disadvantages can be summarised like so:

Advantages	Disadvantages
<ul style="list-style-type: none"> – Access to more information and knowledge; – Higher degree of acceptance of the chosen solution; – More variants can be produced; – Can lead to improved team communication; – In general, better decisions can be made. 	<ul style="list-style-type: none"> – The process takes longer, and is more costly; – Compromised solutions resulting from lack of mutual agreement; – Group can be dominated by one person; – Susceptible to Groupthink and polarisation.

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3. TERRORISM AS A THREAT TO PUBLIC ORDER

3.1 Terrorism: definition and history

3.1.1. Problems with the definition of terrorism

Terrorist activity is not difficult to define, yet the definitions of terrorism are frequently considered controversial, and this sometimes leads to their rejection. One popular type of criticism is neatly expressed in a popular (and deceptive) idiom – ‘*One man’s terrorist is another man’s freedom fighter*’. This kind of criticism reveals a politically-motivated inability to deal with the consequences of defining terrorism, rather than an intellectual inability to properly grasp it. The major problem with the idea of terrorism seems to be that it is frequently used as a tool to delegitimize the enemy and its actions. Calling a group or a person a terrorist excludes them from normal political discourse. The meaning of the word is not neutral – on the contrary, it is pejorative and it is used to totally morally disqualify a (usually violent) political opponent.

To avoid endless and inconclusive debates we should understand acts of terrorism as a *means of political violence*, and try to focus our attention on the specific types of violent activity undertaken by the perpetrators of terrorist acts. Consequently, we must attempt not to apply any moral judgment to the concept, and not to wonder whether a terrorists’ aims are right or wrong. In all states endangered by it, terrorism is simply considered illegal and criminal in nature.

3.1.2. The definition of terrorism

Terrorism, then, is a method of warfare used in violent political conflict, which covers all kinds of activity leading to pre-planned armed attack(s), or credible threat(s) of such attacks on non-combatant targets. The aim of such attacks is to use these acts to achieve political and ideological goals through coercion or intimidation of a public and/or a government.

Regardless of the precise motives of militants resorting to terrorism or justification of their actions i.e. whether they’re fighting for freedom or oppression, if their violent attacks deliberately focus on non-combatants and are aimed at harming them physically and psychologically, they should be considered terrorist entities.

Terrorism is a conscious, organized, clandestine activity that is not limited to carrying out unique terrorist attacks. Such attacks are, per se, crucial elements of the terrorist process. Both individuals and groups involved in terrorist activity must engage in a wide variety of preparatory and organisational tasks in order to carry out an actual attack, or at least credibly threaten a government and society with such an attack, in order to then disseminate their political message and benefit from the political and social reaction to it. Since early anarchist attacks in the late 19th Century, terrorism has often been described as 'propaganda by deeds'. Terrorist plotters usually consider various options for their attacks, but are necessarily involved in fundraising, training, acquiring and assembling materials – including for weapons and explosives – planning and even rehearsing attacks. This also includes maintaining their own security, avoiding detection by security forces, and covertly communicating. Terrorists must also engage in an everyday life, even if they are detached from society, so quite a substantial part of their lives is devoted to non-terrorist activities. Instigation, recruitment and preparation are all important elements of the terrorist's process – without them, terrorist activity would be short-lived and unsuccessful – considering the fact that terrorists must deal with very powerful state institutions, including the police, special services and military.

The essence of terrorism is that it is a deliberate attack on so called non-combatants – civilians and unsuspecting police or armed forces and/or their infrastructure. Surprise attacks on unprepared, defenceless (sometimes randomly selected) victims are obviously a pre-planned activity. So-called soft targets are easy to strike and difficult to protect. Moreover, attacks on ordinary members of the public have a crucial psychological effect of mass fear and intimidation. The alleged randomness of such attacks results in a public feeling that anybody could be a victim of terrorists, and this instigates feelings of shock and awe. Terrorism is far less about creating huge material damage (although this does sometimes happen (e.g. the 9/11 attacks)), but more about creating a damaging mass psychological effect. Terrorism is a weapon of psychological manipulation. Its effectiveness is not measured by the number of people it kills and injures, but the number of those affected by it, and 'scared to death'. Fear is a major driving force and is used by terrorists to manipulate, influence, or even change the minds of decision makers in order to achieve their goals. These goals are of a political and ideological (including religious) nature, even if they ultimately may seem utopian and unrealizable.

3.1.3. Terrorism as tactics and terrorism as a strategy

We should perceive the methods of terrorism as both a set of tactics and a strategy. Tactics is based on attacking defenceless targets (even if actually they are armed, like police or military men and women, but in a situation of a surprising attack outside of the battle field) with the use of various weapons

– today this includes the weaponization of ordinary, every-day items – such as cars, trucks, airplanes etc. As the state cannot protect everyone everywhere, these attacks are relatively easy to pull off and seem to show the ineffectiveness of a state's countermeasures. It is hoped that this will prove the ultimate inability of the state to perform its first and foremost objective – to provide public security. As a result, people can feel defenceless and abandoned by their government, and this can lead to a loss of trust in state institutions – significant social and political damage.

Terrorism as a strategy aims at provoking over-sized and non-proportional reactions from both society and state institutions, changing their behaviour, policies and politics. In their own minds, this gives the attackers' cause a sense of importance and enables them to spread their message for free, gaining world public opinion, and consequently to recruit new supporters and members.

3.1.4. Terrorism and other forms of political violence

Some violent groups tend to limit themselves to terrorist methods, in order to politically influence the government and the public. Others treat it as just one of many forms of violent activity that can be used, amongst assassinations, targeted killings, guerrilla warfare, or even regular war, depending on their means. Hence, some organizations can be called pure terrorist groups (e.g. the Rote Armee Fraction, IRA, ETA, Revolutionary Organization November 17), while others are in fact both guerrilla and terrorist groups (e.g. the Taliban, Islamic State, etc). They can even be considered political parties with their own military wings (e.g. Hezbollah in Lebanon, the Palestinian Al Fatah and Hamas organizations).

