NATO Science for Peace and Security Series - A:
Chemistry and Biology

Counteraction to Chemical and Biological Terrorism in East European Countries

Edited by
Christophor Dishovsky
Alexander Pivovarov

Springer

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Counteraction to Chemical and Biological Terrorism in East European Countries

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Series A: Chemistry and Biology

Counteraction to Chemical and Biological Terrorism in East European Countries

Edited by

C. Dishovsky
Military Medical Academy
Sofia, Bulgaria

and

A. Pivovarov
Ukrainian State University of Chemical Engineering
Dnepropetrovsk, Ukraine

Springer

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Contents

Preface

The terrorist act with sarin gas in the Tokyo underground and the case with the spread of anthrax spores through the U.S. postal system stimulated the development of organization of fight against terrorism on a national and global level. The goal of this workshop was assessment of scientific concepts and practical means for management of chemical and biological agents casualties in the area of terrorist attacks with emphasis on improving the problems and situation in Eastern European Countries.

In this book are included the results of both theoretical and practical research of chemical and biological terrorism presented during the workshop. Different trends of research to fight against terrorism on both local and governmental level including some Eastern European countries are discussed.

The scientific articles are grouped into those areas:

- New approaches in counteraction to chemical and biological terrorism
- Medical treatment and decontamination of casualties from chemical and biological agents
- Diagnosis of exposure to chemical and biological agents
- Development of protection against injuries from chemical and biological agents

In these articles the following are emphasized:

- Some aspects of national and global defense against chemical and biological terrorism
- National action plans and global agreements on combating terrorism
- The characteristics of the major specific injuries connected with chemical and biological terrorism
- Threats of terroristic attacks
- Epidemiological and clinical peculiarities, ways of diagnosing, medical treatment and preventive health care measures

These problems are analyzed from an interdisciplinary perspective.

This book will be interesting and useful for medical and other university students, medical doctors, specialists in the field of personal and social safety, environmental protection experts, chemists, biologists, and specialists of the army and governmental antiterrorist departments.

Professor Christofor Dishovsky, Ph.D., D.Sc. – Co-Director
Professor Alexander Pivovarov, D.Sc. – Co-Director

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Key Speakers

Andreychyn Michail, Corresponding Member of Ukrainian Academy of Medical Science, DMS., Professor, I.Y. Horbachevsky Ternopil State Medical University, 1, m. Voli, Ternopil 46001 Ukraine Tel.: +380352524725; email: *infecdis@ukr.net*

Benschop Hendrik, Ph.D., Professor, Department of Chemical Toxicology, TNO Prins Maurits Laboratory. 45. Rostbox, Rijswijk 2280AA. The Netherlands Tel/ Fax +31153695015; email: *benschophp@planet.nl*

Dishovsky Christophor, DMS., Professor, Military Medical Academy, Department of Military Toxicology, 3 St. G. Sofiisky Str., Sofia 1606 Bulgaria Tel.: +35929226448; Fax. +35929526536; email: *christophord@yahoo.com*

Doan Svetlana, DMS. Deputy Director. L.V. Gromashevsky Institute Epidemiology and Infectious Diseases of Ukrainian Academy of Medical Sciences, Protasov Yar Street, 4, Kiev 03038 Ukraine Tel./Fax. +380442753711; email: *epidemics@ ukr.net*

Grin Grigory, D.Sc., Professor. Prorector of National Technical University "Kharkov Polytechnic Institute" 21. Frunze, Kharkov 61002 Ukraine. Tel.: +380577000135; Fax. +380577000135; email: *gryn@kpi.kharkov.ua*

Heredia Antonio, D.Sc., Professor, Department of Molecular Biology and Biochemistry , Universidad de Mбlaga. Facultad de Ciencias, Campus de Teatinos, Malaga 29071 Spain. Tel. +34952131940; Fax. +34952132000; email: *heredia@uma.es*

Kenzelmann Marc, D.Sc., Head National NBC Protection and Goordination Office SPIEZ LABORATORY Austrasse Str., Spiez. Switzerland. Tel. +41332281636; Fax. +41332281402; email: *marc.kenzelmann@babs.admin.ch*

Kharytonov Mykola, D.Sc. Dnepropetrovsk State Agrarian University, 25 Voroshilov St., Dnepropetrovsk 49600 Ukraine, Tel.: +380563742441; email: *mykola_kh@yahoo.com*

Kozub Pavlo, Ass. Professor. National Technical University "Kharkov Polytechnic Institute" 21. Frunze, Kharkov 61002 Ukraine. Tel.: +380577076745; Fax: +380577000135; email: *pkozub@cit-ua.com*

Kremenchutskyy Gennadiy, DMS, Professor. Dnepropetrovsk State Medical Academy. 4. Octybrskaya Sq., Dnepropetrovsk 49005 Ukraine. Tel./Fax. +380567135188; email: *kremen@dsma.dp.ua*

Kreppel Helmut, Professor, Lieutenant Colonel. Bundeswehr Medical Office, Department of CBRN Medical Defence, 11, Neuherbergstr. Munich 80937 Germany . Tel.: +498931684122; Fax. +498931684129; email: *helmutkreppel@bundeswehr.org*

Kurochkin Ilya, D.Sc., Professor. Chemical Department of Lomonosov Moscow State University, 1, bld. 3. Leninskye Gory, Moscow 119992, Russian Federation Tel./Fax. +74959394391; email: *ikur@genebee.msu.su*

Loza Iryna, PhD, Senior Staff Researcher. Department of Zoology&Ecology, Dnepropetrovsk National University, Gagarina av, 72, Dnepropetrovsk 49005 Ukraine. Tel./Fax. +380567768906; email: *irinaloza@hotmail.com*

Makhaeva Galina, Ph.D. Institute of Physiologically Active Compounds Russian Academy of Sciences Chernogolovka, Moscow Region 142432 Russia, Tel.: +74965242596 Fax. +74965245586; email: *gmakh@ipac.ac.ru*

Marievsky Victor, DMS, Professor, Director. L.V. Gromashevsky Institute Epidemiology and Infectious Diseases of Ukrainian Academy of Medical Sciences, Protasov Yar Street, 4, Kiev 03038 Ukraine Tel./Fax. +380442753711; email: *epidemics@ ukr.net*

Matousek Jiri, Professor. Masaryk University. 126/3 Kamenice, Brno CZ-625 00 Czech Republic Tel.: +420549492860; Fax: +420549492840; email: *matousek@recetox.muni.cz*

Mizin Vladimir, D.M., Ph.D. National Institute of Vine and Wine "Magarach", 31 Kirov St., Yalta, Autonomous Republic of Crimea 98600, Ukraine. Tel.: +380654325591; Fax. +380654320132; email: *yaltamizin@i.ua*

Moeder Monika, Dr. Chemist. Helmholtz Centre for Environmental Research, Department of Analytical Chemistry. 15 Permoserstrasse, Leipzig D-04318 Germany Tel.: +4903412351413; Fax. +4903412351443; email: *monika.moeder@ufz.de*

Moore David, DVM, Ph.D. Battelle Memorial Institute. 1204 Technology Drive, Aberdeen, Maryland 21010 United States. Tel./Fax: 4103068580; email: *moore@battelle.org*

Mutaf Vitalie, Major. Head of the Chemical and Radiological Division. Civil Protection and Emergency Situations Service. 69. Gheorghe Asachi Str., Chisinau MD 2028 Republic of Moldova Tel.: +37322738516; Fax. +37322738501; email: *dse@dse.md*

Nesterenko Valerii, D.Sc., Professor. Ecology Department. Belarusian National Technical University 65, Nezavisimosty Avenue, Minsk 220027 Republic of Belarus Tel.: +375172931695; Fax. +375172327182; email: *v.nesterenko@mail.ru*

Omelchenko Alexander, D.Sc., Institute of Cardiovascular Sciences, University of Manitoba, 351 Tache Ave., Winnipeg, Manitoba R2H 2A6 Canada, Tel.: +2042353350; Fax. +2042336723; email: *omelca@shaw.ca*

Orehovic Zvonko, Professor. College of Velika Gorica - Chrysies Management and College of Security 4. F.Budicoga, Zagreb, Croatia. Tel./Fax. +385915043839; email: *zvonko.orehovec@zg.t-com.hr*

Paul Florin, MD, Ph.D, MPH (1). Chief Medical Support Section Specialist. Support Service Logistic Support Division Department of Field Support United Nations New York 1 UN Plaza, New York 10017 United States Tel.: +12129638663; Fax.+1917367233; email: *paul8@un.org*

Pivovarov Alexander, D.Sc., Professor, Ukrainian State University of Chemical Engineering, 8. Gagarin Av. Dnepropetrovsk, 49005 Ukraine Tel.: +380562385658; Fax. +380562366837; email: *apivo@ua.fm*

Ristanovic Elizabeta, Dr. Ph.D. Military Medical Academy, Institute for Microbiology. Nedeljka Gvozdenovicha br. 1/II-10, Novi Beograd 11070 Serbia Tel.: +381638134129; Fax. +381112660027; email: *elizabet@EUnet.yu*

Savov Encho, Professor. Military Medical Academy, Department of Military Toxicology, 3.G.Sofiiski Str., Sofia 1606 Bulgaria Tel.: +35929522773; Fax. +35929526536; email: *savove@yahoo.com*

Semenov Evgeniy, Ass. Professor. National Technical University "Kharkov Polytechnic Institute" 21. Frunze, Kharkov 61002 Ukraine. Tel.: +380577076353; Fax: +380577076959; email: *esemyonov@kpi.kharkov.ua*

Shiman Leonid, Ph.D. General Director of SE RIC PCP – General Designer of SPRM. Pavlograd, Dnepropetrovsk region 51402 Ukraine. Tel.: +380563266008; Fax. +380563266002; email: *dirphz@mail.pkhz.dp.ua*

Shmatkov Grigory, D.Sc.,Professor. Pridneprovska State Academy of Civil Engineering and Architecture, 24-A Chernishevsky Str., Dnepropetrovsk 49000 Ukraine Tel.: +380562428841; Fax. +380562344029; email. *ecomet@alb.dp.ua*

Shtemenko Natalia, D.Sc., Professor. Department of Biophisics and Biochemistry , Dnipropetrovs'k National University. 72 Gagarin av. Dnepropetrovsk 49050 Ukraine Tel.: +380508596373; email: *ashtemenko@yahoo.com*

Tonkopii Valerii, DMS, Professor, Institute of Limnology, Russian Academy of Science. Sevastyanova str. 9, St. Petersburg 196105 Russian Federation Tel./Fax. +78123887327; email: *chemistry@limno.org.ru*

Tsatsakis T. Aristidis PhD, Professor, Director of the Department of Forensic Sciences & Toxicology Medical School. University of Crete, Heraklion 71409 Greece. Tel.: +3081394679; Fax. +3081542098. email: *aris@med.uoc.gr*

Yaren Hakan, MD. PhD. Gulhane Military Medical Academy, Department of Medical CBRN Defence. Etlik, Ankara 06018Turkey. Tel.: +903123043552; Fax. +903123043300; email. *yaren36@gmail.com*

Zadorsky William, Professor. Pridneprovie Cleaner Production Center, Ukrainian State University of Chemical Engineering. 8. Gagarin Av. Dnepropetrovsk, 49005 Ukraine Tel.: +380567440210; Fax. +38056744557; email: *ecofond@a-teleport.com*

Other Participants and Observers

Balszuweit Frank, Dr., Major. Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, Munich 80937Germany Tel.: +498931682313; Fax +498931682333. email: *FrankBalszuweit@bundeswehr.org*

Bikker Floris Johannes, Dr. Department of Chemical Toxicology, TNO Prins Maurits Laboratory. 45. Rostbox, Rijswijk 2280AA. The Netherlands Tel/ Fax +31152843605. email: *floris.bikker@tno.nl*

Bogadelnikov Igor, D.M., Ph.D. Crimean National Medical University, 5/7 Lenin Bul., Simferopol, Autonomous Republic of Crimea, Ukraine; Tel.: +380652274462; Fax. +380652274462.

Borisova Maja, MD., Military Medical Academy, Dept. Microbiology, 3, "G.Sofiiski" St., Sofia 1606 Bulgaria. Tel.: +35929522773; Fax. +35929526536.

Boyko Alexandra, Professor Assistant. Department of Veterinary, Dnepropetrovsk State Agrarian University, Voroshilova str, 25, Dnepropetrovsk 49600 Ukraine Tel./Fax. +380562463161. email: *boikoalexandra@rambler.ru*

Brygadirenko Viktor, PhD, Associate Professor. Department of Zoology&Ecology, Dnepropetrovsk National University, 72.Gagarina Av., Dnepropetrovsk 49005 Ukraine. Tel./Fax. +380567768906. email: *brigad@ua.fm*

Calancea Alexandru, Captain. Head of the medical-biological protection Division Civil Protection and Emergency Situations Service. 69. Gheorghe Asachi Str., Chisinau MD 2028 Republic of Moldova Tel.: +37322738516; Fax. +37322738501. email: *dse@dse.md*

Domnguez Eva, Ph. D. Biology, Department of Plant Breeding, Estacin Experimental "La Mayora", CSIC, Algarrobo-Costa, Mlaga 29760 Spain. Tel.: +34-952-528990. email: *edominguez@eelm.csic.es*

Dubacheva Galina. Ph.D., Scientific Researcher/Biosensors. Department of Chemistry, Moscow State University, 1/3 Leninskie Gory Moscow 119991 Russia Tel./Fax. +7495 9394042. email: *dubacheva@gmail.com*

Dudka Yeugen, D.Sc. Head of Plant Protection Department of the Grain Crops Institute Ukrainian Academy of Agrarian Science, 14, Dzerzhinsky St., Dnepropetrovsk 49600 Ukraine. Tel.: +38 0562451808.
email: *envteam@ukr.net*

Durau Michel, Director. The NATO Information and Documentation Centre 36/1 Melnykova Kiev 04119 Ukraine.
Tel.: +380444820616/17; Fax. +380444820622.
email: *m.duray@ukrpack.net*

Eremenko Arkady. Ph.D., Senior Scientific Researcher/ Biosensors. Department of Chemistry, Moscow State University, 1/3 Leninskie Gory, Moscow 119991 Russia Tel./Fax. +74959394391.
email: *eremenkoav@yandex.ru*

Haertig Claus. Dr. Helmholtz Centre for Environmental Research. Dep. Environ. Biotechnology 15. Permoserstr, Leipzig 04318 Germany Tel./Fax.: +493412351765; +493412351471.
email: *claus.haertig@ufz.de*

Hubenova Aneta, PhD. Associate Professor. MHATEM N.I. Pirogov Clinic of Toxicology. 21 Totleben Blvd., Sofia, 1606, Bulgaria
Tel.: +3592515357. email: *poison_centre@mail.orbitel.bg*

Isachenko Mykhaylo, Deputy of Chief. Board of External Relations and Foreign Economic Activity, Dnepropetrovsk Regional State Administration. 52. Komsomols'ka Str., Dnepropetrovsk 49005 Ukraine Tel.: +380567445002; Fax. +380567428453.
email: *isachenko@adm.dp.ua*

Kanev Kamen, PhD. Associate Professor. Military Medical Academy, 3.St.G.Sofiiski Str., Sofia 1606, Bulgaria. Tel.: +35929226448; Fax. +35929526536. email: *kam5kan@yahoo.co.uk*

Karayilanoglu Turan Professor Gulhane Military Medical Academy, De-partment of Medical CBRN Defence. Etlik, Ankara 06018 Turkey. Tel. +903123043305.

Kehe Kai, Dr., Lieutenant-Colonel. Bundeswehr Institute of Pharmacology and Toxicology, 11, Neuherbergstrasse, Munich 80937Germany
Tel.: +498931682931; Fax. +498931682333.
email: *KaiKehe@bundeswehr.org*

Kenar Levent Associated Professor. Gulhane Military Medical Academy, De-partment of Medical CBRN Defence. Etlik, Ankara 06018 Turkey. Tel.: +903123043329.

Khudetsky Igor, MD(NBC MED), PhD, Professor. 14/2 Govtneva St. Irpen, Kyiv reg. 08200 Ukraine Tel.: +380449792192; Fax. +380672830011. email: *igor_kh@mail.ru*

Kjoseva Elena. MD., Military Medical Academy, Dept. Microbiology, 3, "G.Sofiiski"st, Sofia 1606 Bulgaria, Tel.: 0035929522773; Fax. 0035929526536 email: *kirien@abv.bg*

Klaus Sebastian M., Dr., Securetec Detektions-Systeme AG, Eugen-Snger-Ring 1, D-85649 Brunnthal/Munich, Germany Tel.: +4989203080-1666; Fax. +4989203080-1652. email:*Klaus@securetec.net*

Kopcha Vasilii, DMS, Professor, I.Y. Horbachevsky Ternopil State Medical University, 1, m. Voli, Ternopil 46001 Ukraine Tel.: +380352229281 email:*infecdis@ukr.net*

Koshevaya Irina, lecturer. Dnepropetrovsk State Medical Academy. 4. Octybrskaya Sq., Dnepropetrovsk 49005 Ukraine. Tel./Fax. +380567135188.

Kovalenko Victor, Prorector of Ukrainian State University of Chemical Engineering, 8. Gagarin Av. Dnepropetrovsk 49005 Ukraine Tel.: +380562466297; Fax. +380562470813. email: *ugxtu@dicht.dp.ua*

Kravchenko Alexander, D.Sc., Director of "Dneprospetzmash" LTD 32. Aerodrom, Dnepropetrovsk 49032 Ukraine. Tel.: +380562395662. email: *drpm@a-teleport.com*

Kuschk Peter. Biochemist. Helmholtz Centre for Environmental Research; Dep. Environ. Biotechnology 15. Permoserstr, Leipzig 04318 Germany Tel./Fax. +493412351765; +493412351471. email: *peter.kuschk@ufz.de*

Macherius Andre, PhD Student, Helmholtz Centre for Environmental Research, Department of Analytical Chemistry. 15 Permoserstrasse, Leipzig D-04318 Germany Tel.: +4903412351430. email: *andre.marcherius@ufz.de*

Mihaylova Gergana. MSc, Military Medical Academy, Dept. Microbiology, 3, "G.Sofiiski"st, Sofia 1606 Bulgaria. Tel.: 0035929225578; Fax. 0035929526536. email: *gmihaylova@gmail.com*

Mueller Henriette. Lieutenant. Bundeswehr Institute of Pharmacology and Toxicology, 11, Neuherbergstrasse, Munich 80937 Germany Tel.: +498931682307; Fax. +498931682333. email: *henrietteulrikemueller@web.de*

Nikolaeva Irina, ecological analyst. Scientist-Production Enterprise «Ecological Audit and Clean Technology», 1. Yaselnaya St., Dnepropetrovsk 49023 Ukraine. Tel.: +380562348299. email: *ecomet@alb.dp.ua*

Noort Daniel, Dr. Department of Chemical Toxicology, TNO Prins Maurits Laboratory. 45. Rostbox, Rijswijk 2280AA. The Netherlands Tel/ Fax. +31152843605. email: *daan.noort@tno.nl*

Ogay Uri, D.T. National Institute of Vine and Wine "Magarach", 31. Kirov St., Yalta, Autonomous Republic of Crimea 98600 Ukraine. Tel.: +380654322248; Fax. +380654230429. email: *enoant@rambler.ru*

Oksamitniy Alexander, Chief of State Administration of Natural Environment Guard in the Dnepropetrovsk area. 69. Laboratornaya St., Dnepropetrovsk 49010 Ukraine. Tel./Fax. +380562464161. email: *postmaster@eko05.dp.ua*

Paul Mariana, Ph.D., Medical Directorate, Ministry of Defense, Romania Izvor Street, no.3 -5,sector 5, Bucharest. Tel.: +12129638663. email: *paul8@un.org*

Petrova Iskra, MSc. Military Medical Academy, 3.St.G.Sofiiski Str., Sofia 1606, Bulgaria Tel.: +35929226448; Fax. +35929526536. email: *christophord@yahoo.com*

Polischuk Elena, Chief of Medical Microbiological Laboratory. L.V. Gromashevsky Institute Epidemiology and Infectious Diseases of Ukrainian Academy of Medical Sciences, Protasov Yar Street, 4, Kiev 03038 Ukraine Tel./Fax. +380442750055. email: *epidemics@ ukr.net*

Popov Tzvetan, PhD. Military Medical Academy 3.St.G.Sofiiski Str., Sofia 1606, Bulgaria Tel.: +35929226448; Fax. +35929526536. email: *tsvtnppv@abv.bg*

Richardson Rudi. ScD, DABT, Professor of Toxicology/ Neurotoxicology. Department of Environmental Health Sciences, School of Public Health, The University of Michigan. 1420 Washington Heights. Ann Arbor, Michigan 48109 United State. Tel./Fax: +17349360769. email: *rjrich@umich.edu*

Samnaliev Ivan, PhD, Associate Professor. Military Medical Academy, 3.St.G.Sofiiski Str., Sofia 1606, Bulgaria. Tel.: +35929226448; Fax: +35929526536. email: *isamnaliev@yahoo.com*

Shendryk Lubov, D.Sc., Professor. Department of Veterinary, Dnepropetrovsk State Agrarian University, Voroshilova str, 25, Dnepropetrovsk 49600 Ukraine. Tel./Fax. +380562463161. email: *boikoalexandra@rambler.ru*

Shtemenko Alexander, D.Sc., Professor. Ukrainian State University of Chemical Engineering, 8. Gagarin Av. Dnepropetrovsk 49005 Ukraine Tel.: +380562474586. email: *ashtemenko@yahoo.com*

Sharun Olesya, DMS, Ass. Professor. Dnepropetrovsk State Medical Academy. 4. Octybrskaya Sq., Dnepropetrovsk 49005 Ukraine. Tel./Fax. +380567135188.

Sigolaeva Larisa. Ph.D., Scientific Researcher/Biosensors. Department of Chemistry, Moscow State University, 1/3 Leninskie Gory, Moscow 119991 Russia Tel./Fax. +7495 9394042. email: *lsigolaeva@genebee.msu.ru*

Steinritz Dirk, Dr., Major. Bundeswehr Institute of Pharmacology and Toxicology, 11, Neuherbergstrasse, Munich 80937Germany Tel.: +498931682304; Fax. +498931682333. email: *DirkSteinritz@bundeswehr.org*

Stepansky Dmitry, postgraduate student. Dnepropetrovsk State Medical Academy. 4. Octybrskaya Sq., Dnepropetrovsk 49005 Ukraine. Tel./Fax. +380567135188.

Stipetic Davor. M.S. Office of National Security Council, 34. Jurjevska. Zagreb. Croatia Tel.: 38598826098; Fax: +38514686049. email. *davor.stipetic@gmal.com*

Stoeva Temenuga. MD., Medical University, Dept. Microbiology, 55, M.Drinov St., Varna 9001 Bulgaria. Tel.: +35952606659; Fax. +35952684252. email: *temenuga.stoeva@abv.bg*

Storch Daniel, PhD, Chemist. Scientific collaborator National NBC Protection and Coordination Office Federal Department of Defence, Civil Protection and Sport, DDPS Federal Office for Civil Protection FOCP SPIEZ LABORATORY. Spiez 3700Switzerland. Tel.: +410332281588; Fax. +410332281404. email: *daniel.storch@babs.admin.ch*

Thiermann Horst, PD Dr., Colonel. Bundeswehr Institute of Pharmacology and Toxicology, 11, Neuherbergstrasse, Munich 80937 Germany
Tel.: +498931682925; Fax +498931682333.
email:*HorstThiermann@bundeswehr.org*

Toutoudaki Maria, Toxicologist, PhD, Department of Forensic Sciences & Toxicology Medical School. University of Crete, Heraklion 71409 Greece.
Tel.: +3081394660; Fax. +3081542098. email: *lawtox@med.uoc.gr*

Trifonova Angelina. MSc., Military Medical Academy, Dept. Microbiology, 3, "G.Sofiiski" St., Sofia 1606 Bulgaria. Tel.: +35929522773;
Fax: +35929526536. email: *angelina3fonova@abv.bg*

Tzatzarakis Manolis, Toxicologist, PhD, Department of Forensic Sciences & Toxicology Medical School. University of Crete, Heraklion 71409 Greece. Tel.: +3081394660; Fax. +3081542098.
email: *tzatzarakis@med.uoc.gr*

Ustimenko Yevgenii, Ph.D., Technical Director of SE RIC PCP, Pavlograd, Dnepropetrovsk region 51402 Ukraine.
Tel.: +380563266008; Fax. +380563266002.
email: *tdir@mail.pkhz.dp.ua*

Van der Schans Marcel Johannes, Dr. Department of Chemical Toxicology, TNO Prins Maurits Laboratory. 45. Rostbox, Rijswijk 2280AA. The Netherlands Tel/ Fax. +31152843605.
email: *marcel.vanderschans@tno.nl*

Yurgel Larisa, DMS, Ass. Professor. Dnepropetrovsk State Medical Academy. 4. Octybrskaya Sq., Dnepropetrovsk 49005 Ukraine.
Tel./Fax. +380567135188.

Zagoruiko Viktor, D.Sc., Ph.D. National Institute of Vine and Wine "Magarach", 31 Kirov St., Yalta, Autonomous Republic of Crimea, 98600, Ukraine; Tel.: +380654325591; Fax: +380654320132.

Zakharenko Olga, Major. The Main Department of Ministry of Emergency of Ukraine in Dnepropetrovsk region. 4, Korolenko Str., Dnepropetrovsk 49000 Ukraine. Tel.: +380567701891.
email: *dnipro@fireman.dp.ua*

Part 1

NEW APPROACHES IN COUNTERACTION TO CHEMICAL AND BIOLOGICAL TERRORISM

Chapter 1

Aspects of a National Defense Against Chemical and Biological Terrorism

Moore D.H.
Battelle Memorial Institute, MD 21010, USA

Abstract. The use of chemical and biological agents as tools for terrorist use has resulted in expanded preparedness and response programs in the U.S. and around the world. In the United States, agencies of the Federal Government have organized to better respond to threats that include terrorist use of weapons of mass destruction. This chapter will discuss several specific aspects of the U.S. program to defend against chemical and biological attacks. Specifically, programs addressing the needs for preparedness, situational awareness, and detection/identification.