Moreover, the states themselves are precisely those organizations which began by using fear and intimidation, incited by attacking so-called non-combatant targets, in order to terrorize their society on a mass scale. This type of action is usually referred to as 'state terror'. In fact, the very notion of terrorism was introduced by the *Academie francaise* in 1798, derived from the dreadful realities of public terror in post-revolutionary France. This French new revolutionary regime applied mass and extreme violence against its own civilian population, in order to prevail in a civil war. The exact number of victims of state terror is unknown, but it does reach into the hundreds of thousands dead and millions intimidated. State terror is perpetrated by government institutions specifically designed to instil public fear. In fact, any totalitarian and authoritarian political regime could be considered a 'leaders' in the mass killing and intimidation of the public – even today, no terrorist entity, be it Islamic State or Al Qaeda, has been able to kill on a scale comparable to those who have perished in state detention camps, gulags, prisons and unofficial 'black sites'. The most lethal act of specific terrorism so far is the '9/11' attack on the World Trade Centre towers and the Pentagon building, which claimed the lives of 2,977 victims in New York, Washington

D.C. and Somerset County, Pennsylvania. But even so, Revolutionary France, Nazi Germany, Soviet Russia, communist North Korea and Latin America's authoritarian states have altogether managed to kill millions, and scare entire nations to death. This is not to mitigate the terrorists' actions, but to put them in a historical context. They definitely break the state's monopoly to use violence but so far have been unable to achieve its deadly scale and effectiveness.

3.1.5. The origins and history of terrorism

The first wave of terrorism in the modern world came with the emergence of an ideology of anarchism that provided active individuals with the motives to attack civilians in a state of non-belligerence. Some currents of anarchism advocated the use of violence precisely against the state and all oppressive regimes, which gave birth to numerous anarchistic organizations and individuals carrying out attacks in the name of the anarchist revolution worldwide. They attempted to 'decapitate' the system by attacking heads of states (kings, presidents, prime ministers, MPs) but ultimately also did not hesitate to plant bombs in restaurants, cafes and theatres, targeting and killing their fellow citizens. Together with other far left revolutionaries, they are responsible for literally hundreds of attacks in the West, harming thousands of people altogether in the late 19th and early 20th centuries. Only World War I ultimately stopped them, and dwarfed the number of victims by millions. The war proves that state-organized violence is much more effective, if not persistent. Anarchists ultimately ceased their political warfare for a while, but the rise of the powerful Bolshevik, fascist and Nazi social movements, which were more than willing to use political violence on a mass scale to terrorize their political opponents, caused a further twist in terrorism's progress.

Soviet Russia, fascist Italy and Nazi Germany proved to be quite efficient in executing their powers through mass, violent intimidation of their public, and after World War II, other ideological currents emerged that helped establish new terrorist-type organizations. Some were motivated by an ideology of decolonization (the best example here is probably the Algerian FLN movement), while others were motivated by the ideology of nationalism, sometimes also called separatism. The two most widely-known European nationalist movements, who became known to be more than ready to utilize terrorist tactics, were the Irish and Basque nationalists (separatists). More commonly known as the IRA and ETA, they are the most recognized and widely-known European separatist organizations to be labelled terrorist groups. The FLN ultimately managed to regain independence for Algeria, though not solely through terrorism, but also guerrilla tactics and political negotiation. Some might also include the Jewish Zionist fighters in the same league of militants, as they have resorted to terrorist acts in order to gain independence from what they consider foreign occupation, as well as

Cypriots from the old EOKA movement, who fought the British for the independence of Cyprus. The Algerians, Zionists and Cypriots all succeeded in their efforts, but terrorist tactics played minor roles in their successes, which were largely achieved through military, diplomatic, political and even moral pressure. It's worth noting that some nationalist/separatist movements have ultimately failed to achieve their goal of liberating their people – the best example here is probably the Tamil's LTTE. In Latin America, a whole plethora of 'people liberation' movements emerged in the late 1950s and early '60s, actively supported by Soviet Russia and Maoist China, with the goal of superseding their far-right military regimes with communist-type governments, or at least to subvert the pro-American military authoritarian rule on the continent. Similar far leftist movements could be found in Western Europe, where communist and Maoist ideologies were connected to the counterculture revolution of the late 1960s. As a reaction to it, far-right movements and even political parties based on a neo-Nazi ideology emerged. Radical Marxist, Maoist, neo-communist groups and (in contrast), neo-fascist and neo-Nazi organizations, all managed to terrorize Western Europe in the 1970s and 1980s, with a third wave of terrorism based on these radical political ideologies. A current, fourth wave, is seemingly connected to the rise of numerous radical and extremist interpretations of the mostly Sunni Islam, based on the Salafi and Wahhabi ideologies, which began its violent existence with the end of the Cold War (i.e. in the 1990s). This wave has lasted until today and seems to yet be flourishing. The most widely-known examples of these religious terrorist groups are Al Qaeda and Islamic State.

3.1.6. Terrorist organizations, networks and movements

Table 1. Terrorist Groups – A List of Terrorist Groups by Type: From Pre-Modern to Present-Day, Author: Amy Zalman, Ph.D.

SOCIALIST/COMMUNIST

Many groups committed to socialist revolution or the establishment of socialist or communist states arose in the last half of the 20th century, and many are now defunct. The most prominent included:

- *Baader-Meinhof Group* (renamed Red Army Faction, defunct as of 1998) (Germany);
- *Popular Front for the Liberation of Palestine (PFLP)*;
- *Red Brigades (Italy)*;
- *Revolutionary Struggle (Greece)*;
- *Shining Path (Peru)*;
- *Weather Underground Organization (United States)*.

NATIONAL LIBERATION

National liberation is historically among the most potent reasons that extremist groups turn to violence to achieve their aims. There are many of these groups, but they have included:

- *ETA (Basque)*;
- *Fatah (PLO) (Palestinian)*;
- *Irgun (Zionist)*;
- *IRA (Irish)*;
- *PKK (Kurdish)*;
- *Tamil Tigers (Sri Lankan Tamils)*;

RELIGIOUS-POLITICAL

There has been a rise in religiosity globally since the 1970s, and with it a rise in what many analysts call religious terrorism. It would be more accurate to call groups such as Al Qaeda religious-political, or religious-nationalist. We call them religious because they use a religious idiom and shape their 'mandate' in divine terms. Their goals, however, are political: recognition, power, territory, concessions from states, and so on.

Historically, such groups have included:

- *Al Qaeda (transnational, Islamist)*;
- *Aum Shinrikyo (renamed Aleph) (Japanese; various influences, including Hindu and Buddhist)*;
- *Klu Klux Klan (U.S.; Christian)*;
- *Abu Sayyaf (Philippines; Islamist)*;
- *Egyptian Islamic Jihad*;
- *Hamas (Palestinian; Islamist) (Hamas is designated by the U.S. and other governments as a terrorist group, but it is also the elected government of the Palestinian Authority)*;
- *Hezbollah (Hezbollah is designated as a terrorist organization by the U.S. and other governments, but others argue it should be considered a movement, rather than a terrorist group).*

Source: <https://www.thoughtco.com/terrorist-groups-a-list-of-terrorist-groups-by-type-3209111>

3.1.7. How does terrorism end?

Individually, there are four common ways out of terrorism: through death, imprisonment, entering regular politics, or entering organized crime. Some ex-terrorists return to a 'normal' life and try to forget their violent past – some even denounce it. This is hard to show with exact numbers because it depends on the specific conflict. Collectively, terrorist organizations can be either violently crushed by the state (LTTE, Baader Meinhof, Red Brigades, November 17),

surrender (ETA), suspend their activities and join regular political process through peace negotiations (the IRA), or even transform themselves into a political party (Hezbollah) or a quasi-state (PLO, Islamic State). Last, but not least, they sometimes even achieve ‘victory’ (FLN, Zionist organizations, EOKA), at which point they transform into a legitimate entity, or disband altogether. Some maintain their violent activity for a long time, transforming themselves according to external needs and conditions (e.g. Al Qaeda).

Exercise 1: Find additional information on the structure and activity of selected terrorist group of socialist/communist, national liberation and religious groups. Use Internet sources.

3.2. The impact of terrorism on the development of EU states

3.2.1. Terrorism and modern politics

Since violence and coercion is visibly present in political relations, both domestically and internationally, it is hard to deny that terrorism is an extension of politics by other means. It seems equally obvious that terrorism is detached from normal, every-day politics, and enters the domain of attracting public attention through ruthless killings, spreading its political message and aiming at submission by extortion. This means that it cannot be accepted as a legitimate form of political interaction, and so is usually fought by state institutions.

3.2.2. Terrorism and the state

Political entities use terrorist methods to attack, destroy or transform a state and force its authorities to alter their policies in areas perceived by the terrorists as key. Ultimately, the government of a state is the principal target of terrorist action – in democratic regimes the public has a chance to influence its own government, and so it is sometimes also held responsible by the terrorists (‘We kill you because you support the government that is killing us’). In non-democratic regimes, terrorist entities attempt to influence government indirectly by showing its inability to maintain public order and provoke harsh repression of large sectors of society, which could enable terrorists to gain more social support. Some EU states have extensive experience with various terrorist entities, domestic and international (the United Kingdom, France, Germany, Spain, Italy), and have introduced a wide variety of countermeasures – legal and organizational – and have at their disposal the staff and infrastructure to deal with this problem more or less effectively. For other countries, the threat of terrorism is a relatively new one and they have yet to adjust their functioning and legal system, or develop

counter-terrorist systems to protect their public. The EU itself actively promotes counter-terrorist co-operation through numerous initiative and policies.

Terrorism is not a new phenomenon in Europe. It poses a threat to our security, to the values of our democratic societies and to the rights and freedoms of European citizens. Between 2009–2013 there were 1,010 failed, foiled or completed attacks carried out in EU member states, in which 38 people died. In addition, several European citizens have been kidnapped or killed by terrorist groups around the world. The phenomenon of fighters from Europe travelling to different locations to fight the jihadists, and the security threat they may pose inside the EU when they return, are also likely to persist in the coming years. Since these threats do not recognise borders, they must be confronted at both a national and international level.

EU counter-terrorism strategy is designed to combat terrorism globally while respecting human rights, and to make Europe safer, allowing its citizens to live in an area of freedom, security and justice. European Union member states are committed to jointly fighting terrorism and providing for the best possible protection of its citizens. To this end, in 2005 the Council adopted the EU counter-terrorism strategy.

The strategy is focused on four main pillars: prevent, protect, pursue and respond. Across these pillars, the strategy recognises the importance of working with third countries and international institutions.

Prevent

One of the EU's priorities in the field of counter-terrorism is to identify and tackle the factors that contribute to radicalisation, and the processes by which individuals are recruited to commit acts of terror. To this end, the Council has adopted an EU **strategy for combating radicalisation and recruitment** to terrorism. In light of evolving trends, such as the phenomena of lone actors and foreign fighters, or the growing potential of social media for mobilisation and communication, the Council adopted a revision on this strategy in June 2014. In December 2014, justice and home affairs ministers across the Union adopted a series of **guidelines** for the revised EU radicalisation and recruitment strategy. These guidelines set out a series of measures to be implemented by the EU and its member states.

Protect

The second priority of the EU counter-terrorism strategy is the protection of its citizens and infrastructure, and reducing its vulnerability to attack. This includes the protection of external borders, the improvement of transport security, the protection of strategic targets and the reduction of the vulnerability

of critical infrastructure. In this area, the EU is currently working on legislation regulating the use of **passenger name record (PNR) data** for law enforcement purposes.

Pursue

The EU is working to hinder terrorists' capacity to plan and organise, and to bring these terrorists to justice. To achieve these goals, the EU has focused on strengthening national capabilities, improving practical cooperation and information exchange between police and judicial authorities (in particular through Europol and Eurojust), and tackling terrorist financing to deprive terrorists of the means by which they mount their attacks and communicate.

In May 2015, the Council and the European Parliament also adopted new rules to prevent **money laundering and terrorist financing**.

Respond

The fourth objective of the EU counter-terrorism strategy is to prepare, in the spirit of solidarity, to manage and minimise the consequences of a terrorist attack. This is done by improving the EU's capability to deal with the aftermath of incidents, coordinate responses, and handle the needs of victims. Priorities in this area include the development of EU crisis co-ordination arrangements, the revision of the civil protection mechanism, the development of risk assessment mechanisms and the sharing of best practices for assisting victims of terrorism.

Priorities in recent years have included:

1. The definition of the arrangements for the implementation by the EU of the solidarity clause, through a Council decision, adopted in June 2014.
2. A review of the EU emergency and crisis coordination arrangements, replaced by the EU integrated political crisis response arrangements (IPCR), in June 2013.
3. Revision of the EU civil protection legislation, at the end of 2013.

Engagement with international partners

The security of the European Union is closely linked with developments in other countries, particularly in the neighbouring states, and so the EU counter-terrorism strategy needs to be on a global scale.

In the strategic guidelines for justice and home affairs, adopted in June 2014, the European Council called for an effective counter-terrorism policy that integrates internal and external aspects. On 12 February 2015, the EU heads of state and government stressed the importance to the EU of engaging more with third countries on security issues and counter-terrorism.

In relations between the **EU and third countries**, the counter-terrorism agenda is present in many ways, through high level political dialogue, the adoption of cooperation clauses and agreements, and specific assistance and capacity-building projects with strategic countries. The EU works on counter-terrorism with countries in the Western Balkans, the Sahel, North Africa, the Middle East, the Horn of Africa and North America, as well as in Asia.

Mutual work with the US is a fundamental component of the EU's strategy. In recent years, agreements have been reached in areas such as the financing of terrorism, transport and borders, mutual legal assistance and extradition. US authorities are working more and more closely with Europol and Eurojust.

Another important part of the external dimension of the fight against terrorism involves working closely with other **international and regional organisations** to build international consensus and promote international standards for fighting terrorism. The European Union works with international organisations including the UN and the Global Counter Terrorism Forum, and regional organisations such as the Council of Europe, the OSCE, the League of Arab States and the Organisation for Islamic Cooperation.