Keywords. Bioterrorism, medical countermeasures, biosurveillance, biodetection

1.1. Introduction

Acts of terrorism are now a fact of modern life. The potential of chemical and biological terrorism are real and images of Japanese commuters poisoned by sarin gas in the Tokyo subway remain reminders of what can happen when toxic agents are deployed against innocent civilians [1]. The spread of anthrax spores through the U.S. postal system has been the impetus for expanded preparedness and response programs throughout the U.S. and planning for the next incidents is a major effort for Governments around the world. Following the events of September 11, 2001, agencies of the U.S. Government were reorganized to better respond to threats to the homeland. The Department of Homeland Security was established as a cabinet level department and over the past decade enormous emphasis has been placed on domestic preparedness for possible use of weapons of mass destruction [2]. In a recent interview, Secretary of Homeland Security Michael Chertoff cited his concern for the possible terrorist use of a biological agent against the U.S. In the interview he highlighted his department's efforts to detect biological agents and to distribute countermeasures to an incident [3].

1.2. Preparedness

1.2.1. The National Response Framework

In the United States, the National Response Framework (NRF) establishes a comprehensive, all-hazards approach to enhance the ability of the U.S. to manage domestic

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incidents [4]. The NRF is designed to help prevent terrorist attacks within the U.S., including terrorist use of chemical and biological agents, and to minimize the damage and assist in the recovery from such incidents. The plan incorporates practices and procedures for security, emergency management, law enforcement, firefighting, public works, public health, responder and recovery worker health and safety, emergency medical services, and attempts to integrate them under a unified structure. The NRF is the basis of how the Federal Government coordinates with state, local governments and the private sector during an emergency. Congressional legislation has specifically directed the establishment of a designated telephonic link to a designated source of relevant data and expert advice for the use of state or local officials responding to emergencies involving a weapon of mass destruction. The National Response Center [5] provides emergency technical assistance from a variety of Federal agencies. Assistance is provided on a wide array of subjects that include personal protective equipment, decontamination systems and methods, toxicology information, and medical symptoms and treatment for exposure to chemical and biological agents.

1.2.2. The Strategic National Stockpile

The Strategic National Stockpile (SNS) was established in 2002 and is managed by the Centers for Disease Control and Prevention of the U.S. Department of Health and Human Services [6]. The SNS program works with governmental and non-governmental partners to upgrade the Nation's public health capacity to respond to a national emergency and give rapid access to large quantities of pharmaceuticals and medical supplies that few State or local governments would have the resources to create and manage.

The SNS is a national repository of antibiotics, chemical antidotes, antitoxins, life-support medications, IV administration, airway maintenance supplies, and medical items. The SNS is designed to supplement and re-supply State and local public health agencies in the event of a national emergency anywhere within the U.S. or its territories. The stockpile now has antibiotics to treat anthrax and treatments for radiation poisoning, chemical agent exposure, and other biological pathogens. There is currently enough smallpox vaccine for every person in the United States.

The first deployable support is the immediate response, "12-hour Push Packages". These are caches of pharmaceuticals, antidotes, and medical supplies designed to provide rapid delivery of a broad spectrum of products for an ill-defined threat in the early hours of an event. These "Push Packages" are positioned in strategically located, secure warehouses ready for immediate deployment to a designated site within 12 h of the Federal decision to deploy SNS assets.

If an incident requires additional pharmaceuticals or medical supplies, they will be shipped to arrive within 24–36 h. If the agent is well-defined, the supplies can be tailored to provide pharmaceuticals, supplies, and products specific to the suspected or confirmed agent.

1.2.3. Development of Countermeasures

In order to ensure that adequate sources of drugs, vaccines and supplies are available to the SNS, the Department of Health and Human Service Office of Emergency Preparedness [7]

utilizes their Biomedical Advance Research and Development Authority [8] to procurer needed countermeasures. To do this, Project BioShield [9] directs the procurement and advanced development of medical countermeasures for chemical, biological, radiological, and nuclear agents. The BioShield program is a partnerships to develop and improve medical countermeasures. Countermeasures include human and animal drugs, vaccines and other biologics, blood and blood products, diagnostic tests, and devices that can prevent, diagnose, and treat illnesses related to a terrorist attack. The main provisions of the BioShield law include (1) relaxing procedures for bioterrorism-related procurement, hiring, and awarding of research grants; (2) guaranteeing a federal government market for new biomedical countermeasures; and (3) permitting emergency use of countermeasures prior to Food and Drug Administration licensure.

Project BioShield establishes a permanent funding source allowing the federal government to buy medical countermeasures from private companies. Examples of countermeasures include "next-generation" vaccines against anthrax, smallpox, and other infectious agents, as well as antidotes against chemical and radiological threats.

Many potential chemical, biological, radiological, and, nuclear terrorism agents lack available countermeasures. Project BioShield also provides funding through the National Institutes of Health to support research and development on medical countermeasures. An example of this support is the Countermeasures against Chemical Threats (CounterAct) Program [10] that involves universities, government labs and industry participants.

1.3. Situational Awareness

1.3.1. Bio-Surveillance–Information Integration

The National Policy for Bio-defense [11] directs the Secretary of Homeland Security to: "establish a National Bio-surveillance Group that capitalizes upon existing surveillance systems" focused on, human disease, food, agriculture, water, and the environment. This group collates, integrates, and analyzes the information from these systems with relevant threat and intelligence information and disseminates this all-source information to appropriate Federal departments and agencies. Under the management of the Department of Homeland Security, this group with representation from intelligence, law enforcement, public health, environment, agriculture and defense communities supports the National Bio-surveillance Integration Center [12]. Using modern data acquisition, analysis and dissemination tools called the National Bio-surveillance Integration System [13–15] this center analyses and integrates data from multiple sources to include environmental data, clinical data and epidemiological data. The center can provide early recognition of biological events of potential national significance, to include natural disease outbreaks, accidental or intentional use of biological agents, and emergent biohazards. The enhanced situational awareness facilitates national decision-making to enable timely response. Hazards of primary concern are human pathogens which can cause high mortality are easily transmitted and could be of significant economic impact. Additionally, animal plant and environmental pathogens that are easily transmitted or

are zoonotic and or capable of causing adverse economic impacts are of concern. The center and the system is not without critics as the new organization and information technologies are not considered by some critics as fully operational [16–18].

1.4. Detection and Identification

1.4.1. Biodetection

In a commencement address in May 2007 President George Bush stated "To help stop new attacks on our country, we launched the BioWatch program, placing state-of-the-art equipment in major U.S. cities to detect biological agents." [19] The primary goal of BioWatch is to provide early warning of a biological attack by rapidly identifying the bio-threat agent and thus minimize causalities. BioWatch will also determine a preliminary spatial distribution of biological contamination, including what populations may have been exposed and aid in establishing forensic evidence on the source, nature, and extent of biological contamination. BioWatch is a program using air samplers to test for threat agents. The samplers are located in undisclosed cities and monitor the air 24 h a day, 7 days a week. Current BioWatch technology is capable of recognizing the release of likely biological agents before the onset of clinical illness, is portable and can be unattended and sense several different threats simultaneously with a low false alarm rate [20–22]. Air is sampled by a network of distributed aerosol collectors. Aerosol samples are continuously collected on filters and filters are delivered daily to a certified analytical laboratory for processing. Samples are processed using several individual TaqMan™-based PCR assays designed to test the sample for different biological agents. The established protocol requires that any individual presumptive screen reactive test be re-analyzed using a much more extensive panel of assays.

Like the biosurveillance programs of the Department of Homeland Security, the BioWatch program is also not without its critics. The criticism is primarily based on the lack of financial support provided to State public health agencies to participate in the programs and laboratory analysis efforts [23] and the choice of technologies to be deployed in next generations of detectors [24].

1.4.2. Laboratory Confirmation

In 1999, the U. S. Centers for Disease Control and Prevention established the Laboratory Response Network (LRN) [25]. The LRN's purpose is to run a network of labs that can respond to biological and chemical terrorism, and other public health emergencies. The LRN is the backbone of the BioWatch Program and the labs receive and analyze the filters from the BioWatch aerosol collectors. The LRN has grown since its inception and now includes over 150 state and local public health, veterinary, military, and international labs. These member laboratories are capable of receiving, analyzing and tracking samples in accordance with strict quality and security standards. Member laboratories participate frequently in training and technology insertion programs.

1.5. Conclusion

In this chapter several specific aspects of the U.S. program to defend against chemical and biological terrorism were discussed. These aspects include projects to ensure preparedness such as development and stockpiling of medical countermeasures to chemical and biological threats, projects to collect and integrate data from many sources in the effort to establish situational awareness as it relates to bioterrorist threats and projects to detect and identify biological agents prior to the appearance of clinical symptoms. Other important aspects of a comprehensive defense include adequate and timely responses to an incident and managing the ultimate consequences of the incident so that a sense of normalcy can be restored. These later aspects are critical, but topics for another chapter.

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Chapter 2

International Legal System on Combating CBRN-Terrorism

Matousek J.
Masaryk University, Faculty of Science, EU Research Centre of Excellence for Environmental Chemistry and Ecotoxicology, Brno, Czech Republic

Abstract. The development of global system for combating international organised crime and its highest and most brutal form – international terrorism is shortly portrayed. Experience with already 13 global agreements on combating international terrorism, starting with the oldest on offences committed onboard aircraft (1963), is thoroughly analysed. Even if some of them deal with tools of violence like that on physical protection of fissile material (1980), on marking plastic explosives (1991), on suppression of terrorist bombing (1997) and on suppression of nuclear terrorism (2005), they are mostly rather target- than tool-oriented and address the chemical, biological, radiological and nuclear (CBRN) terrorism (often depicted as ultra- or super-terrorism) only very exceptionally. The comprehensive Convention against International Terrorism is being negotiated at present. Relevant activities of prestigious regional, sub-regional and other interest organisations in combating international terrorism are reviewed; present state and further developments of political and legal constraints against new forms of advanced terrorism are discussed.

Keywords. International terrorism, global antiterrorist agreements, CBRN events, UN system

2.1. Introduction

International terrorism as a most violent form of organised crime has been developed in connection with trafficking drugs, arms, human beings, money laundering and corruption. This was impetus for gradual responding by international community, creating global system against organised crime and seeking measures for combating international terrorism in the last decade, when international terrorism became one of the undesired consequences of ever increasing economic, social and political divergences (reflected in national, racial, ethnic and religious clashes) in contemporary totally interdependent and globalised world. The main milestones of developing global system for combating organised crime and terrorism as its most brutal form, starting already in the first decade of the twentieth century, can be presented as follows [1, 2]:

1909 – Opium Commission, Shanghai
1912 – First Opium Agreement
1946 – Establishing UN Commission on Narcotic Drugs
1955 – UN Congress on Crime Prevention and Treatment of Offenders

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1961 – Convention on Narcotic Drugs
1968 – Establishing International Narcotics Control Board
1971 – Convention on Psychotropic Substances
1979 – UN Organs for Narcotics Move from Geneva to Vienna
1979 – UN Organs for Crime Prevention Move from New York to Vienna
1987 – UN Adopts Programme for Activities in Combating Drug Abuse
1988 – Convention Against Illicit Traffic in Narcotics and Psychotropic Drugs
1990 – UN General Assembly Adopts Global Anti-drug Programme
1991 – Establishing UN International Drug Control Programme (UNDCP)
1997 – Establishing UN Centre for International Crime Prevention (CICP) in Vienna
1999 – Establishing Terrorism Prevention Branch (TPB) as a Part of CICP
2000 – International Convention Against Trans-national Organised Crime
2001 – Establishing UN Counter-Terrorism Committee (CTC) by UN Security Council [3].

Note: The recent act was evoked by the events of September 11, 2001 (but paradoxically, official UN definition of terrorism is still lacking).

There are already 13 global agreements on combating terrorism, adopted according to the development of executed terrorist acts, analysed below, which are rather target- than tool-oriented, starting with the most frequent acts onboard aircraft. Efforts to draft a global comprehensive antiterrorist treaty (that would include also CBRN terrorism) on the soil of UN already exist but it seems that achieving the final goal is still far. On the other hand, many regional and sub-regional organisations and many states have already adopted legal political, and administrative incentives on combating terrorism in its most brutal forms, oriented to preventive, repressive, protective, rescue and recovery measures.

2.2. Global Agreements on Combating International Terrorism

Thirteen global agreements against terrorism, adopted till now, can be shortly presented as follows [4] – ***Name*** (place and date of signature and entry into force – EIF) scope:

– ***Convention on Offences and Certain Other Acts Committed on Board Aircraft*** (Tokyo, September 14, 1963, EIF December 4, 1969) is the oldest convention applying to acts affecting in-flight safety.

– ***Convention for the Suppression of Unlawful Seizure of Aircraft*** (The Hague, December 16,1970, EIF October 14, 1971) is oriented against aircraft hijackings.

– ***Convention for the Suppression of Unlawful Acts Against the Safety of Civil Aviation*** (Montreal, September 23, 1971, EIF January 26, 1973) applies to acts of aviation sabotage such as bombing aboard aircraft in flight.

– ***Convention on the Prevention and Punishment of Crimes Against Internationally Protected Persons*** (New York, December 14, 1973, EIF February 20, 1977) outlaws attacks on government and international organisation officials and diplomats.

– ***International Convention Against the Taking Hostages*** (New York, December 17, 1979, EIF June 3, 1983) was adopted by the UN General Assembly.

– ***Convention on Physical Protection of Nuclear Material*** (Vienna, March 3,1980, EIF February 8, 1987) is connected with the Statutes of IAEA and nuclear safeguards related to the Treaty on Nuclear Non-proliferation (NPT).

– ***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*** (Montreal, February 24, 1988, EIF August 6, 1989) extends and supplements the Montreal Convention.

– ***Convention for the Suppression of Unlawful Acts Against the Safety of Maritime Navigation*** (Rome, March 10, 1988, EIF March 1, 1992) applies to terrorist acts on ships.

– ***Protocol for the Suppression of Unlawful Acts Against the Safety of Fixed Platforms Located on the Continental Shelf*** (Rome, March 10, 1988, EIF March 1, 1992) applies to terrorist activities on fixed offshore platforms.

– ***Convention on the Marking of Plastic Explosives for the Purpose of Detection*** (Montreal, March 1, 1991, EIF June 21, 1998) provides for chemical marking to facilitate the detection of plastic explosives, e.g. to combat aircraft sabotage.

– ***International Convention for Suppression of Terrorist Bombing*** (New York, December 15, 1997, EIF May 23, 2001) was adopted by the UN General Assembly.

– ***International Convention for the Suppression of the Financing of Terrorism*** (New York, December 9, 1999, EIF April 10, 2002) was adopted by the UN General Assembly.

– ***International Convention for the Suppression of Acts of Nuclear Terrorism*** (New York, April 13, 2005, not yet in force) adopted by the UN General Assembly deals with both radiological and nuclear terrorism.

As it can be seen, vast majority of these agreements was adopted subsequently in response to prior executed crimes of the respective art of terrorism. They are mostly rather target- than tool-oriented. Only those related to protection of fissile material, marking plastic explosives, also to bombing, and of course on nuclear terrorism have something to do with technological tools of violence, addressing them implicitly or even explicitly. The legal and technological problem of marking plastic explosives was developed according to the joint initiative of Czechoslovakia and UK shortly in the aftermath of the Pan-Am crash over Scottish Lockerbie (1988), suspected to be executed (and confirmed recently) by Libyan terrorists using reportedly (but never proved) Czech-originated industrial plastic explosive Semtex^{R}. The problem is that at the time being, many people depict plastic explosives generally as “semtex”. Only two from the named documents, i.e. that dealing with protection of fissile materials (actually belonging to the arms-control issues), dealing with nuclear terrorism, can be considered as explicitly associated with the radiological and nuclear terrorism, not to speak on the latest, that is associated with those both forms of terrorism explicitly (under one summary depiction “nuclear”). The other one, i.e. on suppression of terrorist bombing is intercorrelated with advanced forms of terrorism implicitly. If we look at the wording of this document carefully, we can undoubtedly find this connection, because the terrorist bombing implies by no doubt all possible instruments of killing, implying beside explosives also other moods of intentional causing death including those associated with

toxic chemicals, biological agents and radionuclides even if they are not mentioned explicitly. It is perhaps a good reason, why to address the CBRN threats [5] in one complex document.

A ***Comprehensive Convention Against International Terrorism*** that should fill this gap is now under ongoing negotiations on the soil of UN. An advanced draft was presented at the (closed) International Symposium: Combating International Terrorism: The Contribution of United Nations, held in Vienna, June 3–4, 2002 but it seems that its finalisation and expected adoption by the UN General Assembly and opening it for signature is very far due to still divergent views among negotiating nations on the soil of the respective UN *Ad hoc* Committee, according to actual information from this committee. As a positive shift in reaction of the UN system on CBRN-terrorism, the last UN Security Council resolution on global non-proliferation and counter-terrorism [10] can be mentioned.

2.3. Regional and Similar Joint Political and Legal Constraints Against Terrorism

Consistently with the experience from negotiating any global agreement, e.g. in arms-control issues, it is clear how difficult it is to achieve general consensus of the world community necessary for adopting global general and comprehensive convention for combating international terrorism that seems to be much complex. On the other hand, there are already several regional, sub-regional and other joint political initiatives reflected in bounding documents on combating terrorism. Examples can be found in the first line in America as well as on the Old Continent, where the oldest regional agreements of this art exist for more than a quarter of century:

Organisation of American States (OAS) Convention to Prevent and Punish Acts of Terrorism, Taking the Form of Crimes Against Persons and Related Extortion That Are of International Significance was signed in Washington, February 2, 1971 (EIF October 16, 1973).

European Convention on Suppression of Terrorism was adopted by the (all-European) Council of Europe in Strasbourg already on January 27, 1977. (EIF August 4, 1978). It is worth to be noted that this document has been amended recently by the Protocol, adopted in Strasbourg on May 15, 2003. Many regional and sub-regional political documents were actualised after events of September 11, 2001. Principal point of outcome for some of them was as Resolution of the UN Security Council No 1373 (adopted on September 28, 2001), but most of them were adopted much earlier.

European Union belong to regional organisation, reflecting the new situation in many relevant documents, to name at least those, associated with protection of population [6], deepening the previously adopted action programme of Civil Protection. Another document is worth to be specially emphasised, i.e. the Common Position of the EU Council Concerning International Terrorism [7], because of containing the first “official” concise definition of terrorism and of some other terms (terrorist acts, terrorist groups), not to speak about more than hundred more-or-less “academic” definitions that can be found in the literary sources worldwide in the last 2 decades. This EU paper can be considered at the same time as the first international document tackling also (may be

still in developing and disputable form) problem of nuclear, chemical and biological weapons, including R&D of CB weapons.

Also other regional organisations adopted relevant international legal measures in combating terrorism, all of them long before the September 2001 and before the above mentioned resolution by the UN Security Council. Following documents can be named in this connection:

– ***Arab Convention on the Suppression of Terrorism*** (Cairo, April 22, 1998, EIF May 7, 1999)

– ***Convention of the Organisation of Islamic Conference on Combating International Terrorism*** (Ougadou, July 1, 1999)

– ***Organisation of African Unity (OAU) on the Prevention and Combating of Terrorism*** (Algiers, July 14, 1999, EIF December 6, 2002)

– ***SAARC Regional Convention of Suppression of Terrorism*** (Kathmandu, November 4, 1987, EIF August 22, 1988)

– ***Treaty on Cooperation Among States Members of the Commonwealth of Independent States in Combating Terrorism*** (Minsk, June 4, 1999)

– ***Inter-American Convention Against Terrorism*** (Bridgetown, June 3, 1999).

These activities can be documented by the materials, published by UN [8] and by the mentioned international organisations themselves [9]. Any deeper analysis of the activities and adopted documents by these organisations goes beyond the framework of this paper but it seems, that it was difficult to reach consensus in some of these organisations especially on forms and means of combating terrorist groups, balance between police and military force, use of extensive military force, targets of military operations, approval by the UN Security Council etc., which was valid in the first line for NATO due to the divergent views of the US and most of its other Allies on this issue in the aftermath of September 2001, especially with regard to military interventions in Afghanistan and Iraq most recently.

2.4. Conclusions

The global system for combating terrorism as the highest and most brutal form of organised crime has been developed within the framework of UNO in connection with trafficking drugs, arms, human beings, money laundering and corruption. Otherwise, there are already 13 global agreements on combating various forms of international terrorism adopted in reaction on the most frequently executed terrorist acts. Since the oldest, adopted 40 years ago, they address various problems gradually tabled by the executed terrorist acts, starting with offences and other acts committed onboard aircraft (1963), unlawful seizure of aircraft (1970) and acts against the safety of civil aviation (1971), crimes against internationally protected persons (1973), over the problem of taking hostages (1979), physical protection of nuclear material (1980), safety of civil aviation (1988), of maritime navigation (1988) and of fixed platforms on the continental shelf (1988), to the problem of marking plastic explosives for the purpose of detection (1991), suppression of terrorist bombing (1997), of financing of terrorism (1999), and suppression of nuclear terrorism (2005). As it can be seen, these special global agreements

are mostly rather target- than tool oriented. But at least three of these documents are closely connected with CBRN issues at least implicitly, exceptionally explicitly. A draft of the Comprehensive Convention against International Terrorism (involving the forms of ultra-terrorism) was presented at the (closed) International Symposium on Combating International Terrorism in Vienna, 3–4 June 2002 but it seems that its finalisation is still far. Beside, a couple of political and legal documents have been already implemented into the legal system of many states creating thus a concrete point of outcome for preventive, repressive, protective, rescue and recovery measures connected even with the CBRN attacks.

Many older documents have been actualised pursuant to the UN SC Resolution 1373 (2001) even if there is still lack of any UN-official definition of terrorism. Beside, many relevant documents have been adopted on regional level. In the European space, it is worth to remind the oldest valid (but generally nearly not known even among state officials) European Convention on Suppression of Terrorism, adopted by the Council of Europe (1977) as well as relevant new EC documents oriented to protection of population (2001), reflecting the mentioned UN SC Resolution. Also other prestige-ious international regional entities and defence communities have developed initiatives in combating terrorism, long before the events of September 2001. In this connection, one can mention the OSCE, EU, NATO and other regional and interest organisations, such as the League of Arab States (LAS), Commonwealth of Independent States (CIS), Organisation of Islamic Conference (OIC), Organisation of African Unity (OAU), Organisation of American States (OAS), South-Asian Association for Regional Cooperation (SAARC) and like. Lessons learned from the past development of terrorism show that build-up of a reliable worldwide system for combating international terrorism, encompassing preventive, repressive, protective, rescue and recovery measures is a never-ending agenda in the interdependent and globalised world due to continuous development of instruments of violence by terrorist groups utilising the last achievements of scientific and technological development. This is therefore an actual and extremely complex challenge for crisis management and protection of population.

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Chapter 3

Counteraction to CBRN Terrorism in Switzerland

Kenzelmann M.* and Storch D.
Head National NBC Protection and Coordination Office, SPIEZ LABORATORY, Federal Office for Civil Protection, Federal Department of Defence, Civil Protection and Sport, Switzerland

Abstract. In Switzerland, the planning of security policy measures now increasingly factors in scenarios involving the use of CBRN weapons on Swiss soil. While there have been no terrorist "spectaculars" or dramatic news reports in recent years, this does not mean that the CBRN threat has diminished. In 2003 a project called National NBC Protection was started in order to guarantee efficient coordination of all partners involved in CBRN prevention and incident management at all levels with the aim of improving the NBC protection in Switzerland in the long term. The availability of outstanding expertise in terms of NBC protection therefore remains as important as ever for the Swiss population.

Keywords. National NBC Protection Project, NBC Protection Strategy for Switzerland, Emergency Organisation Radioactivity, SPIEZ LABORATORY, NBC Competence Centre of the Swiss Armed Forces

3.1. Introduction

The end of the cold war-period was paralleled by a marked decrease of the threat of military deployments of weapons of mass destruction (WMD). However, terrorism, violent extremism and the proliferation of WMD as well as transport accidents and technical incidents involving hazardous materials evoked "new" CBRN-threats. Especially the sarin attack in Tokyo (1995), the US Anthrax attacks (2001) and the SARS epidemic (2002/2003) have provoked serious debates about the risks of potential CBRN incidents and their respective management in Switzerland. Consequently the security policy of Switzerland with respect to potential CBRN risks has profoundly changed and called for the availability of consistent professional competence in order to serve an all-inclusive civil-based and military-supported CBRN-protection to the Swiss population. To cope with this new situation it was necessary to reassess all organisations and structures involved in today's CBRN defence.

* Corresponding Author: Marc Kenzelmann, National NBC Protection and Coordination Office, SPIEZ LABORATORY, Federal Office for Civil Protection FOCP, Federal Department of Defence, Civil Protection and Sport DDPS, 3700 Spiez, Switzerland.