As part of its work with the UN, and following a number of UN Security Council resolutions, the EU has adopted certain restrictive measures against persons or entities associated with the Al-Qaeda network.

3.2.3. Possible reactions to terrorist threats

If terrorism is not incidental and involves prolonged militant activity, a nation is forced to react. It can choose a strategy of combating terrorism, or more rarely, negotiating a political solution with the terrorist entities. Even if they refuse to talk to the terrorists, in some cases, tactical negotiations with terrorists are simply a necessity, e.g. during hostage situations. Though usually a state will declare that they won't negotiate with terrorists, some channels of communication with terrorist organizations are often established, particularly during lengthy conflicts. The primary reaction to a terrorist threat, though, is an attempt to eradicate it, or if this is impossible to limit the influence of the incident through police or even military action. Governments consider terrorism illegal as it is usually based on severe breaches of law and order. Armed attacks, the credible threat of such attacks, killings, abductions and other violent actions are simply crimes, and cannot be tolerated by legal political entities, especially when democratically elected. Amongst other things, terrorists will attempt to usurp a state's monopoly on the use of force and coercion. The authorities tend to deem any non-state form of organized violence illegitimate. But 'combating terrorism' is a demanding and tricky activity, and forces politicians to introduce frequently very extensive changes in domestic legislation to make it easier or more effective. This consumes vast financial, human and organizational resources – one of

a terrorists' goals is to exhaust both society (by making them live in a condition of constant fear), and the state, which is forced to use vast resources to counter terrorist activity and ultimately change vital public policies.

3.2.4. The transformation of states by terrorism

Regardless of how a government reacts, terrorism changes the way it functions. The very nature of the threat – small, clandestine units of devoted militants supported by some section of a society's own population – demands development of very special skills and technical capabilities. This includes extended surveillance of the population, including use of so-called SIGINT and HUMINT techniques, establishment of rapid-reaction special forces as a physical reaction to acts of terrorism, vast investigative skills and powers granted to the police, and establishment of detention camps or special incarceration facilities for captured terrorists. Every country, regardless of its political system, develops police, intelligence and military resources to tackle this threat. To ensure it is acting legally, each state is then forced to introduce new counter-terrorist legislation in order not to be involved in illegitimate actions which would undermine its credibility. To protect its own population and institutions, the state simply must resort to special powers, some of which inevitably affect hard-earned civil liberties.

3.2.5. Terrorism and society

Terrorist entities that are alienated from their own society are not usually successful. To be at least partially effective terrorists must be supported by at least some sections of their society, who would be eager to provide safe haven to members of a political group violently opposing the government. The very existence of a prolonged and significant threat by a terrorist group(s) shows the existence of serious divisions within society, which make this kind of conflict possible. These kinds of societal rifts could be based on deep ideological divisions, or ethnic or religious tensions – terrorists are ideologically driven and want to subvert and ultimately change the social and political order within the attacked state. So when there is no social support for this kind of radical, violent action, terrorism promptly fades. But when the threat level increases over the long term, the divisions in society actually widen.

3.2.6. The politics of counter-terrorism

The process of countering global terrorist threats becomes a matter of high politics reaching to the far corners of the world, as can be seen post-9/11. Turning counter-terrorism activity into U.S. foreign policy was precisely what the planners behind the 9/11 attacks were trying to achieve. Thanks to the announcement of

The War on Terror/The Global War on Terrorism) by then-U.S. President George W. Bush, a small group of Sunni extremists, located somewhere in Afghan caves and hardly known prior to that time, became the main enemy of the world's only superpower, dwarfing such threats as Iran, North Korea and even Russia. President Bush's decision to attack not only Afghanistan but also Iraq (a country which had nothing to do with the 9/11 attacks), destabilized the entire Greater Middle East and paved the way for the restoration of the Islamic Caliphate ('Islamic State'), no more than thirteen years after 9/11. This event altered the politics of a superpower, and in this way Al Qaeda managed to hijack the foreign policy of its American opponent. Terrorism is the 'strategy of a mosquito' – a little creature cannot destroy a forest, but by flying into a bear's ear can achieve what it wants by steering the bear's reactions. Al Qaeda, and now Islamic State, have adopted precisely this strategy, transforming the Greater Middle East through the use of the destructive powers of subsequent American administrations. Al Qaeda succeeded in provoking the U.S. to over-react and adopt the wrong counter-terrorist strategy.

Exercise 2. Find information on a selected act of terrorism that happened in an EU state. Present the socio-demographic profile of the perpetrator(s) and the timeline of events leading to the attack, as well as its consequences.

3.3. Modern terrorism and the 'between war and peace' phenomenon

3.3.1. Terrorism as a threat to peace

The very presence of terrorist entities implies the existence of a political conflict, and this kind of conflict endangers the peaceful development of society. Terrorism is also a grave threat to international peace, both directly – through the direct actions of international and global terrorist organisations, and indirectly – through a states' reaction to terrorism. For example, America's so-called 'War on Terror' resulted in American military interventions in Afghanistan and Iraq and an increased military presence in many African and Asian countries. Terrorism endangers peace through its own violent actions, and by provoking nations to exert violent reactions.

3.3.2. Is terrorism a form of war?

No. War is a state of armed conflict engaged in through large-scale military operations. Military forces are distinct from civilian entities, and deliberate and unjustified attacks on civilian targets are usually considered a war crime. Wars are regulated by the international law of armed conflicts and are usually officially

declared by the government. The mortality rate in war is usually high, affecting entire nations in a physically and mentally destructive way, as aggression alongside the use of modern military technology. A state of war is open and visible and has visibly catastrophic results.

In contrast, terrorism is a clandestine activity, and its perpetrators look like ordinary men and women. However, they do not respect the legal codes of conduct of war, and attacking defenceless targets is their primary – usually sole – tactic. Hence, the physical damage achieved through terrorism is usually limited, and the frequency of its armed attacks usually low. ‘Conventional’ army, air-force and navy forces are usually not involved in combating terrorism – they may provide a government with the services of their special forces and intelligence units, but the primary resources used by a state to fight terrorists are the police and other emergency and special services.

So although terrorism is not war, neither is it obviously peace – it is instead a social phenomenon located between war and peace. It is a politically motivated violent assault on a state and society aimed at social and political change, carried out in a way that significantly differs from conventional military warfare.

3.3.3. Terrorism and the local, regional and global order

Depending on the political agenda of the terrorist entity, the results of their activity can be visible at the local, regional and global levels. The IRA and ETA affected Britain and Spain directly, but did not really have a profile elsewhere, even if their terrorists operated in other countries. The Israeli-Palestinian conflict, a continuing series of terrorist attacks and counter-terrorist responses, ultimately managed to transform the entire Middle East and frequently took place beyond its borders (largely in Europe). Today, the influence of Al Qaeda and the Islamic State is truly global, affecting almost all continents.

3.3.4. Terrorism as an act of communication

Terrorism is also presented as an act of political communication – of conveying a meaningful message from one individual or group to another. The intended violence with it amplifies this political message and the seriousness of the attitude of the sender. Hence, modern and post-modern mass media, as well as social media, all play a vital and necessary role in spreading this message – they are an essential part of the ‘terrorist process’. Newspapers, radio, television and now social media have always been extensively used to present the political justification of acts of violence, the demands of terrorists, their views, opinions and attitudes towards the government and public opinion. People willing and able to kill their fellow citizens for political reasons have never been neglected or ignored – on the contrary, even the most obviously unreasonable or senseless

terrorist communiqués were carefully listened to and meticulously analysed. Killing innocents to attract public attention has always proved to be an effective tactic. Perceiving terrorism as propaganda by deed has been as much a thing in the early days of anarchism and newspapers, over a century ago, as it is today, in the era of jihadi fighters and Twitter.

3.3.5. Civilizations and terrorism

In the case of a jihad, terrorism is the preferred tactic everywhere that the jihadists are unable to use other forms of violence, such as guerrilla or conventional warfare, or even genocide. The jihadists perceive their fight as a divine obligation, an act of defence of their sacrosanct Islamic umma (community) and civilization. Their aim is to enforce submission to the one and only 'true' religion. In this way, their aims are non-negotiable and impossible to meet through any kind of partial concession. Total and ultimate surrender is simply needed to meet the jihadists' demands, and this makes conventional politics obsolete. Warfare becomes the only available option in response to this type of violence.

3.3.6. Religious terrorism

Absolute religious goals change the nature of terrorism – it is no longer politics by other means, but rather a method of conquest. In this way, religious terrorism is a punishment for sins, a road to individual salvation, an act of sacrifice in the name of an imagined community, and an attempt to achieve a pure and pious form of society. The supernatural character of jihadi Salafi groups aiming for religious piety and its expansion radically change the very nature of their terrorist threats. It literally precludes the possibility of proposing, then entering, a peace process, and reaching a political solution through negotiations with the terrorists' political wing(s), as happened in the Irish conflict.

Exercise 3. Find information on a terrorist attack perpetrated by Al Qaeda operatives since 9/11/2001. Present a timeline of events leading to this attack and its consequences.

3.4. Terrorist use of CBRN agents

3.4.1. Terrorist acts using CBRN weapons

The following table summarizes known historical instances of the use of chemical, radiological and biological weapons, in reverse chronological order. The listing is limited to events since 1900 (while there were some earlier instances

of chemical/biological warfare, these instances were generally of very limited effectiveness). Note that some incidents are disputed, and casualty figures in some cases are very uncertain. Sources are provided following the table.

Table 2. Historical summary of attacks using chemical, radiological and biological weapons, compiled by Wm. Robert Johnston (last updated 30 November, 2016)

Date	Location	Attacker	Agent	Affected pop	Casualties	Description
21–27 Oct 2016	near Mosul, Iraq	Islamic State militants	sulfur	civilians, soldiers	2 killed , 1,500 injured	sulfur mine set on fire, producing widespread sulfur dioxide plumes
8 Mar 2016	Taza, Kirkuk, Iraq	Islamic State	blistering agent	civilians	1 killed , 600 injured	attack on a town; fatality was 3-year-old child
23 Jan 2015	between Mosul, Iraq, and Syrian border	Islamic State militants	chlorine	Kurdish soldiers	~30 injured	truck bomb with chlorine-filled tanks used against troops
Sep–Oct 2014	Duluiya and Balad, Iraq	Islamic State militants	chlorine, possibly mustard gas	Iraqi and Shiite soldiers	40 injured	bombs with chlorine-filled cylinders used against defending troops
Apr 2012 – Jun 2013	Afghanistan--Takhar province (9), Sar-e-Pul province (4), others	Islamist terrorists	pesticides?	school-children	1,952 injured (including 1,924 children)	23 poison attacks on girls' schools, some cases of water poisoning
Mar 2012 – Apr 2013	Afghanistan	Islamist terrorists	rat poison?	police, other civilians	53 killed , 40 injured	9 attacks involving poisoning of food at police stations/academies

Table 2. cont.

Date	Location	Attacker	Agent	Affected pop	Casualties	Description
Apr–Aug 2010	Afghanistan–Kabul (6), Kunduz (4), others	Islamist terrorists	pesticides?	school-children	672 injured (including 636 children)	20 gas attacks on girls' schools
11 Mar 2007	Iraq	Islamist terrorists	mustard gas	U.S. soldiers	2 injured	failed improved explosive device using chemical weapon artillery shells
Oct 2006–Jun 2007	Iraq cities – Ramadi (6), Baghdad (3), Falluja (3), others	Islamist terrorists	chlorine	civilian targets	115 killed*, 854 injured (including 85 children)	15 car/truck bombings with chlorine tanks used; most fatalities were from the explosions, most injuries from the chemical releases
8 Oct 2006	Numaniyah, Iraq	Islamist terrorists	poison	police-men	7 killed , 700 injured	poisoning of meals on police base; un-confirmed
25 Sep 2006	Baghdad, Iraq	Islamist terrorists	mustard gas	U.S. soldiers	2 injured	improved explosive device using chemical weapon artillery shells

Date	Location	Attacker	Agent	Affected pop	Casualties	Description
15 May 2004	Baghdad, Iraq	Islamist terrorists	sarin nerve gas	U.S. soldiers	2 injured	failed improvised explosive device using chemical weapon artillery shell near Baghdad airport
24 Jun – Jul 2003	near Mosul, Iraq	Islamist terrorists	sulfur	civilians, soldiers	? injured	sulfur stockpiles at mine set on fire, producing widespread sulfur dioxide plumes; at least 41 U.S. soldiers injured
18 Sep – 9 Oct 2001	United States – Washington, DC, New York City, NY, others	Bruce Ivins?	anthrax	government and civilian media individuals; postal employees and customer	5 killed , 17 injured	anthrax-laced letters mailed to federal officials in Washington DC and new media offices in multiple locations; many casualties among postal workers
20 Mar 1995	Tokyo	Aum Shinrikyo cult	sarin nerve gas	Tokyo subway	12 killed , 5,511 injured	nerve gas releases in subway; many permanent injuries
28 Jun 1994	Matsumoto, Japan	Aum Shinrikyo cult	sarin nerve gas	civilians	7 killed , 270 injured	overnight release of nerve gas in city

Table 2. cont.