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3.2. Switzerlands Federal Political Structure

Switzerland has quite a unique democratic tradition and 27 political systems (one federal and 26 cantonal systems). Basic facts and features about Switzerland's political organisation are [1, 2]:

- Switzerland is a Confederation of 26 cantons. Twenty of 26 cantons are considered full cantons, six are considered half cantons because they originated from internal divisions in three cantons. Concerning their institutions, all cantons have equal competences and rights of autonomy and their internal organisation does not depend on whether they are full or half cantons. But as they are different in size (both area and population), cantonal structures differ widely. The distinction between full and half cantons is relevant in voting arithmetics, however: full cantons may send two, half cantons only one member to the small chamber of Switzerland's federal parliament.
- The political philosophy can be described as a form of federalism granting cantons and municipalities a maximum of political self-determination and restricting the competences of the federation to the minimum that is indispensable to run a modern state.
- Governments, administrations, parliaments and courts are organized on federal, cantonal and municipality levels.
- The political system is a Direct Democracy with frequent Referendums on national, cantonal and municipality levels.
- Switzerland has four official languages: German, French, Italian and Romanish.

3.3. The Swiss Civil Protection System

On January 1, 2004, the Federal Council enacted the new "Federal Law on Civil Protection and Protection and Support" (BZG). On October 4, 2002, it had been passed by the federal parliaments (only one dissenting vote). A referendum was subsequently held on the BZG on request by a citizens' initiative, making use of its statutory right. On May 18, 2003, 80.6% of the Swiss population voted in favour of the new law.

The new joint Civil Protection System is the ideal response to current security challenges. It enables a comprehensive protection of the population, its vital resources and cultural property. The joint system brings together the combined forces of five partner organisations. The 'Civil Protection Concept' was adopted by the federal council (Swiss government) on October 17, 2001. The corresponding law on civil protection is based on this concept, which outlines the mission and structure of civil protection in Switzerland.

Civil Protection therefore is an integrated management, protection, rescue and relief system. The partner organisations – police, fire services, health services, technical services and P&S (protection and support) – are in charge of their specific tasks, and provide mutual support. Joint management ensures coordinated planning and preparation, and operational command in case of deployment.

Events that cause damage must be brought under control on site and near to the population affected. Therefore operational responsibility for civil protection lies first and foremost with the cantons and municipalities. The Confederation sees to the coordination of civil protection services and defines its fundamental aspects. For events with a national impact, the Confederation coordinates the deployment of the relevant partners and takes over responsibility for managing the situation.

The Federal Office for Civil Protection (FOCP) supports the cantons and municipalities as well as the partner organisations in their civil protection activities. With the creation of the FOCP in 2003, all areas of the Federal Department of Defence, Civil Protection and Sport (DDPS), which specialise in Civil Protection issues were grouped together. This restructuring reflects the growing importance of civil protection.

3.4. The Swiss Armed Forces

The manifold activities of the Swiss Armed Forces are derived from the three main missions which have been assigned to the armed forces:

- Civil affairs support
- Area protection and defence
- Peace support

Civil affairs support comprises supportive operations of the armed forces on behalf of the population. They are carried out at the request of the civilian authorities, when the resources of Confederation, cantons and municipalities are no longer sufficient. These operations include military disaster relief, maintaining air sovereignty, support of police and Border Guard and protection of conferences and facilities. The operations are carried out in a *subsidiary manner* which means, that responsibility and management are in the hands of the respective civilian authority.

Area protection operations serve to protect important areas and airspaces. Defence is concerned with repelling a military attack against Switzerland.

Peace-keeping operations are aimed at consolidating peace abroad and preventing renewed outbreak of hostilities. They are carried out on the basis of an international mandate (UN or OSCE), usually in cooperation with other states.

The most important legal foundations for the armed forces include the Swiss Constitution, the Armed Forces XXI Guidelines and the Swiss Military Act.

3.5. Modern CBRN Threats

Disasters, emergencies, and terrorist attacks: these are the main hazards Switzerland faces today. Due to our modern and high-tech society, they can cause even greater damage than before. Their likelihood of occurrence is high and may appear without any or only a brief prior warning period. All hazards may also include CBRN aspects. This has also been stated by the Report of the Federal Council to the Federal Assembly on the Security Policy of Switzerland 2000 [3].

3.6. Basic CBRN Tasks Repartition

According to the Swiss Constitution, *the Confederation and the cantons see to the security of the country and to the protection of the population within the limits of their respective competencies*. While the responsibility for nuclear and radiological (NR) events is at the federal level, the management of chemical and biological (CB) incidents is largely a municipal and/or cantonal responsibility (civil protection system). They rely on the relevant cantonal intervention organisations. For CB incidents, the Federal Law on Civil Protection states that the Confederation, in agreement with the cantons, can be asked to coordinate activities and, when necessary, take over responsibility for the management of intervention resources when a disaster impacts on several cantons, the country as a whole, or cross-border regions. Various and different responsibilities that are organised in 26 different (cantonal) ways for different incidents, however, may lead to duplications, misunderstandings and inefficient management of crisis prevention and handling.

3.7. The National NBC Protection Project and the NBC Protection Strategy for Switzerland

In 2003, the Swiss Armed Forces and the Association of Swiss Cantonal Chemical Officers signalled the need for a national NBC protection plan, which would take account of not only all possible threats in this field but also the country's existing political structures. The Federal Council agreed, and asked the Federal Commission for NBC Protection (ComNBC) and the Director of the Federal Office for Civil Protection (FOCP) to carry out a project on "National NBC Protection". The aim of this project was to identify what action was needed, and to propose recommendations on how processes and organisations could be optimised. When examining the "current status", the project team identified several weak points that could seriously hamper the effective management of major incidents. These include a lack of coordination between federal and cantonal levels, the involvement of myriad agencies and individuals, noticeable overlapping, and the lack of a uniform approach to operational planning at the cantonal level. The project has therefore put forward 16 measures (Table 3.1) such as optimised federal command structures, National coordination of strategic and operational tasks in prevention of CBRN incidents, optimised availability of resources, review of responsibilities at the federal and cantonal level, and standardisation of operation doctrines, training courses and material components [4, 5].

On July 5, 2006, the Federal Council ordered the implementation of four priority measures, based on the project findings:

- Development of a national "NBC Protection Switzerland" strategy by the ComNBC
- Creation of a "National NBC Protection and Coordination Centre"
- Expansion of the Radioactivity Steering Committee (LAR) to an NBC Steering Committee (LA ABC)
- Greater federal support for the cantons

The ComNBC was put in charge of developing the national "NBC Protection Strategy for Switzerland" [6].

As part of the national NBC protection project, 14 reference scenarios were developed which the NBC Commission believes cover the entire hazard spectrum (Table 3.2). These scenarios were documented according to their duration, their potential impact and incident management. They provide a valuable tool to identify shortcomings in relation to prevention and intervention, and to develop the appropriate measures to resolve these problems.

Table 3.1. Measures of the project "National NBC Protection"

1. Global strategy (ComNBC)
2. National NBC Protection & Coordination Office
3. Federal command support (Staff of the Federal Council Security Committee)
4. Strategic command, NBC steering committee
5. National report and alert centre (NEOC)
6., 7., 8. Subsidiary deployment – federal
– Personnel
– Monitoring capabilities
– Decontamination
9. Coordination platform/NBC Protection & Coordination Office (cantons)
10. Decontamination capabilities – cantons
11. Availability of monitoring capabilities – cantons
12. Clarification of federal/cantonal responsibilities
13. National B-reporting concept
14. NBC terrorism – operational bases
15. Communications infrastructure
16. Revision of plan on chemical and radiation response units

Several also include variations which involve the threatened deployment of NBC resources. On the whole these scenarios widely correspond to the assumptions found in the Technical NBC Protection Strategy developed by SPIEZ LABORATORY [7].

Table 3.2. CBRN reference scenarios

RN	1. Accident in a nuclear facility: release of radioactivity with prior warning 2. Dirty bomb: spontaneous release of radioactivity causing contamination 3. Deployment of a nuclear weapon: ground explosion close to the Swiss border 4. Attack on a transport carrying highly radioactive waste
B	5. Deliberate contamination of food with ricin 6. Terrorist attack involving the pox viruses 7. Terrorist attack involving anthrax 8. Pandemic (SARS, etc.) 9. Accident in a Biosafety Level 3 laboratory with unintentional release of contaminants
C	10. Transport attack or accident 11. Accident in a chemical storage facility 12. Chemical terrorism: Hydrocyanic acid attack in a shopping centre 13. Chemical terrorism: Sarin attack in an airport 14. Long-range missile attack on Switzerland

The "NBC Protection Strategy for Switzerland" [6] focuses solely on measures which must be implemented if the objectives of the Swiss NBC Protection Strategy are to be met. The Commission also believes that this document should help improve the protection system currently in use. The report also contains additional recommendations:

- Recommendation 1: *Review of the legal bases of NBC security*.
  The ComNBC is responsible for examining whether the existing legal bases adequately prevent the abuse of dangerous NBC substances.
- Recommendation 2: *Evaluation and management of NBC risks based on 14 NBC reference scenarios*.
  The cantons are called upon to re-examine their NBC risk portfolio based on the 14 NBC reference scenarios, to re-evaluate their intervention resources and, where necessary, to adapt them accordingly.
- Recommendation 3: *Further development of the National NBC Protection and Coordination Office and the cantonal coordination platform*.
  The ComNBC will also assist with the introduction of the cantonal coordination platform.
- Recommendation 4: *Promotion of regional cooperation*.
  The cantons are called on to promote cooperation on NBC matters by developing intercantonal regions. Their work could be modelled on the regional biological laboratory network.
- Recommendation 5: *Consensus on NBC intervention resources*.
  Together with the Confederation and the cantons, the ComNBC is responsible for reaching a written consensus on the NBC intervention resources which should be procured and managed at cantonal, regional and federal levels.
- Recommendation 6: *Decentralisation of Federal NBC intervention resources*.
  In line with the aforementioned written consensus, the Confederation is advised to decentralise its NBC intervention resources. These resources must be included in all cantonal plans and training exercises.
- Recommendation 7: *Organisation of a Federal interdepartmental command structure*.
  The cantons demand the introduction of a single point of contact at the federal level for all NBC incidents. The NBC protection tasks of existing staffs and bodies (Staff of the Security Committee to the Federal Council, the Special Staff Unit for Hostage-Taking and Extortion-SOGE, Special Staff Unit for Pandemics, LAR, National Emergency and Operations Centre NEOC etc.) must be reviewed. The Confederation is therefore advised to set up a federal interdepartmental command structure for NBC incidents (14 reference scenarios). This body must also ensure efficient cooperation with the chiefs of staff of the cantonal command bodies. This cooperation will be put to the test during training exercises.
- Recommendation 8: *Updating the Swiss NBC Protection Strategy by the ComNBC*.
  The ComNBC will also be responsible for regularly reviewing and updating the Swiss NBC Protection Strategy (Figure 3.1). It will also be in charge of submitting their proposals for change to the Confederation and the cantons. The legal bases of the ComNBC must be adapted accordingly.

The decision to incorporate the new National NBC Protection and Coordination Office as a special unit within the SPIEZ LABORATORY was motivated by the fact that this would be the ideal environment in terms of shared expertise and would optimise synergies with the Swiss Armed Forces NBC Competence Centre. One of the main responsibilities of the National NBC Protection and Coordination Office is coordinating the establishment of operational principles, as well as liaising with representatives from the "first responders", strategic national partners (especially with the cantons and the relevant federal agencies) and international organisation such as the EU, NATO and the PfP (www.abcschutz.ch).

Since many – if not most – CBRN incidents may not sufficiently be managed by the cantons alone the government must be prepared to support them accordingly. This is actually guaranteed by the government, amongst others, through conducting a C-terror Task Force (EEVBS) which is currently on the point of being restructured to a CBRN Task Force, through carrying on the Emergency Organisation Radioactivity (EOR), the SPIEZ LABORATORY as the Swiss centre of expertise for protection against CBRN threats and hazards, and through arming the CBRN defence within the Swiss Armed Forces.

3.7.1. The Emergency Organisation Radioactivity (EOR)

The Emergency Organisation Radioactivity (EOR) consists of the Steering Committee on Radioactivity (LAR) and its staff; the National Emergency Operations Centre (NEOC) and its military staff; additional departments and bodies of the federal authority, state organisations (Swisscom, SBB), as well as sampling and monitoring organisations. The press centre of the Federal Chancellery supports the EOR with the provision of information to the general public.

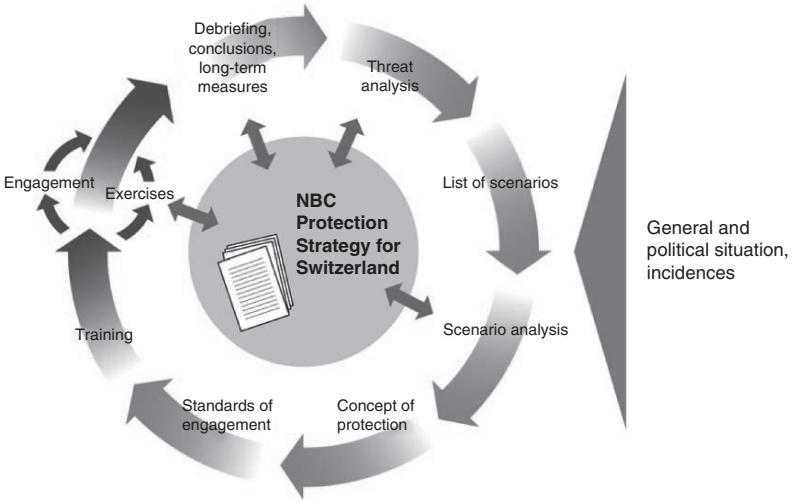

Figure 3.1. National NBC protection as a cyclical process

Should risks arise from accidents in either domestic (Switzerland has five nuclear power plants) or foreign nuclear power plants, the EOR receives additional assistance from the Swiss Federal Nuclear Safety Inspectorate (ENSI - formerly known as HSK).

The LAR is chaired by the heads of the federal offices which may be directly concerned by an incident involving increased radioactivity, namely the Federal Office for Civil Protection (FOCP), the Swiss Federal Office of Public Health (SFOPH), the Federal Office for Energy (FOE), the Federal Office for Agriculture (FOAG), the Federal Veterinary Office (FVO), the Federal Office for the Environment (FOEN), the Federal Office of Transport (FOT), the Directorate of International Law (DIL), the State Secretariat for Economic Affairs (seco), the MeteoSwiss (MCH), the Swiss army command staff (FST A), the Directorate General of Customs (DGC), and the Federal Council spokesperson. In addition to the federal representatives, two members of the cantonal governments sit on the LAR. The LAR checks and discusses the measures developed by the NEOC. These are then submitted to the Federal Council which is responsible for the final decision.

The ComNBC, the Federal Commission for Radiation Protection and Radioactivity Monitoring (KSR), and the Swiss Federal Commission for Nuclear Safety (KNS) support the work of the LAR by giving it access to a number of additional experts.

As one of the four priority measures from the National NBC Protection Project, the expansion of the Radioactivity Steering Committee LAR to an entire NBC Steering Committee LA ABC is now under planning.

3.7.2. The SPIEZ LABORATORY

Routine commissions, laboratory analyses as well as national and international field deployments are central to safeguarding the specialist expertise of the SPIEZ LABORATORY. As the Swiss Centre of Expertise for Protection against CBRN Threats and Hazards, its primary mission is the development and enhancement of the technical and scientific knowledge needed to ensure comprehensive CBRN protection, and to share this know-how with the civilian authorities and the Swiss Armed Forces. The SPIEZ LABORATORY also has the necessary testing and monitoring resources at its disposal and plays an active role in the development of CBRN protection technologies. By building on and preserving its specialist know-how and expertise, the SPIEZ LABORATORY helps ensure that any operational response to an emergency situation will be the right one.

3.7.3. The NBC Competence Centre of the Swiss Armed Forces

The NBC Competence Centre of the Armed Forces ensures operational readiness of the armed forces NBC resources. Apart from managing the armed forces NBC defence, it also ensures various NBC training courses for civilian partners in Switzerland and abroad. After the successful integration of a new CBRN decontamination system, specialist aspects of CBRN reconnaissance and mobile CBRN detection (many parts of the included technical detection equipment have been tested by the experts of the SPIEZ LABORATORY) will now follow.

The NBC Competence Centre in Spiez is mobilised in the event of a major NBC incident which exceeds the capabilities of the on-site civilian operational forces. Thanks to its wide range of resources and services, the NBC Competence Centre can be rapidly deployed in order to provide both civilian and military organisations with subsidiary back-up. If a nuclear or radiological incident occurs, the Radioactivity Monitoring Team of the Swiss Armed Forces (MORA), NBC Competence Centre personnel, and all available NBC Defence militia personnel are called on to offer their specialist expertise in this field. The DDPS Deployment Team (EEVBS) and the SPIEZ LABORATORY will both be deployed during a chemical incident. A request can be sent to the National Emergency Operations Centre (NEOC) for further assistance with field measurements, localisation of contaminated areas, sampling and advice.

3.8. Outlook

Coordinated CBRN protection in Switzerland is still in its infancy. Nevertheless, the introduction of measures is well on track and we shall soon see whether the objectives of this new approach have been achieved. Whatever happens, the foundations for coordination at national level have been laid and there is an undeniably strong sense of commitment among all partners involved.

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Chapter 4

Basic Ways of Prevention of Chemical and Biological Terrorism on the Territory of Ukraine

Shmatkov G.[1], Oksamitniy A.[2], and Nikolaeva I.[3]
[1]*Pridneprovska State Academy of Civil Engineering and Architecture, Ukraine*
[2]*State Department of Environmental Protection Under the Ministry of Natural Resources (MinPrirody) of Ukraine in Dnipropetrovsk region, Ukraine*
[3]*Scientific-Production Enterprise "Ecological Audit and Clean Technology Center", Ukraine*

Abstract. There are thousands of objects in Ukraine, which could be a target of chemical and biological terrorism resulting in chemical (including nuclear) or biological contamination of vast territories, large bodies of water, sources of provision of citizens with drinking water and food products. Such acts of terrorism can give rise to numerous human losses, ecological and social catastrophes in any region of Ukraine or in this country as a whole. Therefore, protection from commitment of acts of chemical and biological terrorism is an integral part of the national security of Ukraine. The Ministry for Emergencies of Ukraine keeps the register of environmentally hazardous objects, from the point of view of risk of man-made accidents or catastrophes thereat. The said objects include pipelines of hazardous chemical substances, tanks and collectors of large amounts of hazardous chemical substances, storage reservoirs for pesticides and toxic chemicals, and enterprises with process cycle using hazardous chemical substances. Criterion of entering the objects in the said register is, mainly, presence of critical mass of stored substance. From the point of view of preventing the acts of terrorism at the objects which are potentially hazardous for committing acts of chemical and biological terrorism (OCBT), this criterion is absolutely insufficient. As far as a threat of biological terrorism is concerned, this problem was neither stated nor solved in Ukraine in any way.

Keywords. Terrorism, hazardous, propellant, storage, melange, protection, risk

Ukraine is the second largest former Soviet republic with population of 47.4 million inhabitants and area of 604,000 km^2. In the modern Europe, it is the largest country, which territory is more than that of the most developed European countries, such as France (543,965,000 km^2) and Germany (356,733,000 km^2).

Ever since formation of Kievan Rus at the end of nineteenth century, Ukraine was the center of Eastern-Slavic culture; however, the country's independence over the period of its history time and again became the subject matter of conflicts.

Geographic position between the West and the East, in combination with ice-free ports on Black Sea and Azov Sea, promoted the Ukraine turning into an important commercial link between the former Soviet republics, the countries of the East and Europe, including Mediterranean countries.

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At present time, we also witness that Ukraine is at the intersection of mutual interests of Western countries represented by European Union and the USA on the one hand, and Russia on the other hand.

Such geographic, economic and political position of Ukraine makes its territory very attractive for commitment of large-scale acts of terrorism, with the use of toxic and poisonous chemical substances, for blackmailing purposes or for destabilization of political and economic situation in the center of Europe.

There are thousands of objects in Ukraine, which could be a target of chemical and biological terrorism resulting in chemical (including nuclear) contamination or biological contamination of vast territories, large bodies of water, sources of provision of citizens with drinking water and food products.

In particular, Ukraine is crossed by oil- and gas pipelines laid from Russia and Caspian region to Central and Western Europe ("Druzhba" and "Eastern Products" oil pipelines; "Siyaniye Severa" ("Shining of the North"), "Progress", "Shebelinka", "Soyuz", "Urengoy–Pomary–Uzhgorod" and "Western-Ukrainian" gas pipelines).

Oil from Bulgaria, Romania, Turkey, Caspian region and from the Near East is delivered by Black Sea to newly built Odessa terminal of "Odessa-Brody" oil pipeline.

There is not large network of product pipelines; among them, ammonia pipeline Tolyatti–Gorlovka–Odessa and ethylene pipeline Kalush–Tiszaújváros (Hungary) should be mentioned. Besides, underground gas storage facilities in the western part of the country, with total effective capacity of more than 30 billion cubic meters, is an important component of transport system of Ukraine.

These objects extend on the territory of Ukraine for tens of thousands kilometers. Naturally, organization of reliable physical system of the said objects' protection from unauthorized access thereto is actually not considered possible.

Therefore, a threat of conscious unauthorized access to these objects and a threat of commitment of chemical terrorist acts always exist.

Even more hazardous and attractive objects for committing the acts of chemical terrorism in Ukraine are the locations of extremely toxic liquid propellant called "Melange". As to chemical composition, this propellant consists of unsymmetrical di-methyl hydrazine.

After dissolution of the USSR, the Soviet Army has left in Ukraine 16,764 t of Melange mixture, very unstable volatile and toxic oxidizing agent of rocket propellant.

At present time, Melange fuel is stored at seven objects located in various regions of Ukraine. Such fuel, in case of leakage thereof and getting to ground water, as well as at direct contact, constitutes a serious threat both to people's health and to the environment.

Noncombustible Melange, nevertheless, reacts with water with production of heat, and creates conditions for ignition in the presence of combustible materials. In case of loss of sealing in storage conditions and ingress of 100 m^3 in the environment, the resulting toxic cloud creates fatal threat to humans in the radius of 2 km from the place of spillage, and hazard for citizens in the radius of 25 km.

Toxic cloud can be moved away by wind to the distance of up to 80 km. Leakage of Melange because of "fatigue" of storage tank metal is observed almost at all warehouses.

In case of major leakage of Melange during commitment of terrorist act from the warehouse located at the distance of less than 10 km from the town of Vinnitsa in Central Ukraine, it poses hazard to 350,000 of residential population (situation here is worsened by presence of large amounts of warehoused ammunition supplies as well).

Terrorist acts at the objects described above may result in local ecological catastrophes, contamination of surface and subsurface waters, including drinking water, for a long time.

In a number of cases, for example, explosion at ammonia pipeline or at propellant storage facility, the act of terrorism may cause numerous human losses, ecological and social catastrophes in any region of Ukraine or in this country as a whole.

Undoubtedly, the list of objects attractive for commitment of terrorist acts causing chemical contamination of the environment should include the storages of unusable and unidentified pesticides and toxic chemicals previously used in agriculture.

In Ukraine, there are more than 20,000 t of such substances in storage. The relevant warehouses are located in all the regions of Ukraine and in the Autonomous Republic of Crimea. These storages are absolutely unsuitable for keeping such hazardous substances, since they appear to be old, often half-ruined premises or open storage locations.

Majority of them are situated in the vicinity of water bodies, being sources of local or centralized drinking water supply. Any physical protection at the above storages is not provided as well.

Thus, commitment of any terrorist act with the use of these chemical substances, for poisoning of drinking water supply sources and injury of hundreds and thousands of people, does not appear to be an intricate problem for terrorists.

Therefore, securing of protection from commitment of acts of chemical and biological terrorism is one of integral parts of the national security of Ukraine.

The Ministry of Emergency Situations of Ukraine keeps the register of objects posing a hazard to environment, from the point of view of risk of man-made accidents or catastrophes thereat. The said objects include pipelines of hazardous chemical substances, tanks and collectors of large amounts of hazardous chemical substances, storage reservoirs for pesticides and toxic chemicals, and enterprises with process cycles involving hazardous chemical substances.

Criterion of including the objects in the said register is, mainly, presence of critical mass of stored substance.

From the point of view of preventing the acts of terrorism at the objects potentially hazardous for committing the acts of chemical and biological terrorism (OCBT), this criterion is absolutely insufficient.

As far as a threat of commitment of biological terrorist act is concerned, this problem was neither stated nor solved in Ukraine in any way.

For guaranteeing security of objects at which any chemical and biological terrorist acts can be possibly committed, it is necessary, at the first stage, to solve a number of tasks listed below:

– Development and adoption of a number of legislative acts on actualization of objects prospective for commitment of acts of chemical and biological terrorism, and on guaranteeing security of the said objects

– Clear identification of those criteria by which any industrial or municipal object, collector, storage reservoir, pipeline etc. should be referred to OCBT

– Drawing up of the regional registers and the State Register of the relevant objects

– Examination of condition of these objects for compliance with prescribed criteria

– Study of safety measures regarding unauthorized access to such objects and probability of any terrorist act thereat

– Assimilation of the world experience in the domain of OCBT protection and guaranteeing security of such objects, and usage of this experience in Ukraine

– Development of the "State Program for guaranteeing security of objects prospective for commitment of acts of chemical and biological terrorism", and approval of the said Program by the relevant Decree of the Cabinet of Ukraine

From scientific and methodical point of view, it is very important to develop the OCBT classification, to draw up and to approve the methods of their examination, and measures on arrangement of standing systems of the above objects' monitoring, with a view to preventing terrorist acts.

Besides, apart from well-known methods of physical protection of environmentally hazardous objects, specific methods of OCBT physical protection should be developed.

One of critical directions in solving the issues of OCBT security is training of specialists of high and secondary level of education at vocational schools and universities of Ukraine.

In view of the above, the scientists and specialists of Ukraine should now perform the great scientific-methodical and law-making work, aimed at achieving guaranteed security of OCBT, within the shortest time possible.