Date	Location	Attacker	Agent	Affected pop	Casualties	Description
21 Jan 1994	Ormancik, Turkey	terrorists	chemical agent	civilians	16 killed	attack on village using chemical grenades
6 Sep 1987	Zamboanga City, Philippines	terrorists	poison	police-men	19 killed, 140 injured	water poisoning with pesticide at constabulary
9–19 Sep 1984	The Dalles, Oregon, United States	Bhadwan Shree Rajneesh cult	salmonella	civilian restaurants	751 injured	food poisoning in several restaurants; was experiment in preparation to interfere with upcoming election
14 Apr 1946	Camp Stalag 13, Nuremberg, Germany	Jewish terrorists	cyanide	SS prisoners	2,283 injured	poisoning of bread for Nazi prisoners

3.4.2. The science of terrorism

In order to increase the deadly effects of their actions, terrorists are constantly seeking innovative ways of killing people, including with weapons of mass destruction. As chemical agents are the easiest to produce, develop and utilise, they tend to concentrate on this aspect of CBRN options. However, it is alleged that Al Qaeda operatives have managed to experiment not only with chemical, but also biological agents, and were actively seeking the means of developing radiological devices.

Nuclear terrorism – towards a real Armageddon?

Assembling a fully working nuclear device is out of most terrorists' reach. As long as they are not given a device or are unable to steal it, use of nuclear devices in a terrorist attack is highly unlikely. More likely is an attack with the use of a radiological bomb – a device often called a 'dirty bomb'.

A 'dirty bomb' is a type of radiological dispersal device (RDD) that combines conventional explosives, such as dynamite, with radioactive material. The terms dirty bomb and RDD are often used interchangeably in the media. But most RDDs do not release enough radiation to kill people or cause severe illness – the conventional explosive itself is more harmful to individuals than the radioactive material. However, depending on the situation, an RDD explosion can create tremendous fear and panic, contaminate property, and require potentially costly clean-up. Making prompt, accurate information available to the public can prevent the panic sought by terrorists.

A dirty bomb is in no way similar to a nuclear weapon, or nuclear bomb. A nuclear bomb creates an explosion that is millions of times more powerful than that of a dirty bomb. The cloud of radiation from a nuclear bomb could spread tens to hundreds of square miles, whereas a dirty bomb's radiation could be dispersed within a few blocks or miles of the explosion. A dirty bomb is not a Weapon of Mass Destruction, but a Weapon of Mass *Disruption*, with which contamination and anxiety are the terrorists' major objectives. RDDs appeal to terrorists because they require limited technical knowledge to build and deploy compared to a nuclear device. Also, the radioactive materials in RDDs are widely used in medicine, agriculture, industry and research, and are easier to obtain than weapons grade uranium or plutonium.

The impact of Dirty Bombs

The extent of local contamination depends on a number of factors, including the size of the explosive, the amount and type of radioactive material used, the means of dispersal, and the weather conditions. Those closest to the RDD would be the most likely to sustain injuries from the actual explosion. As radioactive material spreads, it becomes less concentrated and less harmful. Prompt detection of the type of radioactive material used would greatly assist local authorities in advising the community on protective measures, such as sheltering in place, or evacuating the immediate area. Radiation can be readily detected with equipment already carried by many emergency responders. Still, subsequent decontamination of the affected area can involve considerable time and expense. The health effects of radiation tend to be directly proportional to the dose of radiation received – the higher the dose, the higher the risk of injury.

Sources of Radioactive Material

Radioactive material is routinely used in hospitals, research facilities, industrial activities and on construction sites. These radioactive materials are used for purposes such as diagnosing and treating illnesses, sterilizing equipment and inspecting welding seams. In the United States, the Nuclear Regulatory

Commission, together with 37 Agreement States, which also regulate radioactive material, administer more than 22,000 licenses for such materials. The vast majority of these materials are not viable for use in RDDs.

Control of Radioactive Material

In America, the NRC and state regulations require owners licensed to use or store radioactive material to secure it from theft and unauthorized access. These measures have been greatly strengthened since the attacks of September 11, 2001. Licensees must promptly report lost or stolen risk-significant radioactive material. 'Risk-significant' refers to radioactive sources that could pose a significant risk to individuals, society and the environment if not properly used, protected, or secured. Local authorities also assist in making a determined effort to find and retrieve such sources. Most reports of lost or stolen material involve small or short-lived radioactive sources that are not viable for use in RDDs.

Past experience suggests that there has not been a pattern behind the collection of such sources for the purpose of assembling an RDD. It is important to note that the radioactivity of the combined total of all unrecovered sources over the past 8 years (when corrected for radioactive decay), would not reach the threshold for one high-risk radioactive source. Unfortunately, the same cannot be said world-wide.

The U.S. Government is working to strengthen security for high-risk radioactive sources both at home and abroad. The NRC and its 37 Agreement States – states who have been given authority to regulate nuclear materials within their borders – have worked together to create a strong and effective regulatory safety and security framework that includes licensing, inspection, and enforcement.

The NRC also works with other Federal agencies, the International Atomic Energy Agency, and licensees to protect radioactive material from theft and unauthorized access. The agency has made improvements and upgrades to the joint NRC-DOE (Department of Energy) database that tracks the location and movement of certain forms and quantities of special nuclear material. In addition, in early 2009, NRC deployed its new National Source Tracking System, designed to track high-risk sources in the United States on a continuous basis.

Risk of Cancer

Just because a person is near a radioactive source for a short time or gets a small amount of radioactive dust on themselves does not mean that they will get cancer. Any additional risk will likely be extremely small. Doctors specializing in radiation health effects will be able to assess the risks and suggest what medical treatment, if any, is needed once the radioactive source and exposure levels have been determined.

There are some medical treatments available that help cleanse the body of certain radioactive materials following a radiological accident. Prussian blue

has been proven effective for ingestion of cesium-137 (a radioactive isotope). In addition, potassium iodide (KI) can be used to protect against thyroid cancer caused by iodine-131 (radioactive iodine). However, KI, which is available over the counter, offers no protection to other parts of the body or against other radioactive isotopes. Ultimately, medical professionals are best qualified to determine how to best treat symptoms.

Exercise 4. Find information on a terrorist attack that used CBRN weapons and present a description of the consequences.

3.5. Terrorist crimes in Polish and international criminal codes

3.5.1. Terrorism – is one man’s terrorist another man’s freedom fighter?

This subjectively false dilemma is supposedly responsible for the lack of coherent international legislation on terrorist action that is accepted by all states. As discussed previously, terrorism is a *method* of warfare, and a terrorist’s ‘cause’ is irrelevant here. Hence, terrorists are considered to be all those who are involved in attacking defenceless members of their own or another society, regardless of the ultimate purpose of their fight, or cause. At the same time, the term ‘terror state’ could be applied to any state terrorizing its own or other populations for political reasons. Nevertheless, this view is not commonly held, and so international law was, is and will be, full of differing legal opinions that lack a universal vision, unifying EU legislation in terms of acts of terrorism.