Chapter 5

Preparedness Against Chemical and Biological Terrorism in Turkey and Civilian-Army Collaboration

Yaren H., Kenar L., and Karayilanoglu T.
Gulhane Military Medical Academy, Department of Medical CBRN Defence, Turkey

Abstract. In today's changing world, weapons of mass destruction become one of the most probable threats for states and also for civilian people. Especially after disintegration of U.S.S.R., terrorist groups and some terrorism-supporting countries have desired to get biological and chemical agents/weapons in order to get advantage against their enemies. Because of its geopolitical and geostrategic position, Turkey has been under this kind of threat. In order to provide sufficient response during a biological and/or chemical attack, Turkey has taken necessary measures. In Turkish armed forces, army, navy and air forces work in tight cooperation in terms of intelligence, warning and reporting, protection, evacuation and also medical countermeasures. All the activities are coordinated by Turkish general staff. Medical operations and counteractions are performed by Turkish Armed Forces Surgeon General under control of Turkish general staff. Gulhane Military Medical Academy has a key role in terms of education of all military medical staff, getting military hospitals ready against chem-bio attacks and providing collaboration with civilian hospitals. On the other hand, whole counteractions against chem-bio threats are performed under control of premiership. Ministry of Defense, Ministry of Health, Turkish Armed Forces, Police Department, Ministry of Interior and Local Authority are the other important components of the entire defense system. In the case of any chem-bio attack, event management is performed by "Premiership Crisis Management Centre".

Keywords. Medical response, chem-bio event, organization

5.1. Introduction

Chemical and biological (CB) agents (weapons) have been used by armies as weapons since prehistoric times in wars besides conventional weapons in order to get advantage against their enemies. Their use in warfare has been reported since ancient Grek and Roman times, even if their effect had been rolatively limited due to the knowledge level at that time. Contrarily during the nineteenth century, swift developments in chemistry and the development of the chemical industry were occured and increasing knowledge on their toxicological effects and the possibility for large-scale production of hazardous chemicals provided the basis for their first use as weapons of mass destruction during World War I. This event was the beginning of continuously growing efforts to develop

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more and more effective chemical agents, hazardous toxins and microorganisms including appropriate delivery systems for their usage in warfare. While usage of chemical and biological warfare agents have been continued in battlefield, the development of long-range delivery systems by several chemical and biological weapon possessors promoted the perception of a new dimension of threat also for civilian population. The proliferation of these agents to the terrorist field during the 1990s and the globalisation and escalation of terrorist attacks in recent times resulted in a common awareness of the necessity to include this threat in national and international emergency and risk management plans. Besides intentional uses of these agents, production plant and transport accidents still exist as threats [1–3].

The attacks or accidents with these warfare agents cause mass casualties and/or death, economical and psychological collapse, insufficiency in medical systems, panic and feeling of weakness of authority. The most popular targets to get these effects are; public facilities, military facilities, industrial facilities, schools, shopping malls, bus terminals and airports, subway stations, sporting activities (olympiads) and international meetings (NATO, G-8, etc.), Matsumoto-Japan sarin gas attack (1994), Tokyo subway sarin gas attack (1995), Bhopal-India methylisothiocyanid accident (1984), suicide attack with trucks loaded with Chlorine (2007), Accident on Anthrax, Sverdlovsk (1979), Anthrax spored-letter attack, USA (2001) are well-known events happened in last 3 decades [4].

Because of the growing threat, most of the countries have begun to arise the level of CB preparedness [1, 2]. Turkey, due to its geo-strategic position, is in a high risky situation in terms of CB threat. Considering Turkey's strategic location and the principle "Every Country Should Establish Its Own Health Planning and Organization Depending on Its Own Conditions" [5], the excellent CB defense organization is needed as soon as possible.

Compared to conventional weapons, relatively small amounts of modern chemical and biological agents may cause high numbers of casualties. Therefore, chemical and biological warfare agents have been classified as weapons of mass destruction. Contrary to easy usage of these agents, it is so hard, time consuming and expensive to establish an effective defence system against them at nationwide scale.

5.2. Fundamentals of CB Defence System in Turkey

Whole counteractions against chem-bio threats are performed under the control of premiership. Ministry of defense, Ministry of Health, Turkish Armed Forces, Police Department, Ministry of Interior and Local Authority are the other important components of entire defense system. In the case of any chem-bio attack, event management is performed by "Premiership Crisis Management Centre" which does not exist in peace time.

All the units which function in defense system act in content of (1) Detection, Identification and Monitoring, (2) Warning and Reporting, (3) Physical Protection, (4) Hazard Management, (5) Medical Countermeasures and Support. This content is achieved via pre-incident, during incident and post-incident actions.

Preparedness takes place in pre-incident period. In peace time civilian authority and military authority (General Staff) work in tight coordination. In Turkey, headquarter of police, governorships, coast guard, gendarme and head office of civilian defence work under control of ministry of interior. Directorate of health in counties and most of the hospitals work under control of ministry of health. In counties, police department, regional civilian defence teams and hospitals work under control of the governor. On the other hand, as it has been in most of the counties, Turkish Armed Forces have some special facilities. In case of a CB event, it works under the control of the Governer in coordination with the Turkish General Staff.

The main component of civilian defence organisation is the head office of civil defence and in counties the civil defence teams. The teams have detection equipment, decontamination equipment, reaction plan and also recruit the educated personnel. They not only work in case of CB events but also any kind of disaster such as flood, earthquake and industrial accidents.

In the case of a chemical attack, at first, civil defence teams assess the physicial limits of hot, warm and cold zones. After that police provide the security of whole event zone at outside of the cold zone. Defence teams evacuate casualties from hot zone to warm zone. In warm zone, decontamination of casualties is performed and finally in cold zone immediate medical interventions made by the medical staff. After the stabilization of casualties, they are sent to a hospital dedicated to CB defence or the nearest one according to situation of casualty.

Regarding Turkish Armed Forces, it has its own organisation for CB events according to their structure. Detection, identification and monitoring, warning and reporting, physical protection and hazard management components of CB defence are executed by land forces, airforces and navy under control of general staff. General staff also provide tight coordination between these headquarters. Medical countermeasures are taken by Health Directoriate of General Staff. The hands to execute CB defence of headquarters are: Turkish Armed Forces CBRN Defence School and CBRN Defence Battalion (all headquarters), Yıldızlar Education Center (Navy), CBRN DefenceTeams and EOD/EOR teams (Navy And Air Forces). These units have both educational and operational tasks.

5.3. Medical Organization of CB Defence

It can be said that preparedness, event scene management, detection, identification and education are the main components of medical countermeasures and support.

At civilian side, the medical countermeasers are taken by the Ministry of Health via hospitals and health directoriate of governerships. In every county, one hosptital is chosen particularly for CB defence. Although other hospitals have some preparedness against CB event, this one has special equipment (for detection, identification, decontamination and personal protective equipment) drugs (for active/passive prophylaxis, treatment and rehabilitation) and well educated personnel (doctors, nurses, paramedics, laboratory technicians and other auxiliary staff) for CB defence.

At military side, the medical countermeasers against CB events are taken by healty directorate of general staff via Gulhane Military Medical Academy, military hospitals and infirmaries and dispensaries.

In Turkish Armed Force first level CB defence activities are made by infirmaries and dispensaries. In the case of CB event, these facilities make response including detection, decontamination and medical treatment to some extent. Next, they evacuate the casualties to military hospitals. Second and third level activities are made by Military Medical Academy and military hospital. In medical academy, Department of Medical CBRN Defence coordinates all the defencive activities, about this activities Some concerning departments such as Department of Microbiology, Department of Biochemistry, Department of Infectious diseases Department of Toxicology, Center of Pharmaceutical Sciences, Department of Emergency Medicine helps it conducting defensive activities. Other activities of Department of Medical CBRN Defence in content of medical defence against CB events are; laboratory activities, academic and administrative activities, research and development, training of CBRN medical first-aid and rescue team, education of medical staff. Besides CBRN department, emergency medical service of academy hospital has necessary drugs, fixed decontamination unit and personal protective equipment.

In addition, an operational unit exists as a component of defence capacity of academy called “CBRN Medical First Aid And Rescue Team”. In case of terrorist CB attack in military areas or during any operation in battlefield, the team works according to its task definition and ability. The team consists of doctors, noncomissioned officers, nurses and civil servants. Subunits of the team are; detection and sampling unit, triage unit, decontamination unit, medical intervention unit and support unit. The last one consists of non-medical military personel. The main equipment of the team are as follows; drugs and antidotes, personel protective equipment, detection equipment, decontamination equipment, decontamination trailer and non spesific medical equipment. To achieve the any given ask, the team follow a flowchart of action; first, preparedness (includes getting well educated personel, sufficient equipment and vehicle, communication system) secondly, transportation of the team (via vehicle or aircraft) thirdly, event scene management (includes isolation, detection, decontamination, triage of casualties, medical intervention and evacuation of casualties) and finaly return to duty (self-decontamination, equipment decontamination and reporting).

5.4. Hospital Organisation Against CB Attacks

One of the main parts that constitute the medical defense against CB weapons is the hospital organization. In order to overcome the destructive effects of CB attack, the complete medical management system in hospitals is absolutely required. The preparedness and organization level of the hospital has a vital importance in terms of reducing the damaging effects of CB attack [2, 6]. During a CB attack, there can be an excessive casualty flow to first aid and emergency department of a hospital, or loading on it, which exceed the hospital capacity [6]. From this reason, the preparedness and organization capabilities of the hospital and other involved facilities are the main components

of medical response in the case of such an attack. Because of the potential similarities between accidents of hazardous materials and deliberate uses of biological and chemical agents, it can be said that preparedness style of a hospital are similar for both [7, 8].

In this content, in military hospitals, a defense organization model is applied for CB attack. There are some main elements such as "administrator" "coordinator", "council" and the "team" exist in the organization. The administrator of the defense organization is head physician of hospital who is responsible for conducting the respond system. In particular, during the CB attack, he provides the permanent coordination among the organization elements, in addition to ensuring coordination between the organized hospital and the other health care facilities and military-civilian defense societies.

The execution of the system is the responsibility of "coordinator". Coordinator is chosen by the head physician among the doctors. Defense Council consists of specialist physicians and other health care providers who are experienced on medical CB defense. The council helps coordinator in execution of system and works under control of him/her.

"First Aid and Rescue Team" is the key element of the defense organization and works under the control of the "coordinator". This unit exists in most of the military hospitals. The structure, task definition, equipment and personnel type of the teams are very similar to the team which has been established in Gülhane Military Medical Academy (as mentioned in a detailed manner above).

5.5. Collaboration of Units

In Turkey, in order to get excellent defense system against CB terrorism, all of the units and foundations, both military and civilian sides, work in a tight coordination and collaboration. And they also work in cooperation with the world's leading foundations in the area of CB defense.

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Chapter 6

Lead Contamination as a Factor of Environmental Terrorism: North American and European Perspective

Omelchenko A.
Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Center, Department of Physiology, University of Manitoba, Winnipeg, Manitoba, Canada

Abstract. Despite of strong anti-lead campaign started in 1970s, the world population is still terrorized by the dangerous level of environmental lead. The blood lead level (BLL) among the US population is flattened out at 1 μg/dL suggesting the sustained daily human lead intake of about 25 μg. In a majority of European countries in 2003–2006, BLL among children exceeded the US levels. Such BLL are unsafe since a significant relationship between mortality and BLL was found well below 5–10 μg/dL. A safe BLL level for children has not been demonstrated. Lead from gasoline, paint and water pipes are recognized as major sources of environmental contamination. Even the localized use of leaded gasoline produces global effect as lead aerosol is capable to reach the most remote areas. European data show that, despite unequal atmospheric lead emission by the European countries, atmospheric fallout is normally distributed among them with some countries emitting more lead than taking in. Three million tones of lead remain in the US houses painted prior to 1980s. Lead-based paint is allowed for industrial, military and some outdoor uses. In the US, lead-based paint covers about 465 km^2 of non-residential surfaces and almost 90% of the bridges. Adverse health effects of lead contamination are discussed.

Keywords. Environmental contamination, atmospheric emission, lead, blood lead level, public health, gasoline, lead-based paint, drinking water

6.1. Introduction

Environmental terrorism is usually defined as an act that terrorizes other species and threatens ecological system of the planet. Inevitably, it reduces availability of clean air, safe food and water due to environmental pollution and leads to adverse health effects in human population. From ancient times, human health has been a troubling issue. In 300 BC, the Greek anatomist and surgeon Herophilus wrote: “To lose one’s health renders science null, art inglorious, strength unavailing, wealth useless, and eloquence powerless”. In the multi-national poll conducted by Gallup Institute in 1999, the majority of the respondents have picked a good health as the most valued over other categories,

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such as job, education, freedom, happy family, etc. In the modern society, the population health has become a key determinant of peace and security [1]. At the same time, reduced access to clean environment and associated with that diseases create a solid ground for social tension on local and international levels, which eventually could translate into extreme violence commonly associated with acts of terrorism.

There are accumulating evidences suggesting that exposure to environmental pollutants produce adverse health effects [2]. For example, the incidence cardiovascular diseases (CVD) is not evenly distributed in the US, with West Virginia, Puerto Rico, and Kentucky having the highest proportion of residents with heart disease, whereas Hawaii, Colorado, and the US Virgin Islands have the lowest [3]. In the US, postmenopausal women, regardless of any risk factors for cardiovascular disease, were found to be at increasing risk of fatal and non-fatal cardiovascular events with greater long-term exposure to the fine particulate air pollution, $PM_{2.5}$, i.e., airborne particles <2.5 μm in diameter [4]. Twenty-four percent increase of any CV event, 76% jump in CV mortality, and more than double increase of death from any later diagnosed coronary heart disease were found for every 10 $\mu g/m^3$ rise in $PM_{2.5}$. The study of 4,494 adults aged 45–74 years has shown that living near busy roads increases incidents of coronary atherosclerosis [5]. Recently, first evidence of causal link between brief exposure to diesel fumes during physical exercise and myocardial infarction has been obtained [6]. A short-term exposure to ozone as a component of smog, at concentrations typical of many regions of the US, contributes to premature deaths, especially among people with chronic lung or heart disease or other risk factors [7]. The Canadian Medical Association forecasts that 21,000 Canadians, mostly seniors, will die in 2008 from a combination of short- and long-term exposure to air pollution [8].

Exposures to environmental lead, arsenic, cadmium, pollutant gases, solvents, and pesticides have been linked to increased incidence of CVD [2, 9]. In this perspective, the toxic effects of environmental lead, current levels of human lead exposure and major sources of lead contamination in North America and Europe will be briefly reviewed. The main objective is to show that, despite all the recent efforts, environmental lead is still terrorizing humans and entire ecological system of the planet.

6.2. Health Effects of Environmental Lead: Is There a Safe Blood Lead Level?

Lead is ubiquitous in nature and was one of the first metals smelted and extensively used by humans. Today, lead is the most widely used nonferrous metal, with global annually production of 9 million tons. The total amount of lead that has been dispersed into the world ecosystems through the atmosphere during last 2,000 years is 20 10^9 kg [10, 11]. If that amount was uniformly distributed over the Earth surface, which is 510,065,600 km^2, then the average surface concentration of lead would be about 40 kg/km^2. There is always a possibility that at least a fraction of that substantial amount of dispersed lead could be re-introduced into the atmosphere or dissolved in aquatic systems due to various meteorological events and anthropogenic activities.

Lead is absolutely non-essential for human physiology and even in small amounts is toxic. Only 1 g of lead in 20,000 L of water makes it unfit for drinking. It is thought that lead poisoning contributed into demise of the Roman Empire, due to the damage

inflicted by lead upon the brains of Roman rulers to the extend that they could no longer govern wisely [12]. Romans used lead pipes for plumbing and wine processing. Lead compounds were used as wine sweeteners, food additives, components of cosmetics and glazed pottery. It is believed that some Romans might have consumed as much as 1 g of lead daily [12].

In extreme cases, short-term exposure to high levels of lead can cause vomiting, diarrhea, paralysis, convulsive seizures, coma or even death [13]. Acute lead poisoning causes headache, irritability, abdominal pain, sleeplessness, restlessness, etc. Children may be affected by behavioral disturbances, learning and concentration difficulties; in severe cases, the affected person may suffer from acute psychosis, confusion and reduced consciousness [14].

Symptoms of long-term exposure to lower lead levels may be less noticeable but are still serious. Association between lead exposure and CVD in human population is well documented [15]. Occupational exposure to lead correlates with increased incidence of hypertension, cerebrovascular and cardiovascular disease [16, 17]. Lead causes peripheral arterial occlusive disease and, possibly, direct myocardial injury. Lead poisoning is associated with myocarditis and sinus bradycardia. EKG abnormalities are reported for children and lead-exposed workers. Lead affects both human erythrocytes and lymphocytes [18]. In erythrocytes, it could degenerate the lipid and protein component and suppress hemoglobin synthesis.

Lead has detrimental effects on the central nervous, gastrointestinal, reproductive, renal and immune systems [14]. In the brain, lead has toxic effects on neurons, vascular endothelial cells, astroglia, and oligodendroglia. Long-term exposure to lead causes memory deterioration, prolonged reaction time and reduced ability to understand. It reduced nerve conduction velocity and dermal sensibility. Lead exposure diminishes intellectual capacity in children. For every 10 μg/dL increase in blood lead, IQ decreases by 2 points [19], thereat the mean IQ in the US is 100 and people with IQ less than 70 are considered to be mildly mentally retarded. Notably, there are evidences of strong causative link between the level of children's exposure to lead and violent criminal behavior some 20 years later in their lives [20, 21]. Speculatively, that violent criminal behavior, which comprises aggravated assault, robbery, rape and murder, could be readily translated into terrorist activity.

Other adverse effects of lead include: loss of muscle tone, intestinal colic, anemia, spontaneous abortion, possibly cancer, increase of oxidative stress, down-regulation of nitric oxide production, interference with vitamin D metabolism and magnesium, calcium, iron and zinc homeostasis, etc.

Sufficient evidences assume a casual relationship between blood lead level (BLL) in humans and various pathologies. From geochemical data, natural BLL should be about 0.2 μg/dL [22]. At this level, lead probably does not produce any adverse health effects. Elevated BLL (20–29 μg/dL) correlates with significant increases in all-cause circulatory and cardiovascular mortality [23]. Positive dose-response relationship between low-level exposure and blood pressure was observed at BLL <40 μg/dL [24]. Individuals with average BLL under 63 μg/dL showed signs of peripheral nerve symptoms with reduced nerve conduction velocity and reduced dermal sensibility. At BLL of 33 μg/dL, lead impairs skin wound healing [25].

Although population BLL testing is routinely used for diagnostics and as a control of the status of environmental lead pollution [26–29], it hardly can serve as a direct indicator of human health. There are evidences that current BLLs do not reflect total body burden [30]. In fact, total lead body burden is mainly represented by the bone stores, and bone lead is a better biological marker in chronic lead toxicity. Strong correlation between bone lead and hypertension has been observed [24]. The half-life of lead in bones ranges from a few years to decades [31]. Therefore, bones could serve as an endogenous source of lead and provide elevated BLL comparing to lead from daily uptake in people with increased bone turnover [32]. Blood lead could significantly increase when bones become less stable. During pregnancy, it may double due to mobilization from bones. Notably, fetus actively (i.e. against concentration gradient) absorbs calcium and, simultaneously, lead from mother. Bone loss in postmenopausal women and in aged persons could cause the elevated BLL. In women, lead liberated from the bone as a result of postmenopausal bone loss increased blood pressures and elevated the risk of hypertension [33]. Therefore, bone lead represents a "timing bomb". Thus, a question rises: what BLL is safe if any? The tentative answer is: probably, the safe BLL is the one, below which lead is not accumulated in bones. Simple forthcoming calculations allow evaluation of that threshold BLL.

Lead, entered human body through gastrointestinal tract and respiratory ingestion, is transported through the hydrophobic barrier of cell membranes by mimicking other essential metal ions such as calcium, iron, and zinc and gets into the blood [34]. Blood lead is distributed in various tissues and organs, including kidney, liver, spleen, heart, stomach, intestine, bones, and nervous and reproductive systems, however, 90–95% of adult body lead reside in bones [14]. Human bones mainly consist of calcium orthophosphate (e.g. hydroxyapatite [$3Ca_3(PO_4)_2 \cdot Ca(OH)_2$]), which makes up to 60% of the weight of the human skeleton, comprising 99% of the total calcium of the human body [35]. Roughly, the following equilibrium between blood calcium and bone takes place:

$$3Ca^{2+} + 2PO_4^{3-} \leftrightarrow Ca_3(PO_4)_2\downarrow$$

Blood lead competes with Ca^{2+} and forms less soluble lead orthophosphate:

$$3Pb^{2+} + 2PO_4^{3-} \leftrightarrow Pb_3(PO_4)_2\downarrow$$

Using solubility products, SP, for calcium and lead orthophosphates [36, 37], blood lead concentration at which lead is not deposited in the bones could be calculated from:

$$\frac{[Ca^{2+}]}{[Pb^{2+}]} = \sqrt[3]{\frac{SP_{Ca_3(PO_4)_2}}{SP_{Pb_3(PO_4)_2}}} = \sqrt[3]{\frac{10^{-27}}{10^{-44}}} \approx 5 \times 10^5$$

Physiological blood calcium concentration is about 2.4 mM. Thus, BLL should not exceed 0.1 μg/dL. Although this estimate is oversimplified [38, 39], it probably has certain merits since the result closely relates to the natural BLL from geochemical data

(i.e., 0.2 μg/dL). Notably, current safety standards established by the US Occupational Safety and Health Administration are 40 μg/dL Pb for general population and 10 μg/dL Pb for children and pregnant women [40]. Recent studies have demonstrated a significant BLL-dependence of all-cause mortality, cardiovascular disease, cancer and impairment of children's intellectual functioning at BLL well below 5–10 μg/dL [15, 41–44]. Moreover, recent data [45] suggest that a safe BLL level for children has not been demonstrated. Therefore, a scientifically proven safe threshold BLL is unknown.

6.3. North America: Continuing Lead Insult

Following the ban of lead in gasoline, paint, and the soldering of cans in 1975, geometric mean BLL among general US population decreased from 15.8 in 1976 to 1.6 μg/dL in 2002 [26–29]. The time-dependence of BLL in recent years is plotted in Figure 6.1. It is described by one-exponential function with offset:

$$BLL = 1 + 15 \times \exp(-\frac{t - 1976}{8}),$$

where t is the year (A.D.).

It follows from the equation that, although BLL has been recently declining twofold every 6 years, even in infinite time it will only attain the constant level of ~1 μg/dL corresponding to certain sustained level of lead intake by humans. Such BLL is dangerous since it does not prevent accumulation of lead in bones as was shown above.

Blood lead level of 1 μg/dL in adults corresponds to lead daily intake of 25 μg [46]. Thus, a majority of the US population continues to intake not less than 25 μg of lead on a daily basis. Accordingly, "the average resident of the United States is being subjected to severe chronic lead insult" [22]. Where this lead assaulting the US population is coming from?

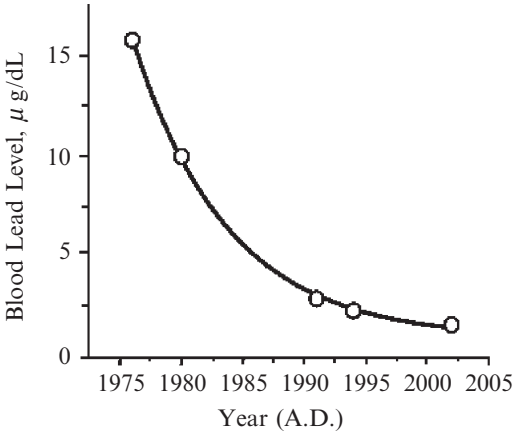

Figure 6.1. Recent temporal trends in geometric mean BLL among general U.S. population

Gasoline, where tetraethyl lead is used as an antiknock additive, is one of the major sources of environmental lead. In the US, leaded gasoline is still legally allowed in aircraft, trains, racing cars, watercraft, and farm machinery [14].

Among the two types of aviation fuels, unleaded one is used in jet-engine aircrafts and leaded avgas is used in piston-engine aircrafts. Avgas, mainly 100LL brand, contains tetraethyl lead, which is the only approved antiknock additive for aviation. Avgas contains 0.56 g/L of lead. In 2006, the annual U.S. usage of avgas was 8.93×10^6 L [47]. The lead emission from this amount is 500 t. Since the population of the US is about 300 million people, that emission could be translated into 4,400 μg of lead per person daily. Obviously, not all that lead will be immediately consumed by people. However, it could be a major source of daily lead intake estimated above, since 25 μg account for only 0.6% of that large amount. Fortunately, the usage of avgas in the US is constantly declining (Figure 6.2). In recent time, the time-dependence is described by formula:

$$Sales = 36480 - 18 \times t ,$$

where sales is given in thousands gallons per day, and t is the year (A.D.).

Importantly, contrary to BLL, the usage of avgas in the US declines linearly, and it will be tentatively terminated in 2027.

Competition vehicles used leaded gasoline participate in the car racing run by the National Hot Rod Association (NHRA), the International Hot Rod Association (IHRA), Champ Car, and the International Motor Sport Association (IMSA). The data on the amount of leaded gasoline used in the US for car racing was not found by the literature and Internet search. However, the import of leaded gasoline for competition vehicles into Canada has increased by 54% between 2002 and 2006 reaching 1.34×10^6 L in 2006 [48]. In Canada, the legislative exemption for use of leaded gasoline in racing cars was extended until January 1, 2009, and the US is not currently planning to ban or restrict the use of leaded fuels in racing events [48].

Speculatively, even unleaded gasoline could be a source of lead. The US Environmental Protection Agency (EPA) allows unleaded gasoline to contain up to 13 mg/L Pb [49] on a basis that this level provides adequate protection for the car catalytic devices and is practicable for the petroleum industry. Although the use of lead additives in the production of unleaded gasoline is strictly prohibited, it may pick up the traces of lead as it passes through refinery and transport systems previously contained leaded gasoline.

Another major source of lead intake is lead-based paint. It contains lead pigments, white $PbCO_3$ or yellow $Pb(CrO_4)_2$, which have been extensively used due to their stability. Since 1978, paint intended for domestic purposes (house paints, toys, furniture, etc.) in the US may contain by law not more than 0.06% Pb. However, white house paint extensively used prior 1980s contained about 50% $PbCO_3$ by weight. Accordingly, 3 million tons of lead remain in 57 million US houses built prior to 1980 [50]. Persistence of lead paint in older homes still constitutes an enormous health hazard to inhabitants. Lead-based paint is still allowed for industrial, military, marine, and some outdoor uses. In the US, lead-based paint covers about 465 km^2 of non-residential surface area and almost 90% of the bridges [14]. Millions of Chinese-made toys were recalled in 2007 due to elevated level of lead in surface paint.

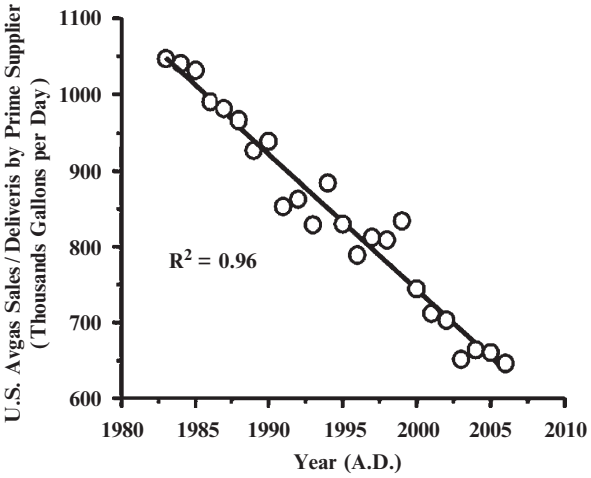

Figure 6.2. Decline of the U.S. avgas usage in 1983–2006[2]

Third major source of lead intake is drinking water. In natural water, typical lead concentration lies between 0.2 and 1 μg/dL [51]. However, drinking water can acquire more lead at the original water source, during treatment and/or distribution through the plumbing system. In the US, about 16–20% of lead body uptake comes from drinking water [14, 52]. The use of lead solder and leaded pipes in public water supply systems was banned in 1986 and lead content in brass plumbing parts was limited to less than 8% lead. Nevertheless, leaded plumbing components continue to be used in schools and daycares creating elevated lead level in drinking water. In many Seattle Public Schools, the elevated water lead concentration (>2 μg/dL) was found in 2004 [53]. In Philadelphia, 57.4% of public schools had water lead levels exceeding US EPA action level of 2 μg/dL, and 28.7% of schools had water lead levels >5 μg/dL [54]. Interestingly, the EPA jurisdiction does not extend to drinking water in public school buildings and only the non-enforceable guidelines are provided [55].

6.4. Europe: Lead Atmospheric Emission *vs.* Fallout

In a majority of European countries in 2003–2006, BLL among children exceeded the US levels [56]. Similarly to North America, the major sources of lead exposure of the Europeans comprise air emissions, dust from lead-based paint and lead in drinking water. However, lead atmospheric emission and fallout represent the greatest health concern, because of the quantities involved and the widespread dispersion. Airborne

[2] Data of the US Energy Information Administration. U.S. Prime Supplier Sales Volumes of Petroleum Products, January 30, 2008

lead can be deposited on the water bodies, soil and crops, thus entering the animal and, eventually, human food chain. Up to 40% of the blood lead is airborne by origin [57].

The detailed analysis of the database on the atmospheric lead emission and fallout over Europe [58] shows that European countries non-equally emit lead (Figure 6.3). The atmospheric lead emission in most countries in 2004 was less than 0.5 kg/year/km^2. Contrary, the area-specific lead fallout is not proportional to emission and strongly depends on meteorological conditions. An average residential time of lead in the atmosphere is about 4–6 weeks [59], during which most lead can be uniformly distributed over vast space.

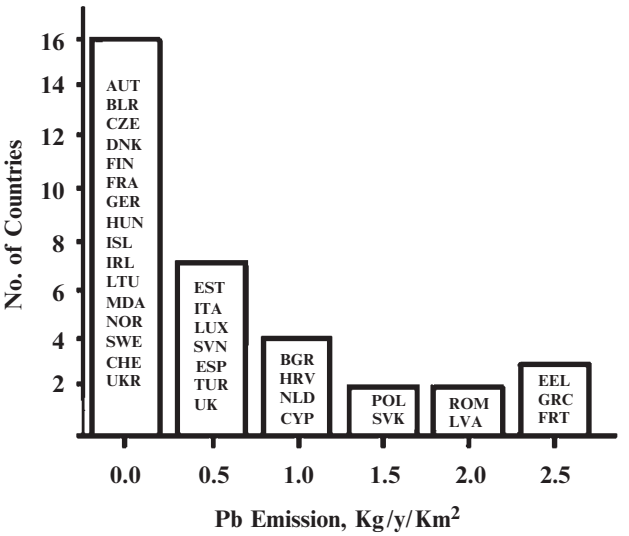

Figure 6.3. Distribution of European countries by the amount of atmospheric lead emission[3]

The evidences of this are found in ancient and modern times. For example, lead contents of ice layers deposited in Greenland between 500 BC and AD 300 were about four times that of background, implying widespread pollution of the Northern Hemisphere by emissions from Roman mines and smelters [60]. Correlation between chronology of anthropogenic lead emission and the lead content in seal hair, penguin droppings and snow was found in Antarctica [10]. In individuals living in remote regions of the Himalayas with no known lead exposure, BLL was found to be 0.78–3.2 μg/dL [14]; similar to the current US levels. Indeed, atmospheric lead fallout is *normally* distributed

[3] AUT-Austria, BEL-Belgium, BGR-Bulgaria, BLR-Belarus, CHE-Switzerland, CYP-Cyprus, CZE-Czech Rep., DNK-Denmark, ESP-Spain, EST-Estonia, FIN-Finland, FRA-France, GER-Germany, GRC-Greece, HRV-Croatia, HUN-Hungary, IRL-Ireland, ISL-Iceland, ITA-Italy, LUX-Luxemburg, LTU-Lithuania, LVA-Latvia, MDA-Moldova, NLD-Netherlands, NOR-Norway, POL-Poland, PRT-Portugal, ROM-Romania, SVK-Slovakia, SVN-Slovenia, SWE-Sweden, TUR-Turkey, UK-United Kingdom, UKR-Ukraine

over 34 European countries with most countries getting about 1 kg/year/km^2 (Figure 6.4). This suggests pretty good air mixing over the Europe. Figure 6.5 shows the "*lead gain*" for each country, i.e. the ratio of atmospheric fallout over the emission. It is seen that most countries acquire more lead than they emit. Notably, Iceland gets 276 times

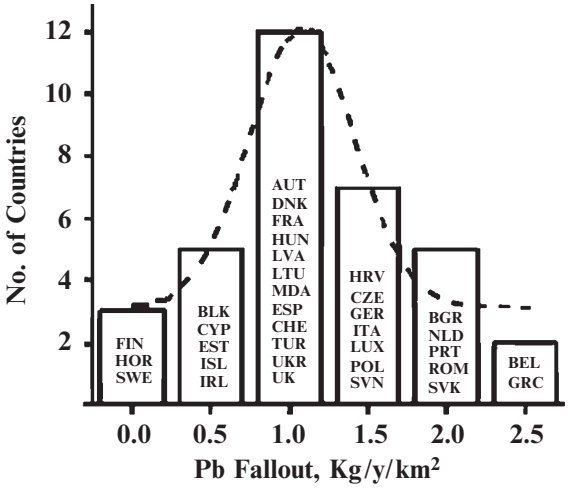

Figure 6.4. Distribution of European countries by the amount of atmospheric lead fallout. Dashed line represents the best least-square non-linear fit to the normal distribution function

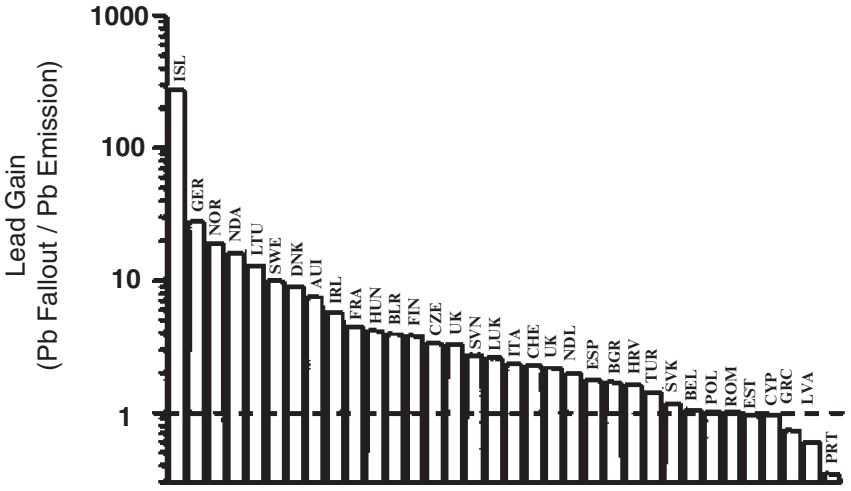

Figure 6.5. The "lead gain" by the European countries. The dashed line corresponds to the ratio that equals unity

more of atmospheric lead than emits, whereas, Portugal gets three times less than emits. Interestingly, entire atmospheric lead fallout over Europe (7.8×10^6 t/year) exceeds lead emission (4.6×10^6 t/year). To find culprits for this misbalance, it is probably necessary to look at the neighboring geographical areas, such as Mediterranean, Sub-Saharian and Middle East countries, Russia, etc. Fascinatingly, a thin grey-white dust originated from the Sahara desert in North Africa could travel as far as Great Britain. On one particular day, about 1 million tons of Sahara's dust was deposited over southern England and Wales [61].

6.5. Conclusive Remarks

The sources of lead exposure are not limited to above-mentioned ones. Traces of lead are found in almost all food, in lead-based glazes on ceramics, in vinyl lunchboxes and mini-blinds, in lead solder used in canned food, in leaded crystal glassware [14, 52, 62]. Lead is present in some pharmaceutical products and dietary supplements [46], especially in those produced in China, India, Middle East and Saudi Arabia [14, 63, 64]. An enormous amount of lead was used as fishing sinkers and jigs, and lead shots for hunting and target shooting [65]. Although lead weights for fishing and waterfowl hunting have recently been largely phased out, lots of lead was previously released into environment with no chance to be recovered. Besides, the lead use for upland hunting, shooting sports, and in fishing tackle remains common [66]. It was reported that in the areas of extensive angling activity the elevated level of mortality was observed in turtles, waterfowls, and eagles, including California condors [65, 66].

In a social sphere, dissemination and popularization of obtained scientific results on lead toxicity is necessary to raise the level of public awareness, since the awareness of ill effects of lead is not widespread. Also, in political sphere, better regulations and tighter collaboration between countries is needed on a global scale, especially to eliminate transboundary distribution of atmospheric pollutants.

Finally, environmental pollution and associated with it adverse health effects could create sense of insecurity among population and, in extreme cases, a political tension, violence or even war. That is why lead pollution as a factor of environmental terrorism should be closely attended on the national and international levels.

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Chapter 7

The Czech National Action Plan to Combat Terrorism: Political and Legal Point of Outcome in Responding to CBRN-Terrorism

Matousek J.
Masaryk University, Faculty of Science, EU Research Centre of Excellence for Environmental Chemistry and Ecotoxicology, Brno, Czech Republic

Abstract. In the Czech Republic, a complex approach and broad institutional co-operation involving inputs of scientific research to analyse endangered critical infrastructures and to adopt respective countermeasures had led to strengthening national measures in implementing respective international agreements dealing with WMD non-proliferation under deepening the co-operation within EU and NATO. The concrete complex programme of harmonised effort of all state organs in combating international terrorism resulted in the Czech National Action Plan to Combat Terrorism (2002). This (bi-annually updated) binding political document identifies threats to all sectors of society and contains active measures to be undertaken by involved organisations and institutions in all aspects of prevention, repression, protection, rescue and recovery for cases of terrorist attacks. Its contents is discussed with special emphasis on the aspects of CBRN terrorism and role of Integrated Rescue System in responding the threats.

Keywords. Terrorism, CBRN events, Czech Republic, national plan

7.1. Introduction

The last third of the twentieth century was marked with increasing frequency of peaceful incidents and accidents endangering human life and environment that have led to the change of previous systems of civil defence aimed mainly to address military threats to the systems of civil protection addressing the new challenges. In the last decades of this century, increased threats to civilised societies appeared with the shift of "classical" forms of organised crime and its highest form – international terrorism, i.e. from strikes with explosives and incendiaries to chemical and biological terrorism with expected radiological and nuclear terrorism with remaining potential of "classical" forms. After the events of September 2001 starting new era in the global terrorism, new security threats were identified and needs to fight against international terrorism were stressed pursuant to the known UN Security Council Resolutions and common position of the international community.

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In the current security policy situation of any country, the only possibility of diminishing the vulnerability of the society to terrorism is to establish a wide framework of measures dedicated to all aspects of potential risks. The approach of the Czech Republic is shown on the complex document adopted for the first time in 2002.

Basic impetus for this document were obviously the events of September 2001, starting new era in the global terrorism as well as reactions of the international community, mainly reflected in the UN SC Resolutions 1368, 1373, 1377 (2001), and 1540 (2004), leading to cautious identification and analysis of threats and to development of appropriate measures. This work was performed by state organs under leadership of the Ministry of Interior, together with research institutions and a couple of experts. It is to be noted that 1 year earlier, the complex legislation dealing with protection of population had been adopted, changing the already developed former system of civil protection into Integrated Rescue System of the Czech Republic with General Directory of Fire Rescue Corps under Ministry of Interior as the steering organ.

The above mentioned measures, summarised in the National Action Plan to Combat Terrorism, clearly declare the resolution of the Czech Republic to comply with its obligations towards the international community. The mentioned Action Plan (current wording for 2007–2009) [1] is arranged in thematic sections that besides the introductory and concluding passages describe the current situation in specific areas and give reasons for further measures. The Action Plan is updated bi-annually; its latest version was adopted as an Annex to the Resolution of the Czech Government No. 129 (February 11, 2008).

7.2. Route to the Czech National Plan to Combat Terrorism

7.2.1. Previous Changes in Protecting the Czech Population

Preparations for adopting the National Action Plan to combat terrorism (NAP) were proceeding under already executed profound changes in the Czech legal and administrative environment that had significantly altered previous system of original civil defences to civil protection in the 1980s based on gradual changing perception of threats and establishing respective priorities. Also splitting of the Czechoslovak Federation in 1992/1993 had some impact on the system of protecting population.

The most important change was transfer of leadership from the Main Office of Civil Protection (at the Ministry of Defence of the Czech Republic) to the Directorate General of Fire Rescue Corps (at the Ministry of Interior of the Czech Republic) as a result of adoption a series of basic laws that together with changes in organisation paved way to the National Action Plan.

To the fundamental legal documents adopted in the year 2000 belong:

- Act No. 238/2000 Coll. on the Fire Rescue Corps
- Act No. 239/2000 Coll. on the Integrated Rescue System of the Czech Republic
- Act No. 240/2000 Coll. on the crisis management
- Act No. 241/2000 Coll. on the economic measures for crisis events

Beside these Acts and respective decrees to implement them, a couple of other relevant legal documents were issued associated with implementation of the core multilateral agreements dealing with disarmament and arms-control related to weapons of mass destruction, i.e. Chemical Weapons Convention (CWC), Biological and Toxin Weapons Convention (BTWC), Treaty on Nuclear Non-proliferation (NPT), Comprehensive Test-Ban Treaty (CTBT) and other relevant documents on preventing threats by radionuclides, ionising radiation, huge accidents and like.

7.2.2. Impact of International Co-operation

It is without any doubt that important impetus for elaboration of operational measures was the known resolutions of the United Nations Security Council (UN SC) adopted shortly in the aftermath of the events of the terrorist attacks on the United States in September 2001, such as No. 1368, 1373 and 1377 (2001) and on the further versions of NAP also UN SC R No. 1540 (2004).

Czech Republic ratified also all 13 multilateral treaties dealing with suppression of various forms of global terrorism (adopted in 1963–2005).

Important were consultations within NATO (due to the Czech membership since 1998) and pursuing relevant incentives adopted within the European Union (EU), the Czech Republic had signed the Association Agreement since 1994 and became full member since 2004.

It is also worth to mention the Czech active membership and co-operation with a couple of Intergovernmental organisations (IGOs), such as IAEA, OPCW, WHO, FAO, UNEP, WMO, WTO, ILO etc.

For adopting some measures in responding threats and protecting population in cases of trans-border reaching consequences of events, the bilateral co-operation on several levels with the closest neighbours is extremely important, i.e. with Germany and Austria, and within the Visegrád Group, i.e. with Slovakia, Poland and Hungary.

7.2.3. Research in Identifying Threats and Responses

Prior to issuing the National Action Plan, a couple of profound research studies was elaborated and reviewed with the main aim to

– Identify critical structures important for the life of society that might be attacked by terrorists, and to
– Propose adequate measures for protecting these structures as well as to
– Respond to potential attacks in appropriate time, with adequate force and effects

These works were organised by the Ministry of Interior and encompassed co-operation by a couple of ministries and other central state offices, various research institutes, academic organisations and individual experts.

The assessment of critical structures involved *inter alia*:

– Production, storage and transport of dangerous goods
– Transport infrastructures of all kind (including oil and gas pipelines systems)
– Energy production and delivery infrastructures

- Water management of all kind
- Chemical, pharmaceutical and petrochemical industry
- Agriculture and forestry
- Food production, storage, transport and distribution
- Health and social infrastructures
- Settlements, including communal waste management
- State, regional and communal administration
- Security systems
- Safety systems
- Communication systems
- Media

7.2.4. Characteristics of the Czech National Action Plan

The first issue, adopted in 2002 was rather long but the aim from the beginning was to adopt complex harmonised programme for all state sectors to combat all known forms of international and domestic terrorism with a clear identification of concrete obligations and tasks for all state sectors involved taking in consideration international commitments.

The NAP that was firstly issued in 2002 is updated bi-annually. Last update is valid for the time period 2007–2009.

The Plan clearly identifies actual threats to society and to its infrastructures. It involves international obligations of the Republic on WMD non-proliferation and deepens international co-operation within UN, with relevant IGOs, within NATO and EU and especially bilateral contacts with the neighbor states.

The NAP comprises a balanced system of preventive, repressive, protective, rescue and recovery measures, reflecting the legislation about crisis management and protection of population pursuant to which the Czech Integrated Rescue System had been created in the year 2000.

7.3. Structure and Contents of the National Action Plan

Latest version of the NAP (current wording for 2007–2009) was issued as an Annex to the Resolution of the Czech Government No. 129 (February 11, 2008). This document is rather long (30 pages) to be presented here in detail but it is possible to look at least on its structuring into main sections that can be considered as its main pillars in order to be familiar with the whole concept of this binding governmental document.

The document is structured in Introductory Part and four sections, representing the Plan's four main pillars (specific areas of measures).

7.3.1. Contents of the Current Version of the Plan

Introductory Part characterises endangering by terrorism analysing actual situation worldwide and especially in the Czech Republic and presents terrorism as one of the fundamental security challenges. At the same time it points out new dimensions of

terrorism, i.e. misuse of CBR agents and nuclear materials, information and communication technologies and continuously increasing threat by terrorism. This part refers on basic principles of combating terrorism and reminds very important feature, i.e. balance between main values, such as security vs. freedom of citizens and democratic order. Also importance of implementing international obligations is mentioned, in the first line co-operation with international entities, especially within European Union.

Section I. Improving communication and co-operation among the subjects of antiterrorist system. This section stresses ability of security services to gain information on intents of terrorists. Information technologies and their potential misuse are mentioned while preventive measures are underlined. Necessity of actual information from the environment of risk groups of population belong to important task. Otherwise, this section defines concrete tasks for police, intelligence services, legal measures, internet servers and electronic mail, monitoring of radio spectra, financing, specialisation for combating international organised crime and terrorism, education and training, etc.

Section II. Protection of population, critical infrastructures and environment. This section seems to be the most important from the point of view of combating CBRN-terrorism. It deals with crisis management, Integrated Rescue System and protection of population. It stresses necessity of protecting specific groups of population which are potential targets of terrorist attacks. Here are also explained principles of co-operation with general public, information and media policy and also development of desired conduct of people in the events of emergency.

Section III. Prevention against the development of non-transparent immigrant communities and the radicalisation of their members. This part of the Plan is relatively new in comparison with previous editions and it reflects the fact of increasing immigration wave and necessity to analyse the communities of immigrants from specific regions with potentially increased appearance of various radical types and ideologies of behavior.

Section IV. Foreign policy in the area of the fight against terrorism. This section summarises international commitments of the Republic and concrete actual tasks of implementing obligations of international agreements and other areas of international co-operation.

7.3.2. Anchoring of the Action Plan in the Political and Legal System

After 6 years since the first wording of the National Action Plan to Combat Terrorism, this document became a standard framework in political, legal and operational activities.

Within the legal framework, it influences penal law and other legislation connected with human rights, extradition of criminal prosecution abroad, the fight against financing of terrorism, etc.

In the institutional framework, it has impact on the activities of the President and the Parliament and mainly on the Government because all important ministries and other central authorities fulfill their tasks and obligations as defined according to the Plan. Both, civilian and military intelligence services are heavily involved in the activities predicted by the Plan. The whole institutional system of protecting population, the Integrated Rescue System is regulated by the Plan. Obviously, organisation providing assistance to victims of terrorism act accordingly.

International co-operation is reflected by ratification by the Republic of all 13 UN Conventions, and also by ratification the European Convention on the Suppression of Terrorism and signature of the Amending Protocol to this Convention in 2007.

Public awareness is by no doubt an important phenomenon heavily influenced by the Plan.

7.4. Conclusions

The Czech National Action Plan to Combat Terrorism is a bi-annually updated governmental programme, harmonising efforts of all state sectors in combating relevant forms of international terrorism containing preventive, repressive, protective, rescue and recovery measures.

The protective, rescue and recovery measures are based on previously established new system of crisis management and protecting population pursuant to a series of state acts that have created *inter alia* the Czech Integrated Rescue System.

The National Action Plan stresses international co-operation within UNO, IGOs, NATO and EC and concrete co-operation with neighbour states at events which consequences trespass state borders. Beside, the NAP reflects international commitments of the Czech Republic stemming from its membership in multilateral agreements on WMD counter-proliferation and other documents relevant to CBRNE materials and their potential misuse for terrorist attacks. The Plan contains balanced legal and practical measures in crisis management, Integrated Rescue System and protection of population, consistent with the present challenges of modern civilised societies, including the CBRNE threats.

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Chapter 8

Military, Technical and Defence-Security Standards on Industrial Facilities Protection in Case of Terrorism and Military Attack

Orehovec Z.
Military Academy of NBC Defence, Zagreb, Croatia

Abstract. Industrial facilities, which use toxic chemicals in their production processes, are tempting targets for military and terrorist strategists. They know that these facilities when attacked could produce effects not realizable with conventional weapons. The resulting legal, policy and political consequences would be minimal as compared to that of disseminating toxic chemicals or chemical agents as weapons on enemy territory. At this time there is no clear definition of the legality or illegality of these types of actions used against specific industrial targets for the purpose of mass destruction or disruption. Without clearly defined international regulations covering these actions, we must depend solely on national defense systems. Not only are these regulation not defined, there are no implementation tools, which would be available if the various treaties (CWC/BWC) etc., were able to incorporate needed legislative action. Consequently we must depend on and put into practice technical and defense security standards for industrial facilities for protection against both possible terrorist and military attacks. In this paper and presentation we will show the results of 15-years of study concerning all military and non-military attacks on industrial facilities in the ex-Yugoslavia territory. The analyses include all of the most important mistakes that took place at the factory level, local community level and on the regional and international levels in processes of technical, defense-security and rescue measures. Likewise, we will propose military, technical, and defense-security standards for protection of industrial facilities and their people at the factory and in the local community. These standards can be considered part of the disaster management plans for the industry, facility, and community. Finally, we will discuss many useful and important conclusions, and the information they are based on, for national and international institutions responsible for protection and response in case of terrorist attacks on chemical, oil, petrochemical and pharmaceutical industries.

Keywords. Industry safety, chemical warfare, safety procedure, first response

8.1. Introduction

Because of weapons inferiority and undeveloped chemical, petrochemical, oil and pharmaceutical industry there is no evidence of chemical catastrophes as direct warfare damage before Second World War. Significant changes had happened during the Second World War when almost each factory with military importance had been target of heavy bombers.

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However, dramatic changes happened since industrial facilities had been unavoidable chosen first time by military planers for military targets because of military, economic, policy and criminal reason as well on the end of eighties last century. Results had been enormous human victims and material damage and big social disorder as well. After those first attacks which had been noted in Iraq – Iran war, followed I and II Golf wars, wars on an Ex Yugoslavia territory and NATO air attacks on Serbia and Monte Negro territory.

During the war on Croatian territory, beside military, first time terrorist attack on oil, chemical and petrochemical industry had been noted. Croatian State Commission for inventory and estimation of war damage (War in Croatia 1991–1995) established that Croatia had sustained damage of $37.1 billion (wit note that this is not finished estimation) where from had been; 40% material damage – $14.84 billion (30% of all economic capacity had been damaged or destroyed with 41% less production on 1992 then before of war), 26% living cost, 33% live and health of people. Since 1.4 million of houses and flats, 217,000, or 15% had been destroyed. Demographic deficit was over 270,000 citizens, with 15,538 death and missing and 37,180 wounded [1–4].