3.5.2. Terrorism and guerrilla warfare

There is a fundamental difference between guerrilla warfare and terrorism. The former implies an armed assault delivered by small *paramilitary* units against *military* targets that are actively involved in (or needed to conduct) military operations. Terrorism implies an assault on non-combatant, defenceless targets. Even soldiers and policemen can be considered non-combatants when they are off duty, or not taking part in combat operations. When *guerilleros* choose to attack civilians, they abandon the path of an irregular (yet legally recognised) type of warfare, and enter the path of terrorism.

3.5.3. Terrorism and the rules of war

Put simply, war implies attacks on legitimate military targets. Terrorism implies unlawful attacks on non-combatants.

Table 3. The 10 Rules of War

The rules of war, also known as international humanitarian law	
1.	Protect those who are not fighting, such as civilians, medical personnel and aid workers.
2.	Protect those who are no longer able to fight, such as injured soldiers or prisoners.
3.	Prohibit the targeting of civilians. Doing so is a war crime.
4.	Recognize the right of civilians to be protected from the dangers of war and receive the help they need. Every possible care must be taken to avoid harming them or their houses, or destroying their means of survival, such as water sources, crops, livestock, etc.
5.	Mandate that the sick and wounded have a right to be cared for, regardless of whose side they are on.
6.	Specify that medical workers, medical vehicles and hospitals dedicated to humanitarian work cannot be attacked.
7.	Prohibit torture and degrading treatment of prisoners.
8.	Specify that detainees must receive food and water and be allowed to communicate with their loved ones.
9.	Limit the weapons and tactics that can be used in war, to avoid unnecessary suffering.
10.	Explicitly forbid rape and other forms of sexual violence in the context of armed conflict.

Source: <https://www.icrc.org/en/document/10-things-rules-of-war-Geneva-Conventions>

3.5.4. The legal approach to terrorism

Every state involved in counter-terrorist activities has developed its own legislation to solve the legal questions in combating and preventing terrorism.

3.5.5. Terrorism in Polish and international legislation

Since the 1st of May 2004, the Polish penal code has adapted the term “terrorist related crime”, in §20 of art. 115. On the 2nd of July 2016, Poland brought into force its very first Counter-terrorist Act, which provides Polish state institutions with special powers to investigate, prevent and react to terrorist threats. Amendments to Polish Criminal Law have been made to punish the financing of terrorism, participation in terrorist training, the spreading of terrorist propaganda, the provision of assistance to terrorist activities and even refraining from informing the police about possible terrorist attacks. In contrast, international law lacks a unified convention on terrorism and is based on bilateral and multilateral agreements and conventions, as well as several sectoral U.N. conventions dealing with a variety of different terrorist-related activities.

Table 4. International Conventions on Terrorism – Related Crimes

1. Convention on Offences and Certain Other Acts Committed On Board Aircraft (1963) <http://treaties.un.org/doc/db/Terrorism/Conv1-english.pdf>
2. Convention for the Suppression of Unlawful Seizure of Aircraft (1970) <http://treaties.un.org/doc/db/Terrorism/Conv2-english.pdf> Protocol Supplementary to the Convention for the Suppression of Unlawful Seizure of Aircraft (2010) http://legacy.icao.int/DCAS2010/restr/docs/beijing_protocol_multi.pdf
3. Convention for the Suppression of Unlawful Acts against the Safety of Civil Aviation (1971) <http://treaties.un.org/doc/db/Terrorism/Conv3-english.pdf>
4. Convention on the Prevention and Punishment of Crimes Against Internationally Protected Persons (1973) <http://treaties.un.org/doc/db/Terrorism/english-18-7.pdf>
5. International Convention against the Taking of Hostages (1979) <http://treaties.un.org/doc/db/Terrorism/english-18-5.pdf>
6. Convention on the Physical Protection of Nuclear Material (1980) <http://treaties.un.org/doc/db/Terrorism/english-18-5.pdf>
7. Protocol for the Suppression of Unlawful Acts of Violence at Airports Serving International Civil Aviation, supplementary to the Convention for the Suppression of Unlawful Acts against the Safety of Civil Aviation (1988) <http://treaties.un.org/doc/db/Terrorism/Conv7-english.pdf>
8. Convention for the Suppression of Unlawful Acts against the Safety of Maritime Navigation (1988) <http://treaties.un.org/doc/db/Terrorism/Conv8-english.pdf> Protocol to the Convention for the Suppression of Unlawful Acts against the Safety of Maritime Navigation (2005) https://www.unodc.org/tldb/en/2005_Protocol2Convention_Maritime%20Navigation.html
9. Protocol for the Suppression of Unlawful Acts against the Safety of Fixed Platforms Located on the Continental Shelf (1988) <http://treaties.un.org/doc/db/Terrorism/Conv9-english.pdf> Protocol to the Protocol for the Suppression of Unlawful Acts against the Safety of Fixed Platforms Located on the Continental Shelf <http://cil.nus.edu.sg/2005/2005-protocol-to-the-1988-protocol-for-the-suppression-of-unlawful-acts-against-the-safety-of-fixed-platforms-located-on-the-continental-shelf/>
10. Convention on the Marking of Plastic Explosives for the Purpose of Detection (1991) <http://treaties.un.org/doc/db/Terrorism/Conv10-english.pdf>
11. International Convention for the Suppression of Terrorist Bombing (1997) <http://treaties.un.org/doc/db/Terrorism/english-18-9.pdf>
12. International Convention for the Suppression of the Financing of Terrorism (1999) <http://treaties.un.org/doc/db/Terrorism/english-18-11.pdf>
13. International Convention for the Suppression of Acts of Nuclear Terrorism Nuclear Terrorism Convention (2005) <http://treaties.un.org/doc/db/Terrorism/english-18-15.pdf>
14. Convention on the Suppression of Unlawful Acts Relating to International Civil Aviation (2010)

Source: http://legacy.icao.int/DCAS2010/restr/docs/beijing_convention_multi.pdf

Exercise 5: Find the text of a selected legal convention or act regulating the fight against terrorism. Present the most important issues.

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4. ANTI-TERRORIST ACTIONS INVOLVING CBRN MATERIALS

The antiterrorism action in CBRN environment is very complicated issue which must involve various sources and services. The main component of that type of operation are Police special units with PPE and special detection equipment, fire fighters with decontamination pathway as well as other services responsible for CBRN agent neutralization. Exercises in the field of responding to the CBRN event in the context of a terrorist CBRN episode are aimed at systematizing knowledge and focusing the process of preparation of practical services, entities in the area of supporting CBRN specialized activities carried out in the course of counterterrorism activities. The main goal should be to indicate the necessary elements in the process of preparing and conducting AT / CBRN activities, including improving the CBRN detection capability and individual command skills necessary to perform functional duties in the process of preparing and conducting operations in a crisis situation in accordance with the intended use.