Analyzes of had up wars showed us that own chemical, oil, petrochemical and pharmaceutical industry started to be powerful chemical weapons of enemy and that will happened nothing in international area what could stop military and terrorist planers in planning war plans because there are much more reason to make an attaching plans than fright for consequence [5–9].

8.1.1. Global Reasons for Attaching Industrial Production Plants and Stockpile

Global reason are not in more and more human neglect and immorality even more because of pragmatic reasons comes from world globalization process, and more important are [10–12]:

- Technological development make country defense possibility dramatically dependent of energy sources, infrastructure, telecommunications and the others.
- Possibility and capacity to built an powerful defense system depend of developments contemporary democratic society, which development depends about economic success, more correct, about quantity and regularly money income, which depend about filling of investment security, money and capital flow and business security on a same time.
- International low regulation, include international conventions of WMD prohibition, prohibits use, development, stockpiles technology transfer and the others, but they are not recognize and do not punishes attacks on industrial plants and their stockpiles which use toxic chemicals as method and kind of war.
- Increased needs for bigger and bigger standard carried enormous development of industry, which uses toxic and explosives chemicals in production processes. These chemicals had began security problem because are attractive military and terrorist target. Practically, with input small quantity of energy into system of high thermodynamic potential, discharges enormous quantity of kinetic energy and enormous quantity of toxic chemicals where standard military protection equipment does not protect.

8.1.2. Practical Reason for Attack on Industrial Plants and Their Stockpiles

There are so many and more important reasons for military and terrorists attack on facility then reasons for preventing of attacks, but for this paper the most important are the next [13].

According to more analysts, military reasons, strategic and tactical are the most important reasons for attack because of direct influence on war plans and mobilization plans, direct soldiers exposure to toxic chemicals (military protection mask – filter, does not protect against industrial toxic chemicals), to interrupt logistic routs and defense zones and lines, to challenge scare on soldiers whom have family close to attack facility, challenge insecurity, fury and unreasonableness and the others.

Economic reasons are: momentary production stopping and preventing its production as long as possible in a purpose to decline defense and economic properties and capacity; to expose people, animals, plants; to make material and finance damage; to cause unemployment and social insecurity.

Politic reasons are: to create perception of insecurity and incapability of policy and politicians; to produce insecurity in state and region.

In criminal reason of attack on facilities could be put all terrorist activity whatever who, in whom name and for each reason has been made. They include in themselves all military and economic reasons and could be more and more attack by terrorist motivation in a future because terror and terrorism has been started method and kind of warfare.

8.2. Technical and Defence-Safety Standards in Facilities Protection

Specifics of industrial catastrophes challenged by military and terrorist attack are [14–16]:

- Speed of catastrophe because of extreme pressures and temperatures challenged by relatively small quantity of explosives in before planed positions in a plants production
- Speed of intensities and dispersion of toxic chemicals out of facility, depending of meteorological conditions
- No possibility to evacuate without special protection equipment for present kind of toxic chemicals
- No possibility to remove toxic cloud from factory area without a big quantity of water

Preventing measures of industrial plants are very well known (city-planning, architecture, organization, technical and technological, educational, finance, information…), but there are few practical experiences in providing them. The main intention of this paper is exchange experiences according to 5 years war experience and analytical data and conclusion of last 25th years wars research.

8.2.1. Deficiencies and Common Mistakes in Protection and Rescue on Local, National, Regional and International Level

It is absolutely understandable that during the cold war period protection and rescue system had been built due to the doctrine of nuclear, chemical and biological warfare. Under those conditions industrial catastrophes had been appeared like game and less dangerous. Protection and rescue measures which had been attempted seemed sufficient [7, 8].

8.2.1.1. Deficiencies and common mistakes in protection and rescue on local level

Unfortunately, that assumption proofed to be wrong for industrial production plants because of following reasons [9]:

- *Lack of experience in similar war situations, non existing realistic plans for protection and rescue operation, and a number of wrong actions especially at first reaction*.

  A few experiences were based on the classical industrial accidents where the accident development time is relative slow compared to a war or terrorist act, and where the path of reaching critical point of an accident is predictable and measurable. At the same time all planed activities around an accident (informing public, alarming, evacuation, sheltering, and accommodation of the displaced, decontamination...) can be executed over a longer time period. In case of a terrorist act against industrial objects the time of the accident and the critical point of the accident are almost at the same time and therefore there is no time to conduct protection and rescue operation. Main reason for that is in a fact that at the moment of an attack on an industrial object, that object is operational and running production. As such, that industrial object is in a state of higher thermo-dynamical balance and serves as a bomb loaded with energy and an externally initiated explosion is just detonator for a self-explosion.
- *Inadequate and insufficient funds and equipment for detection, lowering and neutralizing emission of toxic chemicals (toxic cloud), protection, notification and alarming and accommodation, and software for damage assessment is also inadequate*.

  Estimations on NBC danger, military units, police, firefighting units as well as civil protection possessed equipment for detection, dosimetry, protection and decontamination of RBC war agents. Unfortunately that equipment was mostly inadequate for industrial toxic chemicals. Only in a factory level could be find adequate and organized technical protection service units with adequate equipment and adequate trained as well, but not enough to put toxic cloud down without inside help.
- *Inadequate educated personnel and inadequate training were cause for wrong decisions and confusions*.

  Based on two previous groups of lacks, it is normal that inadequate educations and training had been established what caused wrong decisions and orders which were late in a time and space area, or were absolutely out of reality and suitability.

- *Inadequate place and quantity of oil and gas energy and chemicals planning, as well inadequate technologic solutions of production processes*.

  At a moment when war had begun, management didn't order quantity reduction of oil, gas and chemicals as well as finalized products because of scare for profit and market area. In such situation was impossible expect order to stop production, or to drastic production reduction. That happened only when management calculated that damage could be bigger in case of total facility destruction on a long period, or when Government ordered that. Same logic has to be used during facility projecting process because happened that facility element (power plants, production plants, stockpiles of chemicals and finalized products) were built so close to each other with short pipelines what is main reason for chained explosion reaction and quick toxic chemicals exposure in around area. On a same time such production made obstruction to build efficacy protection measures like water barriers and enough water pressure for it. On the end such facility is easy military and terrorist target.

8.2.1.2. Deficiencies and common mistakes in protection and rescue on national level

It is natural that deficiencies and mistakes on a local level are source for such *deficiencies on a national level*, and the most important could be next:

- As on a local level, insufficiency of experience had been reason for *inadequate plans and wrong standard operation procedures in equipped, preparation and planning process on national level as well to forward firefighting, civil protection and military teams and units, and police*, what resulted by confusion and very often without any decision (what in the end was quite better then to made bed decision so far of reality).
- But, as the biggest lacks shoved *inadequate national doctrines and defense strategies as well as protection, rescue and first response doctrine*.

  All national doctrines had been building on cold war concept of prevention and revenge against weapons of mass destruction, with big and mass conventional armored armies equipped and trained for NBC war. Under that condition and philosophy had been building civil protection too, but because it system wasn't so important, very often with old and non perspective cadre members for the army. It is evident that military strategists minimized facilities according to NBC dangerous. May be it was truth for that period, but in globalization area when each destabilization means insecurity for investment and business transformed facilities, especially oil, chemical and petrochemical, into interesting military and terrorist target.
- *Inadequate country defense preparation, equip by inadequate equipment and war plans which do not recognize a new kind of expose*.

  Inadequate country preparation could be shown across inadequate facilities preparations for war or military destructions. There are no adequate plans for decreasing chemicals, oil, gas and final products, as well as decreasing production level or totally stopping production processes in case when and where dislocation

is impossible. Exactly that had been decided by Croatian Government during the war period what was main reason for few human witnesses.

Furthermore, inadequate war plans didn't recognize real dangerous from facilities because very often happened mobilization area, logistic routes and combat zones under possible spread routs of toxic clouds. Under scope that soldiers do not have adequate protection equipment for such kind of dangerous, this affirmation is very significant.

8.2.1.3. Deficiencies and common mistakes in protection and rescue on regional and international level

Lack of experience and conscience on national level about facilities as military and terrorist target resulted by *lack and mistakes on international level particularly under international agreements of WMD and agreements of international response, protection and rescue*, where are [17]:

- Under strong international policy maker which armies still recognize facilities as one legal strategic military targets, as well as under international capital of oil, chemical and pharmaceutical pressure which does not like that facilities could be defined as potential chemical weapons, *catastrophes challenged by attacks on declared facilities according to CWC weren't defined adequate as well as aggressor adequate punished* [18, 19].
- *Lack of international protection and rescue doctrine, as well as strategic decision of international community about minimal administrative, material and personnel criteria which the equipment, teams and units should fulfill before they are given at disposal to international organizations* [20].

  International organizations specialized for assistance in case of human catastrophe, like UNHCR, UNOCHA are not equipped and trained adequate for protection and rescue in case of industrial catastrophes. Attempt to built international teams, units and protection equipment storage under EU, NATO and OPCW umbrella has a lot of administrative, law, organizing barriers, since a time for national governments approval, pass across borders of third countries, regulations of method and kind of leads and orders, to the policy questions like: which are objectives, to whom and when the assistance and protection should be given, which are the units and means, how are the objectives of providing assistance realized. International community still does not recognize that such attacks are very cheap and simple method of avoiding the international laws, rules and regulations considering the CWC, Geneva Protocol, International human and war law and the others.

8.2.2. Proposals for Amelioration Protection and Rescue on Local, National, Regional and International Level

A couple of years war experience on protection and rescue as well as research of all wars happened last 25 years, shoved us that so many changes has to be done under national and international defense doctrine, under protection and rescue system and its

technical and tactical operational measures, under personnel policy, under education and training system, equipped of teams and units, in a process of building efficacy national and international system against facilities as military and terrorist target as well as protection and rescue system in case of chemical disaster. Most important, which some of them are under Croatian protection and rescue system after war experience, are [5, 19–21]:

- ***On a factory level***

In a projecting phase important is that big factory wit a huge quantity of toxic chemicals should be out of population area on a distance which guarantee alerting, declension, evacuation on time and proper way that should be out of mobilization areas, combat areas, logistic routes, with god communication connection.

Inside arrangement of power plants, production plants and stockpiles has to be on a way to prohibit chaining explosions and fire. Between of factory segments have to be god communication, very strong water pipes with foam systems and system for producing 10–25 m high water barrier spread like mist. Factory has to have automatic detection system, automatic software for damage assessment, alert and information system, as well as semi-automatic system for close pipes on a distance.

In a case when factory exist and it is not possible to remove it, or make project changes under production processes, it is necessarily to impose law regulations to provide protecting measures include build above quotation.

When factory are in population area or so close to, it has to participates in cost of evacuation, protection and rescue plan building as well as in participate of equipment cost (alert and information system, protection equipment …).

Each factory has to built lesson learned lecture and put it on disposal to the others factories.

- ***On a local level***

Very important is to evaluate and to pass present plans of alert, evacuation, protection and rescue due to lessons learned from last 25 years experiences and evaluations, renovate it as well as include a news like: new alert system which explain by voice what is going on and what is necessarily to do; equipped people in a first zone by proper protection equipment; to hermetic houses and flats by proper material for windows and doors and by overpressure.

- ***On a state level***

The most important is to evaluate and to pass national defense doctrine and strategy, national preparation plans, national law regulations, defense and rescue system, role of security and intelligence systems (police, inspectorates, intelligence services…).

Obligations are helps and controls on a factory and local level, as well on armed defense system, police and intelligence – secure system, with purpose to built one integrated, compatible interoperable national defense system according to a new damage.

Each country has to built lesson learned lecture and put it on disposal to the others countries and international organizations.

- ***On an international level***

On this level has to be built and accepted international doctrine of assistant delivery which should clearly respond and determine the directions of practical activity and give clear answers on a questions put on articles [1–3]. Minimal administrative, material and

personnel criteria has to answer on question like: time of readiness of the teams/units for engagement from the time of the call until the readiness for transport; choice and education of members of the teams and units; size and ringing out the teams and units with regard to the tactical possibilities and regard to the length of engagement.

8.3. Conclusion

The twenty-first century is a century of unconventional "asymmetrical" warfare and special attention has to be addressed to the army reorganization. In this new warfare the most important will be protecting the environment, ecological protection. This includes physical surroundings, water, air, soil, energy sources and their exploitation, critical infrastructures and industries, transport and logistics, safety of air and naval sectors, chemical, biological and radiological contamination, and explosive materials. Once the environment has been disrupted, subsequent military and economic threats and actions can follow. Ecological protection is a new target for many contemporary threats. The goals of war are no longer simply the loss of territory, relieving royal families from their throne, or conquering or retaining new colonies. Those who control life's environment are real masters of all forms of business, of their nation's prosperity, and of its democracy, culture and lifestyle. Terrorism threatens life's environment. Those who do not have a safe life environment cannot count on the continuing prosperity of their nation and state [6].

The main characteristic of chemical disasters is huge quantities of toxic chemicals in a short time period which cannot be discharge by any armed system any world army. Classical defense systems, as well as protection and rescue systems are not enough efficacies without changes presented into this work.

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Chapter 9

Crisis Management in Bioterrorism Attack: Medical Approach

Paul F.[1] and Paul M.[2]
[1]*Medical Support Section, Department of Field Support, United Nations Headquarter, New York 10017, USA*
[2]*Medical Directorate, Ministry of Defense, Romania*

Abstract. Murder or terrorism with biological or chemical agents is not a far-fetched scenario – it has been used before. The particular risk of biological agents, when used as weapons, is their extreme uncontrollableness. The biological threat is very different from the threat posed by chemical or nuclear weapons in terms of needed preparedness and response actions and in terms of appropriate research and intelligence requirements. The control of such an epidemic requires a coordinated effort of the entire public health community. Local public health experts – physicians, nurses, and other medical personnel – would use their epidemiological tools to detect, identify, and investigate a suspected biological agent and formulate proper decontamination procedures.

Keywords. Biological agents, bioterrorism, public health, epidemics

9.1. Introduction

In the wake of the 2003 outbreak of Severe Acute Respiratory Syndrome (SARS) preparedness for public health emergencies was propelled into worldwide consciousness [1]. The appearance and rapid international spread of SARS demonstrated to all- including global leaders how an infectious disease can rapidly spread and cross borders and continents, and jeopardize health and economical and social life.

It was a good example how a biological agent deliberate released can behave and what is the magnitude of the crisis it may produce.

The scale, range, complexity and variety of threats re-emerging or emerging disease may have call for new approaches in order to preserve and maintain the health status of population in large areas, and not only in a specific country or territory.

In the last 15 years a large number of complex outbreaks have occurred all over the world: epidemics of plague in India, Ebola hemorrhages fever in Central Africa, avian (H5N1) influenza in Asia, SARS outbreak, and many others. Investigation of these outbreaks provided a number of important lessons, many of them being reinforced by the threat of bioterrorism [2].

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There is a critical need to strengthen surveillance capacity in clinical and public health settings. The outbreaks have illustrated the impact on economic and social life; disruption of commerce, travel, education process occurred every time raising the threat at a large scale, posing national and international security in a very difficult situation. Almost the same will happen if a biological attack or a bioterrorist attack occurs.

There is a general consensus that criminal use of biological agents is a challenge for any public health authorities and medical care providing system. Nevertheless, not only medical authorities are involved as all aspects of normal life are disrupted.

9.1.1. Role of Public Health and Medical Community in Response to Bioterrorist Attack

Biological weapons have at least seven characteristics that make them ideal for terrorist attack [3]: easy and low cost of production; easy of dissemination as aerosols; efficient exposure of great number of population through inhalation; delayed effects from the moment of release; high potency; high subsequent mortality and morbidity; their ability to wreak psychological havoc.

It is very easy to understand that effects of such attack are catastrophic. With a bioterrorist attack, the public health and medical communities are frontline response. They are the ones who must first detect the incident, identify what biological agent was used, decontaminate the environment, and probably the most important provide preventive measures and treatments.

The public health and medical approach in response to a bioterrorist attack must begin with the development of local, county, state and national plans, which may include all major players: public health, emergency response, law enforcement communities, veterinarian and social workers etc. All of these must work closely together to mitigate the effects of the crisis.

To have an effective medical response to a terrorist attack, a focused educational effort for health care professionals, especially emergency physicians, nurses, and out-of-hospital emergency medical services personnel is paramount. Only through training and practice will health care professionals develop the expertise and degree of suspicion necessary to initiate an effective response [4].

A very suggestive research was conducted in 2001 by Wetter and all [5]. They fund that hospital emergency departments in US generally were not prepared in organized fashion to treat victims of chemical and biological terrorism. To achieve an efficient level and capability substantial additional resources at the local level were required.

There are four goals, each of which has direct relevance for preparedness of medical response [6]:

- Disease surveillance and outbreak response
- Applied research to develop diagnostic tests, drugs, vaccines and surveillance tools
- Infrastructure and training
- Disease prevention and control

From public health perspective, continuous surveillance, expanding of laboratory diagnostic capacity, training of clinicians to recognize the syndromes potentially resulting from biological attack, existence of adequate facilities to treat casualties are critical.

The last but not the least, ensuring availability of pharmaceuticals for treatment and prophylaxis is vital and probably the most challenging and expensive task.

Detection and surveillance are essential for ensuring a prompt response to bioterrorist attack. To achieve that goal there are demands for developing new mechanism for detection, evaluation and reporting of suspicious event. Existing rapid assay technology for identifying critical biological agents and developed rapid toxic screening in population must be available at local level. These local laboratories must have as backup county/regional laboratories, capable to confirm the suspicion. Public health authorities must develop guidelines and quality assurance standards for the safe and secure collection, storage, transport, and processing of clinical samples.

As a part of detection and surveillance system emergency departments should be integrated into a network, and medical personnel must be adequately trained. Strengthening communication among medical community, including clinicians, emergency rooms, infection control professionals, hospitals, pharmaceutical companies, pharmacists, research laboratories, and public health professionals is of paramount importance [7].

Special measures should be addressed to assist casualties. That includes patient care and hospitalization, possibly quarantining patients if needed, availability of requested pharmaceuticals (antibiotics, other antimicrobials, vaccines, antidotes). An adequate stockpile of drugs must be available. No matter what type of incident, the local community must respond immediate and adequate. It is also crucial the self-sustainability in crisis situation, until support will be provided by regional, national capabilities. This may take 24–72 h.

9.2. Conclusions

For an effective response to a bioterrorism attack there is a need for a realistic response plan. This must include not only activities requested during crisis situation, but also coherent and permanent preparedness activity. Expanded public health laboratory capacity, increased and improved surveillance and response to outbreaks capabilities and capacities, communication within medical and pharmaceutical communities, along with strong connections at all level of local and public authorities are necessary to ensure a coherent and efficient response in case of bioterrorist attack.

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Chapter 10

Evidence of Bio-Terrorism as Form and Method of Warfare

Orehovec Z. and Stipetic D.
Colleague of Velika Gorica – Chrysies Management, Velika Gorica, Croatia

Abstract. In 1995, during the war in ex Yugoslavia, reports indicated that bio-agents and chemical agents were used to create illness among the defending soldiers in an attempt to destroy their combat effectiveness. In one case there was an apparent successful operation, Code named "Sword-!" by these regular forces to contaminate food (flour, sugar, oil...) with bioagents in a convoy bound for populated areas in one part of Bosnia. According to Operation Plan, bioagents had been tested on a captured soldier. What this event means for us? This is very strong evidence for the thesis which the CB MTS series had stated many times during the 1990s and into the beginning of this twenty-first century: Terrorism is evolving; the perception of the idea, tactics, forms and methods of terrorism is evolving. Previous research on terrorism as a phenomenon from the end of twentieth century, including actual terrorist acts, leads to a conclusion that today's terrorism is an instrument or form and method of warfare. This means that in the future we will have to face the fact that terrorism could be used not only by those who previously used it, but also by the states or organizations that practice using war as an instrument to achieve their goals. The reasons for this lie in the following facts:

- Globalization has brought many changes in social, economic, psychological, technical and informational aspects that have changed life and culture. These changes make it easier to achieve changes in economic, political and social insecurity without classical warfare.
- Scientific and technological progress has made it easier to produce or procure instruments for performing terrorism (including WMD) than before.
- Industry increasingly uses toxic chemicals and microorganisms in the production processes and has thus created many more potential targets for terrorism and military actions.

During presentation and in a paper, will be discuss the specific evidence of the case presented, as well as fully discussed bio-terrorism as an instrument or form and method of warfare.

Keywords. Bioterrorism, war and terrorism, food poisoning

10.1. Introduction

"By combining the application of OB SVK methods ensure full counterintelligence and physical protection of the convoy on the G – LPS route and entering CZ in order to strategically harm the units and commands by XY biological weapon and the method of poisoning consumer goods which shall be through illegal trade delivered to XY"... [1]

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"After the incubation period and the spread of dysentery use the same intensively within the psychological-propaganda plan which is to be encompassed in the plan of disinformation and data within the operation "Sword""...Inject a smaller dose of poisonous substance into the food of the illegal HV deserter MG who is located in F prison in order to test the said substance"... "Incubation lasting 5-7 days. The substance is in the form of a powder produced in XX. In case it is taken in larger quantity and depending on the immune system the consequences may be lethal. During normal usage it causes stomachache, diarrhea, headache and stomach cramps [1]*."*

The above quotes are taken from the original Counterintelligence plan for "Sword-1" operation and from the report to then President of one of the entities in the territory of former Yugoslavia. This is the first time since the testing of biological substances on people in Manchuria during WW II by the Japanese, that the usage of biological agents in a warfare and as terrorist means is proved. As stated by the authors of the Plan, the goal was to minimize the combat capability of the adversary by secretly causing disease in the total population in the area, meaning soldiers and their families. One of the pathogens was chosen as biological agent which is transmitted by food and water and which includes *Salmonella sp.* and *Shygella dysenteriae* (dysentery), and both are endemic diseases of the area.

The intention of the author of this work is not to call upon any entity, or any individual – who took part in this terrorist act in the territory of former Yugoslavia. By analyzing this case, and all the other proved cases of non-traditional and untypical usage of conventional weapons for attacks to chemical, petrochemical and oil factories, which, in the production procedure use dangerous and toxic chemicals (cases were described in earlier works in series CB MTS), the attention wants to be drawn to the following dangerous occurrences [2–4]:

- International legislation, including conventions on banning some types of WMD, by its definitions and provisions cuts down RCB agents and their usage to a very limited list and due to that it is necessary to look at WMD as WMD in a broader and in a tighter sense. According to the author's opinion, it is especially important for biological agents.
- Current definitions of terrorism usually recognize terrorists and define them within subjects that do not belong to institutions or states, and states and their subjects are recognized as institutions which have the legal right for their protection and fight against terrorism. To be more precise, states and their armies, the police and secret services are never mentioned in definitions as possible perpetrators of terrorist acts.
- According to the current definitions of terrorism there is a possibility that some secret usages of non-war chemical and biological agents are not recognized as terrorist acts and that today's statistics does not show the real number and extent of terrorist acts. Due to that, terrorism, and especially the type of it which uses any biological, chemical and radiological agent, should be viewed in the light of division to traditional terrorism and silent (covert) terrorism.
- Globalization has brought many changes in social, economic, psychological, technical and informational aspects that have changed life and culture. These changes make it easier to achieve changes in economic, political and social insecurity without classical warfare.

10.2. Goals of Biological and Toxic Terrorism

In the globalization era the race for profit resembles the athletes' race, where only the first three get medals and rewards and the rest are consoled by their name and photo next to the winners. In order to eliminate someone form the race one does not need to use a club, a tank or a rocket, it is enough to take part in the race and block the competition. This is exactly what one person does to another these days. It is every expensive to start the war machinery, and it includes a lot of human loss, international contempt and an uncertain end. Therefore, one turns to new methods, tactics, forms, means and targets in fulfilling their goals, and these are terrorism, organized crime, prostitution, drugs, human trafficking, money laundering etc.

Terrorism is not the phenomenon of twenty-first century. It is as old as humanity itself. Terror has always served as a method, means and form for achieving goals. In the new century, oversensitive to any threat to living environment, the information about the threat creates a panel for manipulation ranging from criminal to religious and fanatics. Users and executioners of terrorism and its accompanying occurrences such as organized crime and other new types of threats are not target groups or organizations. To achieve their goals both the rich and the poor use it, as well as educated and uneducated, individuals, groups and states, people in legal uniforms and without those, religious and national fanatics, entrepreneurs and scientists. Methods used for terrorist acts, as well as the scenarios, are numerous and depend on human imagination only. One can only count the means and the targets.

The authors and makers of the series of CB MTS Industry congresses have on numerous occasions emphasized the possible new division of RBC terrorism, to overt and covert terrorism [4, 5].

Overt terrorism is a notion used for conventional and unconventional terrorism done by terrorist and extremist political, religious and criminal organizations independently or under the aegis of an interested government. Mainly the goal of such terrorism is to attract attention to a terrorist group which then proclaims its political or criminal goals which, if the mentor is a state, then coincide with the goals of the said state.

Covert terrorism is mainly planned and performed by states within a framework of a special war against a particular state by own "special" forces or through an extremist or criminal organization and in ultimate secrecy because the goal is to weaken, "soften" the state which is then followed by other, overt forms of conventional or unconventional war. The characteristic of this covert terrorism is that it uses means and methods which are masked by endemic weaknesses of the attacked state, such as causing an ecological accident due to technical reasons in a factory which generally has problems with old equipment and bad security system. An example may also be intentionally causing disease among animals, people and plants (trichinosis, mad cow disease, flu, salmonella, potato beetle, poisonous spiders, etc.) which usually occur in such areas. An example may also be intentionally causing hostility, conflict and violence, and an example is intentionally launching false bank notes, stock market frauds and crime during privatization…. [6]

There are many examples and methods and means may also vary, from economic to chemical and biological with one constant rule – *they just instigate what already exists in reality*. Results can usually be exploited very quickly, there might be an article

in the newspapers of the said country or third party that it is not good to spend holidays somewhere in the coast because the hotels are full of salmonella, the food is prepared from meat which contains trichinosis, there are poisonous spiders in the camping sites… and sometimes there is a quick reaction, whether diplomatic, military or economic in order to protect the interests of their minority since the "victim" country is not able to do it on its own [7–9].

- Scientific and technological progress has made it easier to produce or procure instruments for performing terrorism (including WMD) than before.
- Industry increasingly uses toxic chemicals and microorganisms in the production processes and has thus created many more potential targets for terrorism and military actions.

This type of terrorism is difficult or impossible to discover, it can be suspected, it should be foreseen and using the system of national security the potential causes and goals should be eliminated.

Contemporary goals of RBC terrorism, which every contemporary state should take into account, might be grouped in the following way [7–9]:

Political:

- Put the credibility and functioning of legitimately elected government into question (it can be done by foreign states due to various interests, and it can be also done by internal subjects such as anarchists, fanatics, the opposition, entrepreneurs, etc. and the purposes may also differ, from covering up economic crime (privatization), early elections, or blocking the government in lobbying for a very important international decision by temporarily occupying them at home)
- Put into question the position of the state on the international political scene by making it seem incapable and/or unready to fight new forms of threats, which can also be done by various actors
- Use the terrorist activity in the territory of a country in order to make it seem as the source of new types of threats

Economic:

- By performing a terrorist act in the territory of a country a strong message is sent out that nobody is safe in that country which can lead to collapse of tourism as a branch of economy.
- Controlling all or some of the elements of the living environment.
- In a smaller scale economic interests may be shown in eliminating the competition, lowering the price of the company which is up for sale etc.
- Through economic goals, by destroying the branch of economy in one region only or in the region occupied by a particular national group, their social revolt might be caused, and that can serve as an excuse to a neighboring country to start political and other measures in order to protect their nationals.

Religious and fanatical:

- From the wish and need for domination of one religion or some other idea, to usage of this type of attack on the prior goals

Criminal:

- From the wish for easy money by human trafficking, drugs or other dual-purpose goods to the wish to control a certain company, production branch…. This goal is also reflected to political and economic stability of the country.

Anarchistic:

- Those that are particularly prone to it are politically extremist individuals and groups that are not satisfied with ultimate rightist or leftist political programs or that are disappointed with the membership in such groups.

10.3. Sources of Biological and Toxin Threats

Natural disasters may be expected in biological area through epidemics and pandemics of diseases of plants, animals and humans. It is necessary to differentiate between epidemics of an endemic disease from the disease that is not typical for the area because measures of prevention are usually taken for endemic diseases. Normally, therefore the competence and professionalism of undertaken measures of prophylaxis come into question, as well as prophylaxis in general, since the disease is not expected and there is no necessary quantity of vaccines or medicines. The danger is multiplied in case epidemics turn into pandemics when states where vaccines and medicines are produced treat those products as strategic.

In case endemic diseases are used as a cover up for their intentional additional spreading, we must talk about covert terrorism.

Natural threats include all other natural disorders (floods, earthquake, storms, changes of climate…). These situations can easily be used for "silent action" by disseminating agents of endemic diseases or by further initiating problems that were started by a catastrophe, such as food and water poisoning, lack of hygiene, spread of diseases typical for catastrophes.

Natural disasters are an ideal platform for all new forms of threats, and not only for terrorism. Therefore, natural disasters must not be viewed solely as a concern of National Protection and Rescue Directorate, but must be encompassed by anti-terrorist plans and measures against proliferation of special types of threats such as information-psychological and economic influence and new types of threats, first and foremost the organized crime [7, 8, 10].

Anthropogenic threats include, apart from warfare, which should be analyzed by a special study within the Strategy of armed defense, all threats caused by human action or threats which stem from the facilities built by men for their own purposes. These are technical and technological accidents in industry and energy plants, accidents during transport of goods and passengers by sea, air or land, caving in of buildings, etc. Terrorism, especially chemical and biological terrorism, whether the stated objects are

targets or serving as means for terrorist acts, is extremely dangerous for economic, political and social stability of the country. There are an increasing number of experts for contemporary threats who believe that terrorism is a means and form of contemporary warfare. As already stated in the introduction, it is very expensive and uncertain to start the war machinery and classic armed actions for the proclaimed purpose. We are witnesses of the said thing when speaking about wars in the last 20 years of which none has achieved the proclaimed goals, but also about terrorist activities which have greatly harmed the stability of big countries such as the USA, Great Britain, the Russian Federation, etc. Terrorism today, when a person as an individual and part of the community is very sensitive to their own security, is a very easy and cheap way to achieve goals, whether political, economic, religious or that of self-interest, profit, cutting down the value of a facility or plant, cutting down or increasing the value on the local and world market. In such situation, terrorism is initiated on the level of well prepared information sent at the right time, and ends as a well planned series of secret and public activities with clearly defined goal or goals.

For the above mentioned reasons both means and methods for terrorist acts are various, as well as the perpetrators. While considering the strategy of defense from terrorism, one must not start from the presumption based on the following:

(a) Traditional definition of enemy based on geographical, political or religious factors.
(b) Traditional belief that any unresolved territorial issue, pretension or unsolved international issues can and must be resolved only by armed force. On the contrary, there are a number of situations which, in accordance to definitions of international laws of war, point to the conclusion that terrorism as new-modern type of warfare is used equally by the states (directly or indirectly through informal groups and organizations) and individuals, formal and informal groups and organizations that we call terrorists, but also interest groups which are related to capital, criminal and production.

As a conclusion, if the contemporary notion of the state, statehood and national interests – as a presumption of the existence of state, starts from the fact that the state and nation must have sovereign control of the elements of the living environment such as infrastructure, finance, economy, natural surroundings, etc.; then the anthropogenic threats themselves cause innumerable damage to the state, and in case they are the target, means or the goal of terrorism, then we can say with certainty that the state is at war and that its sovereignty in the modern sense is at risk.

10.4. Potentially Most Interesting Targets

Based on everything that has been said while looking at the national security of the state and within the framework of strategy for defense against terrorism, it is necessary to start from the above mentioned facts and to foresee all new types of threats as current threat, and terrorism, even the biological one, is just one of them and the means to achieve them – in order to achieve or harm the control of elements of the living environment of the attacked state. In that, both overt and covert methods are equally likely

to happen, and the war in the form of low intensity conflict can only be the cause, goal or consequence of the new-specialized types of threats, where the most likely methods are the following [11–16]:

- **Tourist destinations and tourists themselves in order to ruin the tourist season** (sea pollution, food and water poisoning, fires, road accidents, car thefts, etc.). In that sense, agents are not necessarily war agents, but also, for example, regular "salmonella" which can be explained by low hygiene and care, etc.
- **Food industry** where, again because of "bad care", one can find "salmonella", "trichinosis", "mad cow disease", etc.
- **Agricultural areas** where, by new parasites and diseases which can significantly cut down the crops, and with the additional psychological factor, we can speak about food poisoned with pesticides, after which the tourists in the said country are at risk.
- **Cattle of the said country** or imported meat with various diseases, such as "anthrax", "swine plague" with the goal of cutting down the quantity and putting tourists at risk again by "trichinosis", "mad cow disease", "salmonella", etc.
- **Chemical, petrochemical, oil, pharmaceutical and similar industry with dangerous substances** which, apart from the human and material losses, causes the pollution of the target state (agriculture and tourist destinations) and travel to the said country becomes unsafe.
- **Critical infrastructure objects, such as airports, sea ports, train and bus stations and their infrastructure for transport, storage and handling goods and passengers** where, intentionally or unintentionally infected passenger and intentionally disseminated biological agents, especially with agents belonging to category I of biological agents, mean complete blockage of transport and putting the infected passenger and all who have been in contact with them in quarantine, and in case of late reaction there might also be blockage-quarantine of a wider area, and even the state itself.
- **Critical infrastructure objects, such as theatres, concert halls, cinemas, sports halls, shopping centers etc.;** which due to a large number of people in a limited area and very limited exits are especially convenient for all types of terrorism, including RCB terrorism by silent dissemination of RCB agents, especially biological.

If necessary, this type of terrorism (as silent action) may also turn into an open one with loud paroles such as some used by some of the organizations for the rights of the oppressed, deprived, refugees, etc.

Apart from these types of possible threats to a country by radiological, chemical and biological agents, the threat may happen in a number of other ways, some of which are the following:

- Open aggression to industrial plants
- Natural disasters, technical omissions, human factor
- Chemical, nuclear and biological accidents in neighboring countries
- International terrorism
- Local and regional war in neighboring areas

- Epidemics and pandemics in neighboring areas
- Accident in handling NCB weapons in neighboring areas
- Smuggling of NCB materials through the territory of a country

Any of the above mentioned types of threats may, in case the state is not ready, have consequences for its population and economic and political stability.

10.5. Means for Terrorism by Biological and Toxin Agents in a Wider Sense

Biological and toxin terrorism is the usage and spreading of biological agents and toxins in population areas, with the aim of causing epidemics and mass casualties and destroying moral. Biological and toxin weapons are generally not created for terrorist attacks but their main purpose are mass casualties. The effects of biological terrorist attack become visible only after a few hours or days (after one of the victims leaves the scene of the attack). This may make finding the perpetrator or the target harder in case of a terrorist attack. As opposed to biological weapons, or biological agents defined as war biological agents in the Biological Weapons Convention, there are a number of other agents and toxins which can be used for terrorist attacks. Biological and toxin terrorism may be performed in a number of ways with a number of biological agents and toxins and their transmitters. In better equipped microbiological labs dangerous biological agents may be developed and they can be used for terrorist purposes. Special laboratories for development of war biological agents and toxins are therefore not necessary.

The most dangerous biological terrorism is the covert one, so called “silent action”, when endemic causes of diseases in humans, animals and plants are used and for which it is very difficult to differentiate between natural epidemics or intentionally used biological agent.

According to CDC Atlanta, critical biological agents which can be used in bio terrorism from the point of view of the national security, public health system and urgent response teams include pathogens and toxins which are divided into three categories according to the potential risk and certain criteria:

Category I of terrorist biological agents and toxins meets the following criteria:

- Possibility of easy dissemination or transfer from one person to another
- Cause of high level of infection and casualties and strong influence on unprepared public health
- Possibility of causing mass panic
- Special requests and preparation of public health institutions

Category I includes the following biological agents and toxins:

- Small pox
- Bacillus anthracis (anthrax)
- Yersinia pestis (plague)
- Botulism
- Francisella tularensis (tularemia)
- Ebola virus

- Marburg virus
- Lassa virus
- Hemorrhagic fever

Category II of terrorist biological agents and toxins meets the following criteria:

- Medium possibility of dissemination
- Medium infection and lower level of casualties
- Calls for specific preparation of diagnostic capacities and health care

Category II includes the following biological agents and toxins:

- Coxiella burnetti (Q fever)
- Bricella sp. (brucellosis)
- Burcholderia mallei (maleus)
- Venezuelan horse encephalomyelitis
- Eastern and western horse encephalomyelitis
- Ricin
- Clostridium perfrigens toxin
- Staphylococcus entherotoxin B

Into this category, according to our experience, also could be included pathogens transmitted by food and water:

- Salmonella sp.
- Shygella dysenteriae (dysentery)
- Escherichia coli o157:H7
- Vibrio cholerae (cholera)

Category III of terrorist biological agents includes new and already existing pathogens, which can be prepared for mass dissemination in the future and which meet the following criteria:

- Easily available
- Easily produced and disseminated
- Strong impact on the unprepared public health system

Category III includes the following biological agents:

- Nipah virus
- Hemorrhagic fever with kidney syndrome
- Viruses of tick-borne hemorrhagic fever
- Tick-borne encephalitis
- Yellow fever
- Causes of tuberculosis which are resistant to medicines

It is generally accepted that the first information of a biological attack shall be the large number of infected people, mass dying of animals and wasting away of useful plants. There are a number of problems for the anti-biological defense system during

bio-terrorist attack, especially within the framework of public health care, such as recognition of the attack, determining the applicable biological and toxin war agent, determining the way of taking care (triage, medical and evacuation characteristics, treatment).

In the beginning of the epidemics or after the bio-attack while the cause is still unknown, treatment is decided according to the clinical-epidemiological (syndrome) diagnostics, and the choice of therapy (antibiotics) is determined according to the therapy diagnostics. Antibiotics should be given to practically everyone, even without the firm diagnosis. The biggest number of bacterial diseases is successfully treated by antibiotics. The choice of the medicine depends on the clinical circumstances but antibiotics of a wider specter are given in full therapy doses and if possible intravenously. The therapy should be started as early as possible in the earliest period of health care. Symptomatic therapy should be done when lowering body temperature, decreasing pain and suffering, keeping spontaneous breathing and securing intravenous application of fluids and medicines. Isolation procedures with patients who suffer from contagious diseases are important for the protection of doctors and nurses and for spreading of disease after the bio-attack or incident.

Unfortunately there is no reliable medicine treatment against many of the biological and toxin war agents, not today and not in the foreseeable future either, even with the most famous medicine technologies and procedures. Vaccines are not the solution although they are the best protection we have today. Even the best vaccines are partly efficient. They cannot protect us from the new microorganisms which were altered by genetic engineering techniques. The new treatments such as mono and polyclone antibodies are still to be developed.

Prophylaxis, as a specific method for the protection from bio weapons, is performed urgently as urgent prophylaxis and includes the usage of immune preparations and antibiotics before the bio attack (pre-prophylaxis) or after the first patients appear (post-prophylaxis). Since the bio-attack is recognized after the first patients occur, prophylaxis shall be post-exposition, since mass protection cannot be performed due to a large number of causes without specific protection (vaccines).

Methods of personal and collective protection, which are used for the protection from chemical weapons, may generally be used for the protection from biological and toxin weapons, which is transmitted by air (aerosol). However, requests for the protection from biological and toxin war agents are much higher than for the protection from chemical war agents.

10.6. Measures of Protection from Biological and Toxin Terrorism

Most of the countries wish to prevent future usage of CBN weapons. Global conventions and verification systems in different stages of development will significantly decrease the future risk from mass usage of the existing weapons and disable the development of new and technically advanced weapons, *but not from their terrorist application* [17–19].

In our modern information society the existing knowledge on weapons is further spread to other countries or terrorist organizations, probably even unconsciously, but also intentionally by unscrupulous individuals. This type of covert development is very

difficult to discover. From the technical point of view it can be foreseen for the development of "classical" biological and chemical weapons, but also for possible primitive nuclear weapons. It is theoretically possible that technologically most developed countries and terrorist organizations may develop new types of biological and chemical weapons, e.g. by using fast developing genetic engineering and biotechnology.

From the point of view of protection, the technological discrepancy between offensive and defensive possibilities is already disappearing and in the future this difference will be erased. This can be achieved because the existing international conventions with verification systems enable the development of protection, and forbid offensive research. However, the threat from genetic engineering and biotechnology may come as an exception.

There is the already mentioned need for preparation during peace time against nuclear-bio-chemical incidents due to possible sabotage and terrorist attacks. The increasing participation in international peace keeping operations may also increase the risk from WMD attacks.

Contemporary democratic states, due to the above mentioned facts, must take into account the wide specter of threats by developing the state system against nuclear-chemical-bio attacks, which must be based on the following presumptions:

- Security-intelligence assessment of threat and assessment of threat to facilities and institutions
- Security-intelligence monitoring in the country and abroad
- Fundamental and applied research in the area of prevention and protection
- Diplomatic activity in all international efforts to ban weapons and methods and forms of warfare
- Applied education on all levels and in all segments of society, especially of those who should actively take part in the protection system
- Developing concepts of general measures in all segments of state based on individual and functional responsibility
- Developing concepts of special measures based on plans of participation of MoD, MoI, health, industry and agriculture, and especially MoD, MoI and health and their forces in case of intervention

Special attention must be given to the structure and equipment of the following segments of protection and defense and to erasing the deficiencies:

1. Creating special forces for fast intervention in case of a threat by nuclear, radiological, chemical, biological, toxin and explosive devices. This force should resemble CBRE units, HazMat units and should include pyrotechnics, NCBO experts, antiterrorist combat part, doctors, etc. It should be equipped and trained for emergencies in case of terrorist or any unknown threat, and the task is to decrease or remove the danger. Their task is not to protect and save the population.
2. Creating labs of III and IIA classification for the needs of the state, and it should include representatives of all interested state subjects and it should perform wider education and specialization of doctors, NCB officers, members of special forces, etc.

3. Procurement of means and equipment for NCB protection should be given full priority and it should be planned and implemented for the needs of all segments of protection and defense of the state.
4. Introduction of strict control and oversight of the companies that are registered for production and/or procurement of dual-purpose goods.
5. Creating expert – not political – body for the oversight of the implementation of measures of the fight against terrorism and all new types of threats. This body should include prominent experts from different areas, from the police, army, medicine, natural and technical sciences, law, economy, psychology and sociology. Team members should be expert, mobile and available!
6. Thorough examination of the present state of state reserves which should be enough for the duration of quarantine of a city of an average size.
7. Serious approach to introduction into education of new types of threats with the stress on terrorism in all defense and protection subjects in the state, from the police, army, rescue and protection to health and environment protection.
8. The system of national security should include all serious security guards' companies and their employees and that should be done by training planning and engagement planning.
9. Seriously consider finding a solution which will enable all high school population training and education for defense and protection from all types of natural and anthropogenic threats.

10.7. Conclusion

In the future the risk from mass usage of nuclear–biological–chemical weapons shall decrease, while the risk from the usage of it locally at war or at peace might increase. Especially dangerous is the fact that the risk from misuse of chemistry and biology in the action against a state through so called "silent" action is exponentially increasing, and it could happen as a part of a special war during which not only classical chemical and biological agents might be used, but also toxic and contaminating agents and microorganisms which can be found in the territory of the said state during peace time production or have already caused, during a certain period, epidemics with people or cattle (pathogenic microorganisms). This means that the protection will be necessary in the near future, for the army and for the police, medicine and civil protection and civilians, but not in form of a physical protection of people and goods but as a system of antinuclear, anti-biological and anti-chemical security on the state level.

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Chapter 11

Ukrainian Corruption and Non-ruled Market Economy as Sources of Chemical and Biological Terrorism

Zadorsky W.
Ukrainian State University of Chemical Engineering, Ukraine

Abstract. Today already all of us know, that corruption is extremely diverse, in various degrees it amazes practically all layers of our society – from the beggar – to the large government official or the deputy whom for a good bribe much can make. A characteristic sign of modern corruption is the conflict between actions of the official and interests of its employer or the conflict between actions of the elective person and interests of a society. In process of strengthening of political parties and the state regulation, episodes of arrangement of political elite and large business began to cause growing concern. Globalization has led to that corruption in one country began to affect negatively development of many countries. Not casually for propagation of knowledge of United Nations corruption founded the International day of struggle against corruption. Because Ukraine has got the status of the country with market economy, corruption scales as a result only have increased. In our country all areas, both state management, and local government are subject to corruption practically. Unfortunately, corruption actions in sphere of protection of environment and maintenance of safety of ability to live of the person are especially extended. In the report typical corruption schemes in these areas are considered and classified, the reasons of increase in scale of corruption are analyzed and possible directions not elimination are defined, it essentially while is hardly possible, and at least decrease in harmful influence of corruption in spheres of management, a science, the services, are connected with ecology and safety of ability to live. Real successes can achieve if to pay the basic attention thus to realization of principles of the concept of a sustainable development (SD) with the decision of ecological, economic and social problems at the expense of orientation to development of an average and a small-scale business, use of high innovative potential and market mechanisms of managing on the basis of the system analysis and modern information technologies. Especially effective there will be in struggle against the corruption phenomena use of the indexes of stability of development offered in SD for an expert estimation of correctness of the chosen decision and competitive, tender mechanisms of a choice of the project. Besides, it is necessary to provide the maximum transparency, wide scientific discussion of the most important directions of the development, most ecologically dangerous projects and specific proposals on their realization. As an example of a successful anticorruption policy, they often mention the Swedish experience. There the independent and effective system of justice has been created. Simultaneously Swedish parliament and the government have established high ethical standards for managers and their execution began to achieve. After all some years honesty became social norm among bureaucracy. Salaries of the high-ranking officials first exceeded earnings of workers at 12–15 times, however eventually this difference has decreased to the

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double. For today Sweden has one of the lowest levels of corruption in the world. And, nevertheless, in Ukraine tactics of decrease in corruption should be constructed on a number of vertical measures: regulations of actions of officials, simplification of bureaucratic procedures, strict supervision over observance of high ethical standards. Probably, by experience of other countries, it is necessary to create a special Bureau on investigation of cases of corruption in which citizens could address with complaints to state employees and demand the indemnification. It essentially would increase at once filling of the budget of the country. Simultaneously with it followed toughen the legislation, to raise independence of judicial system, to enter economic sanctions for bribery or refusal of participation in anti-corruption investigations.

Keywords. Non-ruled market economy, sustainable development indexes, corruption, market mechanisms of managing, economic sanctions, Chemical and Biological Terrorism

Do not appropriate illegally property each other and do not bribe judges intentionally to appropriate a part of the property of other people.

The Koran

11.1. Introduction

At first it is a little about the term. Today we already know, that corruption (from lat. *corrumpere* – to spoil) – use by the official of the imperious powers and the entrusted rights with a view of the personal benefit, contradicting the established rules, we know, that corruption is extremely diverse, in various degree it amazes practically all layers of our society – from the beggar – to the large government official or the deputy whom for a good bribe much can make. A characteristic sign of modern corruption is the conflict between actions of the official or elective person and interests of ones employer. Not only State machinery is subject of corruption in our country, but also local governments. In process of strengthening of political parties and the state regulation, episodes of arrangement of political elite and large business began to cause growing concern. Because Ukraine has got the status of the country with market economy, corruption scales as a result only have increased. Globalization has led to that corruption in one country began to affect negatively development of many countries, and not casually the United Nations have founded the International day of struggle against corruption.

The list of kinds of activity of a hired labor which can be qualified as corruption becomes simply immense. Really, it is difficult to find at least one segment of our life where feelers of a corruption tumor would not get:

- Medicine: payment of priority service; additional medical services at all levels, purchase of the equipment, conditioning agents and medicines in the overestimated quantities, under the overestimated prices; payment of medical certificates inappropriate to the validity.
- Customs services: the admission through border of the goods forbidden to transportation; return of the confiscated goods and currency; understating of the customs duties; unreasonable delays of customs payments.

- Car inspection: unreasonable granting of a driving license, inquiries on checkup passage; a payoff from lawful punishment by infringers of instructions for use by roads; falsification of data and conclusions about road and transport incidents in favor of interested persons.
- Judicial bodies: prejudiced consideration of circumstances of business; acceptance of illegal decisions; infringement of a legal procedure; opposite decisions of various courts on the same business; use of courts as the raider tools.
- Law machinery: excitation and the termination of criminal cases, and also their direction on additional investigation; absence of lawful punishment for offences of various weights.
- Tax bodies: non-levying of taxes in full; VAT returning; the check provoked by competitors and a manufacture stop, etc.
- Licensing and registration of enterprise activity – about well-known «permissive documents», «regulator politics», and about uniform window for registration for 1 day in general just right a poem to write - here where revelry of the corrupted officials!
- Delivery of permissions to placing and carrying out of bank operations with budgetary funds.
- Reception of credits.
- Reception of export quotas.
- Use of budgetary subventions.
- Building and repair at the expense of budgetary funds.
- the Notarial certificate of transactions.
- Supervision of observance of rules of hunting and fishery.
- Clearing of an appeal on military service in Armed forces.
- The State registration, certification and accreditation not state (and already and state!) higher educational institutions.
- "Device" on the service, allowing to have the considerable illegal income of a post.
- Sphere practically all utilities, for example the ritual.
- At last, rather new segment of the corruption market – formation of party service records.

This list of segments of the corruption market can be continued infinitely, but scales and high speed of development of illness are already obvious.

Unfortunately, corruption actions also in sphere of protection of environment and maintenance of safety of ability to live of the person are very extended. Corruption acts last years promoted realization of the increasing quantity of ecologically dangerous projects menacing to the life of people in Ukraine. "Thanks to" corruption in our country the considerable part of the state resources meaningly goes to channels where they are the easier for plundering or using as a bribe. The policy of any ruling elite, anyhow, is directed on suppression of mechanisms of the control over corruption: freedom of the press, independence of system of supervision over legality and justice. Such policy is promoted today by absence of the National program of a sustainable development of

Ukraine. Assiduous resistance of the authorities of Ukraine to occurrence of such program amazes any more only us. For 16 years the country (may be already unique in the world!) has not executed the summit decision in Rio decision about its working out. On last elections again any of more, than 100 parties has not made its imperative. And after all anything super difficult in this concept is not present. It only provides balance between works on economy, nature protection subjects and social questions. But we are limited 16 years to attempts to develop not the national program of a sustainable development of the country, and … national Ukrainian terminology. Absence of the National program of a sustainable development in Ukraine is one of the factors promoting development of corruption for leads to that decision-making under the project of any scale is spent not at the expense of use of objective quantitative indexes of stability as it is accepted all over the world, and is frequent as a result of corruption actions of the persons making of the decision. Instead of the quantitative indicators, allowing to choose the best decision, instead of professional examination other methods and means are used absolutely.

For ecological examination in other countries specially they create the professional expert organizations and employ in them highly skilled experts. At us examination has already ceased to be for a long time independent and, it is not a secret, that in expert judgments frequently simply *trade*.