The proper scenario of this type of action will be presented based on Polish Anti-terrorist exercise in National Centre of Nuclear Research (NCBJ) in Świerk called Patrol 2015 which verified the readiness of the KSWSiA Contamination Detection & Alerting system.

The scenario of the exercise was that some radioactive material was released from the MARIA nuclear reactor in result of a terrorist attack. Objectives of the exercise included to check readiness of the relevant services/offices/forces (National Atomic Energy Agency's Radiation Emergency Centre, Otwock County Police Department, Polish Police Headquarters' Duty Officer, Polish Police Headquarters' Bureau of Counter-Terrorist Operations) to react to this threat.

1. The aim of the exercises was to perform CBRN detection activities, CBRN event management at the operational level in the most-real-world conditions and to develop the right skills and habits that are necessary in counterterrorism activities.

2. In order to increase the realism of the exercise, the actions of the crisis management entities were played, while the terrorist activities were simulated by the police anti-terrorists.

3. The content of the exercises was the execution by participants of the detection in the course of simulated counter-terrorist actions, effective use of the

acquired knowledge acquired in the course of specialized courses, commanding, planning and conducting logistics activities.

The basic principles of the exercise were:

- unity of individual and team training;
- realism;
- continuity of the exercise.



Figure 1. March of arrested people assisted by BOA KGP officers to the place of initial decontamination

The scenario of the episode played in NCBJ assumed the invasion of an armed group of terrorists on the premises of the institute. The service of the facility, which was to constitute a bargaining chip for unknown perpetrators of the offense, was captured. Immediately after the incident was noticed by the patrol of the internal security service, the response procedures provided for such a circumstance were activated. Thanks to that, only a few minutes after the notification, specialized services arrived: the Police, the State Fire Service and the locally based Radioactive Waste Utilization Plant.

After conducting preliminary police arrangements by local police units, a unit specialized in CBRN counter-terrorism operations, Bureau of Counter-terrorist Operations of the Police Headquarters (BOA KGP), was established *via* the Police's duty system.

In accordance with the applicable procedures, the headquarters of the police operation command office was established in a safe place to conduct command and coordination activities at the scene.

The composition of this command office include:

- directing anti-terrorist activities along with officers carrying out planning and coordination tasks (in this situation);
- the administrator of the nuclear facility;
- commanding the activities of the State Fire Service;
- Police spokesman.

During the action the following activities had been performed:

- evacuation of personnel from the object at risk was carried out;
- establishing the zones with a division into hot, transitional and so-called clean (safe) with the location of possible press bands;
- a safe place has been set for the dislocation of equipment and service necessary to perform initial decontamination;
- the tactics of action for the responding services have been defined, taking into account the means of individual protection and the provision of tactical equipment, including dosimetry devices;
- defined possible action scenarios, including the possibility of spreading contamination and exposure to possible exposure of ionizing radiation.



Figure 2. The securing decontamination tents by the officers of the BOA KGP

The initial decontamination was installed by the State Fire Brigade, which had arrived in place, in a configuration enabling safe decontamination of officers,

hostages and perpetrators of the crime. With particular emphasis on tactics that allow continuous surveillance during its operations over detainees (Fig. 3). An important element was the organization of security for contaminated firearms and specialized equipment of officers carrying out anti-terrorist tasks.



Figure 3. The initial decontamination process under the supervision of the officers of the BOA KGP

In the meantime, monitoring of possible contamination of the action zone was carried out through the use of permanent dosimetry systems located on the site of NCBJ and based on individual equipment of policemen securing access to the danger zone. The officers of BOA KGP also used a robot armed with devices signaling elevated gamma and neutron radiation. The vehicle moved remotely through the hot zone and also enabled the vision to be provided through the cameras in which it was equipped.

A police helicopter equipped with highly specialized optical equipment enabling satellite transmission of a registered online vision directly to the commanding police operation was also ordered. There have been established shooting positions aimed at supporting officers who perform counter-terrorist tasks. In the meantime, it has secured a place for medical assistance to victims that could possibly be contaminated.

At the time of note shot in the facility, where were terrorists and hostage head of counter-terrorism activities decided to launch an immediate assault on

the building occupied by the perpetrators. Anti-terrorist officers equipped with individual CBRN protection measures (upper respiratory tract protection, body surface) and equipment outside firearms and tactical equipment in an individual dosimeter started a concealed approach to the object.

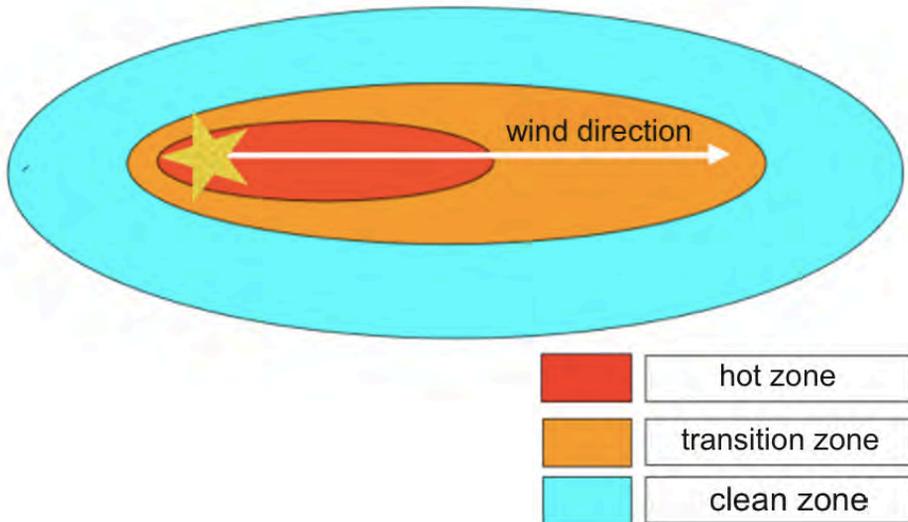


Figure 4. An example of the designation of zones during CBRN counterterrorism activities

After the action, in accordance with good practice, the personnel involved in the handling of the decontamination area first measured the contamination of the body surface of the officers together with the detainees to determine possible contamination. Due to the assumption that application was contaminated in the object being attacked, steps were taken to remove the harmful agent from the body surface of people exposed to it. The whole activity was correlated with protective actions on detained persons and deposited equipment, i.e. firearms, ammunition, tactical equipment.