Thus Concept of Sustainable Development (CSD) is not applied at all as criterion, the indicator to definition of stability of development of a city, region, the country. And all world only consider so. If we have gone on this way then a lot of projects which contradict this concept , would not introduce. Because it leads to gross infringements of principles of ecological safety and according to safety of ability to live. I will result examples. Only in near Dniepr Region there are some objects where ignoring of principles of the concept of a sustainable development is simply scandalous.

- Start-up of a copper for burning of sunflower husk at enterprise “Oleina” – a typical example of the administrative decision that is mismatching not only bases of CSD, but also to positions of the Kiotsky protocol. Hundred thousand tons sunflower lusk are burning literally in city centre. You represent, how many thus occurs emissions of harmful substances to the smoke gases dumped practically without serious clearing of harmful substances? And after all of installation of this copper in due time very much were proud, it everywhere advertised as the big achievement in energy saving area. I consider, that it, without exaggeration, an ecological crime.
- The Second example – active use in Ukraine of the fuel “enriched” by a chemical-recovery plant waste (about that I told at our last conference). Pretty often in fuel the mix of aromatic connections – in the form of numerous Ukrainian chemical-recovery factories waste (BTXS fraction) – benzene, toluene, xylol and solvent are added. There are only 20% of the real gasoline, all the rest is fraction BTXS. For this reason in our country already there is no concept “gasoline”, and there is only a modest name of this mix – ersatz – «fuel motor». Thus doctors still search for the authentic reasons of growth of disease of tuberculosis in Ukraine. And may be, it is necessary to analyze a curve of disease and to pay attention that the beginning of abrupt growth suspiciously coincides with the beginning of introduction of “aromatic”

fuel. We simply poison people, adding in fuel these aromatic connections. After all not only they are toxic, but also at their incomplete combustion in the engine the toxic connections, which influence on a human body, are formed while was studied by nobody. And this already direct infringement not only principles of the concept of a sustainable development, but also human rights.

- The Third example – recycling of rockets in Dnepropetrovsk and Pavlograd.
- The Fourth – building in immediate proximity to inhabited files in the same Dnepropetrovsk several turns of manufacture of lead accumulators "Ista".

These are examples – only from Near Dniepr Region. Examples across Ukraine it is possible to result much more. And the conclusion can be made the simple. We should understand only one simple true: only the complex decision of questions of development of economy, improvement of a condition of environment and social problems is capable to deduce the country from a vicious circle that exists today.

There are three whales of our sustainable development. And if to add to them still computer science and the system approach, we will receive to what so we aspire, – advancing development of Ukraine. Deputies of Dnepropetrovsk city council do not understand it, alas, even. Therefore the sustainable development program in our city is not present. But, should tell, that in Ukraine there was a whole network of cities where try to realize the sustainable development concept – them nearby 70. Dnepropetrovsk, unfortunately, in this network does not appear. But, to honor of our area, in it there is Dneprodzerzhinsk and Pavlograd.

So, realization of CSD can become one of effective counteractions of corruption in sphere of ecology and safety of ability to live. However even if CSD suddenly will appear in Ukraine, for its realization it is required to solve questions of creation of practically new effective system of an independent expert appraisal, consulting and audit. ***The essence*** of modern scientifically – proved approach to the decision of problems of sustainable dynamic development of economy of our country ***is reduced to the simultaneous complex decision of problems of development of manufactures, creation and realization of new perfect technological processes, with the simultaneous decision of energy saving problems and maintenance of ecological safety on the basis of use of the system approach and the sustainable development concept***. Such complex approach allows, having refused the independent decision of problems of perfection of technology, energy saving and decisions of environmental problems to provide the simultaneous complex decision of this triune problem. After all, means and decision methods on each of these directions, optimization parameters are uniform and are realized, eventually, by the same experts in manufacture sphere, that is "in the pipe beginning", instead of in its end.

We have variety of successful examples of realization of it enough the non-standard approach for our country. For example, still Branch laboratory of reactors and mass-transfer devices of USSR Minhimprom at our institute has been approved and realised variety of technological installations at the chemical enterprises of the former USSR (dimethylformamide, isopropyl alcohol, 1,4-dioxane, dimethilacetamide, metaphenylenediamine, o-toluil acid, metaphenylenediamine manufactures, etc.), positive results of operation or which approbation have confirmed expediency of the concept set forth above.

For a conclusion of economy of Ukraine from technological, ecological and energy crises in which it now is, it is expedient to return to realization of the given complex approach not only in chemical, but also in other branches of manufacture connected with technological processes of processing of raw materials.

The working mechanism for realization of the given approach is perfection of a technique and development of complex ecological – technological – energy complex audit. Audit (instead of corruption mechanisms!) should provide modern level both at perfection operating, and at creation of new objects. As it is noted above, in Ukraine absolutely independent three audits are spent and they are not connected among themselves, each of which requires perfection. For example, ecological audit which is spent in Ukraine, mismatches the international norms and rules for usually contains only fixing of lacks and infringements and does not contain recommendations about ways of the decision of the environmental problems revealed at audit. Actually audit is replaced by examination and does not contain even consulting elements. Usually it is connected with low level of professionalism of auditors, absence of knowledge and an operational experience in that branch of manufacture which audit they undertook. Similar approaches are characteristic and for power and technological audits.

Because ecological, technological and power audits are interconnected, spent by close techniques, should lean against the same methods of the system analysis, in the light of the concept of the complex approach set forth above to perfection of technological objects, in our opinion, it is necessary to spend ecological–technological–energy complex audit that will essentially lower their cost and will raise efficiency and productivity. As the first step for this purpose, it is expedient to create the Ukrainian scientifically-methodical, information and educational centre for ecological–technological–energy complex audit which primary goals for the first period of work are:

- Creation of the interbranch scientifically proved technique ecological–technological–energy complex audit based on use of methods of the system analysis and the parameters of optimization recommended by the Concept of a sustainable development.
- Creation of computer-assisted databases on methods and techniques of perfection of operating and again projected manufactures with use of modern information technologies.
- Working out of methods of training and improvement of professional skill of auditors in area of ecological–technological–energy complex audit with use of modern educational technologies (trainings, the master-classes, remote formation, etc.).
- Realization of pilot projects on performance of ecological–technological–energy complex audit, duplicating of results.

The problem of creation of the working, not corrupted system of audit in our country is closely connected with elimination or at least essential restriction of corruption in such spheres, as a science, manufacture and education. Here it is necessary to carry to corruption acts, first of all, receiving of science degree by practically all political and official elite. Only laziest of these persons did not become recently by «pro***ff***essors», doctors of sciences and academicians. Diplomas and certificates already are for a long time not a professionalism measure, and mastering measure corruption receptions. But there is in a science one more variant of corruption acts which anything, except condemnation, does not cause. I mean developed system of state budgetary financing of

scientific researches. Fondly to hope, that the state will give the finance on development of a science or its reforming. The former government which in words has chosen innovatively – investment model of development of the country, in practice all "free" means of the poor budget has directed to the oligarchic sector of the economy which have "not soiled" not those that by development of a science, but even cooperation with it. Whether it is corruption acts on the scale of the country!

Attempts of National Academy of sciences to monopolize a science and to incur a role of the unique scientific organization of Ukraine have slightly corruptive smell. It is thought, and the operating government will not manage to pull out the country from a deep hole if it is guided only by those intentions which have been sounded during the recent expanded bureau of presidium of National Academy of sciences devoted to the major directions of scientific researches and workings out. First of all, it is absolutely not enough «the Ukrainian authorities to start to respect science», it is necessary to understand, that «the Ukrainian break» can be successful only in the event that the science becomes its basic platform, instead of «scientific support of development of all sectors of economy» …. The Science of Ukraine has to become one of the major components of economy. "Help" to Academician Science, revival of a branch science does not help us. Only integration of arising innovative technological business into average and small venture business will be effective. The Prime minister has offered National Academy of sciences (absolutely not clearly why only it, instead of frequently to more effective not academic science – high school, corporate, even private) – has to develop system model of "existence" (very well-aimed word!). There are proposed the list of the major directions of scientific researches and workings out. The main direction among them became: nanotechnology development, information technologies and resources, technologies in nuclear power, projects on energy saving, working out of new technologies of an oil recovery and the gas recovery, new biotechnologies for public health services, the projects directed on increase of productivity of agriculture, working out of new materials and processing methods, rational use of nature-resource potential, and also politico-legal, economic, administrative and social–cultural aspects of strengthening of competitiveness of Ukraine on a world scene. And all it is without wide discussion, without scientists from peripheral academic institutes and high schools, without inventors, businessmen etc. Behind each point – "ears" of the academicians who are responsible for these directions. Whether it is corruption? Have gathered, have distributed budgetary money, having forgotten even to place priorities, and have dispersed from feeling of the executed debt to wait from a feeding trough budgetary funds!

We take at least our ill-fated chemistry which has forgotten to include in general list of progress directions. Ukraine in the USSR was one of most chemically developed republics. Today the basic chemistry in the country practically is not present. Those oil refining and petrochemical factories, which else remained also which problems, by the way, is in the list of directions, for a long time belong to Russia and other countries. And to revive the chemical industry, judging by the list, our scientists do not gather. Meanwhile the Ukrainian break is simply impossible without new directions in the chemistry, successfully developing in industrially developed, instead of the banana countries. I mean manufactures of chemical reactants, especially pure substances for micro radio electronics, the fiber optics, substances for nuclear power, manufacture of

new composite materials with absolutely fantastic properties, unique heat-carriers without which the decision of energy saving problems, etc. is impossible. To us, it will not be possible to restore those numerous manufactures of the basic chemistry which worked in Ukraine in days of Soviet Union the next decades. It is simply impossible in the conditions of market economy for years of idle time there were new technologies and the equipment, providing manufacture of substances of better quality with ready more low expenses of raw materials, energy, at last, expenditures of labor. Old manufactures never will be profitable and competitive in case of their resuscitation – the world has left far forward. It is necessary to create absolutely other, competitive chemistry. Most likely, it will be multi-nomenclature chemistry, quickly transformed according to requirements of the market chemistry based on block-modular installations of high flexibility with a wide admissible range of loadings on raw streams, the big running start of change of admissible parameters of process. Our chemists successfully were engaged in due time in this direction of development (Dnepropetrovsk, Kharkov, Cherkassy, Rubedgnoje, Shostka, Gorlovka), and is far not all professionals have left. But in Ukrainian Academy it is not known about it – such works there were not conducted. Therefore the whole direction also has disappeared, can, the most important for the country. Here only one example of that the academic corruption can seriously do much harm to the country and withdraw it aside from the decision of strategic problems.

There is only one exit that (no, not to eliminate, it essentially while it is hardly possible), to lower harmful influence of the academic corruption on process of resuscitation of a science. The transparency, competitiveness, and wide scientific discussion both the most important directions of development and specific proposals on their realization are necessary. To the majority of experts for a long time it is already clear, that reform only sciences is senseless, if it is not coordinated to reform economic, social and ecological (in full conformity with the sustainable development concept). Realization of innovative strategy is possible only on the basis of new tactical receptions with the complex decision of social and economic and ecological problems at regional level. Clearly also, that it is possible for a science and it is necessary to hope only for itself, on own intellectual forces supported with possibilities of market economy. And the power should provide legislation change, first of all in the field of intellectual property, the fiscal policy. Anybody naturally does not bring an attention to the question on liquidation of the state academies, in particular greatest of them – National Academy, or their formal association with universities. It is a question of ripened and their absolutely necessary transformation into powerful structures of technological business. This approach will bring at once to nothing the academic corruption. If, of course, it will be possible to solve personnel questions. After all simply competent professionals and the creative experts capable operatively to solve questions of revival of a science and on its base of economy of Ukraine are necessary not. Thus the higher school of Ukraine should become, as well as all over the world, not only a smithy of creative scientific shots, but also the centre of creation of an innovative product. To whom as not to students and post-graduate students to do it, proceeding from known wisdom: to float, it is necessary to float.

I will risk from provincial high school to formulate the motto of revival of the Ukrainian science which it can realize if it manages to bridle the academic corruption: for Ukraine realization of principles of a sustainable development with the decision of

ecological, economic and social problems at the expense of orientation to development of an average and a small-scale business, use of high innovative potential and market mechanisms of managing on the basis of the system analysis and modern information technologies is represented to the most perspective. Especially effective there will be in struggle against the corruption phenomena use of the indexes of stability of development for an expert estimation of correctness of the chosen decision and competitive, competitive, tender mechanisms of a choice of the project. Besides, it is necessary to provide the maximum transparency, wide scientific discussion of the most important directions of the development, most ecologically dangerous projects and specific proposals on their realization.

In Ukraine tactics of decrease in corruption should be constructed on a number of vertical measures: regulations of actions of officials, simplification of bureaucratic procedures, strict supervision over observance of high ethical standards. Probably, by experience of other countries, it is necessary to create a special Bureau on investigation of cases of corruption in which citizens could address with complaints to state employees and demand the indemnification. It essentially would increase at once filling of the budget of the country. Simultaneously with it followed toughen the legislation, to raise independence of judicial system, to enter economic sanctions for bribery or refusal of participation in anticorruption investigations.

Thus, a number of directions on decrease in corruption of activity on restriction at the expense of it Chemical and Biological Terrorism is above offered. It is possible to reach only at the expense of transition from the concept of struggle against emissions of harmful substances" on the pipe end" to the concept of pure manufactures (CP Concept). High requirements of this concept to the organization of manufactures will make practically impossible corruption receptions at the decision of this problem. As the major principle of cleaner economy, the systems approach is taken that deals with perfecting any nature-technology system at the various hierarchic levels, from environmental pollution sources to consumers. This type of analysis will reveal relationships between the ways to improve processes and the challenges of risk management and nature conservation. The main task is therefore to harmonize the nature-technology relation and, ideally, to engineer high-performance systems featuring desired environmental characteristics at each hierarchic level, so that the favorable environmental background is not impaired and, where possible, even restored.

Following are the basic assumptions underlying the cleaner economy concept for Ukraine:

1. At this time of a deep economic crisis, the economic and environmental challenges must be
2. Met simultaneously, in keeping with one strategy of cleaner economy.
3. A move towards a cleaner economy must focus not on consumption, but rather on
4. Perfecting those entities that are actual or potential polluters.
5. The success of a cleaner economy policy will be largely determined by the availability
6. Professionals well trained in the theory and practice of "economy clean-up" and
7. Environmental management

8. No cleaner economy will be possible without creating a civilized environmental market

These strategic principles determine some tactical measures for pursuing them. Such measures are applicable to any industry and include:

- No waste due to improved selectivity
- Neutralizing wastes directly at the origin, rather than at the exit
- Flexible technologies
- Recycling materials and energy, industrial symbiosis
- Conservation of resources
- Waste treatment, etc.
- These tactics must be combined with certain design and process engineering techniques, such as
- Providing a considerable excess of the least hazardous agent
- Minimizing dwell times
- Recirculation of materials and energy via closed loops
- Concurrent reactions and product separation
- Introduction of heterogeneous systems
- Adaptive processes and apparatuses
- Increasing throughputs
- Multifunctional environmental facilities, etc.

It is necessary to joint techniques development with restructuring in the area of material production to be based on:

- Developing a socially oriented market economy that would guarantee a proper life standard for the population, cleaner production, minimizing environmental loads, material conservation, adoption of new types of activity grounded on environmentally safe technologies
- Making a more balanced economy by shifting from production of means of production to consumer goods, and environmental impact assessment and auditing for all economic projects

Part 2

MEDICAL TREATMENT AND DECONTAMINATION WITH CHEMICAL AND BIOLOGICAL AGENTS

Chapter 12

Analytical Methods to Characterize the Composition of Surface Lipids of Helophytes Exposed to Contaminated Water

Macherius A.[1], Hartig C.[2], Kuschk P.[2],
Shtemenko N.[3], and Moeder M.[2]
[1]*University of Applied Sciences, Faculty of Mathematics/Natural Sciences, Zittau, Germany*
[2]*Helmholtz Centre for Environmental Research UFZ, Department of Analytical Chemistry, Leipzig D-04318, Germany*
[3]*Dniepropetrovsk National University, Ukraine*

Abstract. The epidermis of plants is coated by a thin cuticular membrane protecting the plant from water loss and UV radiation. This lipid layer includes long-chained fatty acids, esters, hydrocarbons, alcohols, aldehydes and ketones. The composition of these compounds depends on the species, their ages and growing conditions. Also chemical stress seems to change the qualitative and quantitative composition of the lipid layers caused by pollutant interfered plants biosynthesis. Particularly the chain length, saturation and branching of fatty acids change during pollutant exposure as previous studies indicated. In this project especially the wax layer of juncus effusus and phragmitis communis were studied under chemical stress caused by chlorobenzene and hydrocarbon contaminated water. The extractable surface lipids were isolated from the plants and analyzed by gas chromatography-mass spectrometry after derivatization. For data processing a pattern recognition program was used that based on a costume made data base containing typical plant lipid substances. First results indicate that in comparison to the control plants the composition and particularly the amount of the wax components changed clearly in the exposed plants. Statistical evaluation of the data confirmed the alterations determined as significant influence of the pollutants.

Keywords. Plants, chlorobenzen, carboxylic groups, pollutants, evaluation, hydrocarbon

12.1. Introduction

During evolution plants developed on their surfaces extracellular, highly hydrophobic membranes of 1–15 μm thickness, called cuticle. The creation of the lipophilic interface between plant and surrounding air became necessary to survive at conditions with strong water gradients between the inner plant and the environment. The cuticle of plants is therefore an evolutionary adjustment of plant physiology on the living conditions at the continents.

C. Dishovsky and A. Pivovarovi (eds.), *Counteraction to Chemical and Biological Terrorism in East European Countries*,

This cuticle as outer stratum protects the plant efficiently from the loss of water and essential nutrients. The cuticle serves as mechanical barrier reflecting dangerous UV-irradiation and prevents pest attacks. Particularly the extremely hydrophobic part of the cuticle, the so called lipid or wax layer, regulates the permeability of the cuticle for water and polar substances [1]. On the other hand, the wax layer with its high hydrophobicity is predestined as sorbent for environmental pollutants which can damage the cuticle and as a result the whole plant. Are pollutants up-taken by plants able to interfere the plants' biosynthesis especially the formation of the lipid layer? This is a major question asked in the project entitled "Influence of Contaminant Stress on Plant Surface Lipids" (NATO CLG No. 983031). An important prerequisite to answer this query is an informative analysis of the wax layer. The detailed qualitative and quantitative determination of the components of the wax layer is the basis to recognize the changes caused by pollutant influence.

For this study, rush (*juncus effusus*) and reed (*phragmites communis*), were selected as plants for exposure experiments. These helophytes are frequently used in wetland systems for bioremediation of contaminated water as shown actually in pilot plants tested in the former chemical industry areas of Bitterfeld and Leuna (Germany). Former intensive opencast-lignite mining and petrol chemistry had caused groundwater contamination characterized by high concentrations of chlorobenzene (to 30 mg/L) and benzene as well as methyl-tert.butylether (MTBE) at concentrations of about 12 mg/L and 3 mg/L, respectively (Figure 12.1).

Figure 12.1. Pilot wetland plant of the Helmholtz Centre for Environmental Research at the petrol chemical industry centre at Leuna, February 2008. Photo: Möder 2008

On-site treatment with planted soil filters use reed to eliminate the hydrocarbon pollution. The wax layers of the corresponding plants were investigated in comparison to non exposed reed growing at the greenhouse.

In additional model experiments in greenhouse, rush plants were exposed to water loaded with chlorobenzene of 2 mg/L concentration (Figure 12.2). Pots with non exposed rush plants served as control.

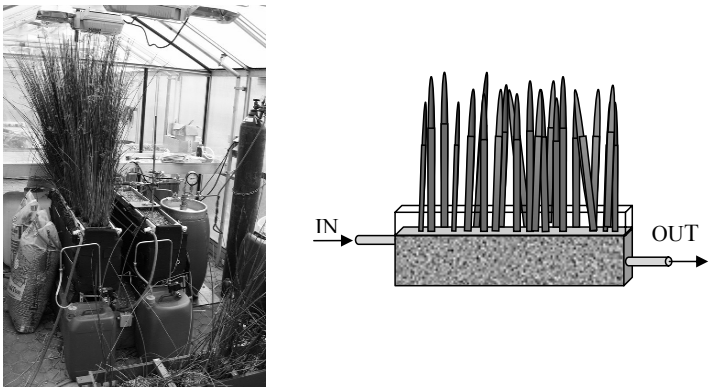

Figure 12.2. Model wetland experiment in greenhouse for the exposure of juncus effusus growing in grit and contaminated water designed for project "SAFIRA II", concentration of chlorobenzene = 2 mg/L, horizontal flow. Photo: Macherius, 2008

In the following an overview is given on the established analytical methods to investigate the wax layer of plants and first results of the study are presented.

12.2. Analytical Methods to Investigate the Wax Layer of Plants

Generally different approaches are used to get information on lipid layers of plants. The investigations of plant surfaces allow an overview on the intact surface of the organism. The microscopic picture of the surface let estimate the general healthy or strained state of the cuticle. The study of wax conglomeration provides information on stress input on the plants such as caused by pollutants. On molecular level infrared spectrometric methods (IR) are able to depict functional groups included in the wax molecules. Since oxydative stress increases the formation of hydroxyl, carboxy and carboxylic functional groups, in Attenuated Total Reflection Infrared Spectrometry (ATR-IR) elevated IR signals typical for these groups can be observed clearly [2].

Apart from the non destructive IR methods gas chromatography-mass spectrometry (GC-MS) is most commonly used to investigate the composition of the plant wax layers. However, prior to analysis the waxes have to be extracted from the plant surface as complete as possible. The hydrophobic compounds mostly long chain alkanes, fatty acids, alcohols, aldehydes, and steroids are extracted by an appropriate organic solvent like chloroform. The compounds with polar functional groups such as OH and COOH need special pre-treatment to become analyzable by gas chromatography. Derivatization procedures are required to transfer carboxylic groups into methylesters and hydroxylic groups into trimethylsilylethers. Both derivatization steps are carried out subsequently before GC-MS analysis can be performed. The scheme showing the preparation of the wax layers for analysis is given in Figure 12.3.

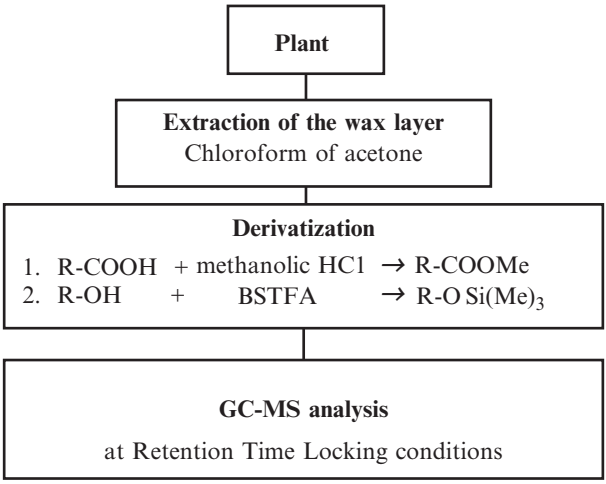

Figure 12.3. Scheme of sample preparation and analysis of plant wax layers

Highly stable gas chromatographic separation conditions are essential for reliable results and the comparability of chromatograms. A special commercially available software "Retention Time Locking" (RTL) offered by Agilent Technologies, enables the standardization of retention times in different analysis. The retention times of all components in a chromatographic run are locked finally to the retention time of hexadecanoic acid methylester which is a common reference compound for this purpose. The chromatographic conditions fixed guarantee highly reproducible retention times of a long series of analysis. This is the basis for performing automated data processing using a modified fatty acid ester data base established by C. Härtig [3].

A typical total ion chromatogram obtained from the GC-MS analysis of the wax layer of an exposed reed (leave) is shown in Figure 12.4.

The chromatographic pattern was found to be typical for the plant species. Mainly the quantity of individual components varied in dependence on the plant age but more clearly after exposure to the pollutants chlorobenzene and the mixture of hydrocarbons.

In order to facilitate and accelerate data processing an automated procedure on the basis of a fatty acid ester data base was applied. Within the project it became apparent that the data base has to be extended and modified by plant specific components such as long chain alkanes, alcohols and aldehydes and carboxyl acids with carbon numbers over 24.

12.3. Changes in the Wax Compositions After Pollution Exposure

Both exposure experiments with rush and reed showed significant changes in the surface layer composition of the plants. The investigations utilized plants of approximately the same age (correlates with the high of plant stems).

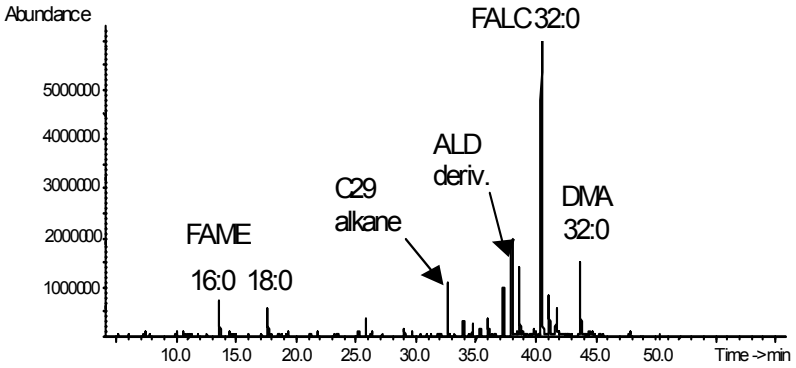

Figure 12.4. Total ion chromatogram of a derivatized wax extract of an chlorobenzene exposed reed plant FAME = fatty acid methylester, ALD = fatty acid aldehyde, FALC = fatty acid alcohol (trimethylsilylether derivative), DMA = dimethylacetale (chain length: 32 carbon atoms, zero double bond)

The result of the comparison between exposed plants and corresponding controls is demonstrated on the example of rush samples exposed to chlorobenzene (Figure 12.5).

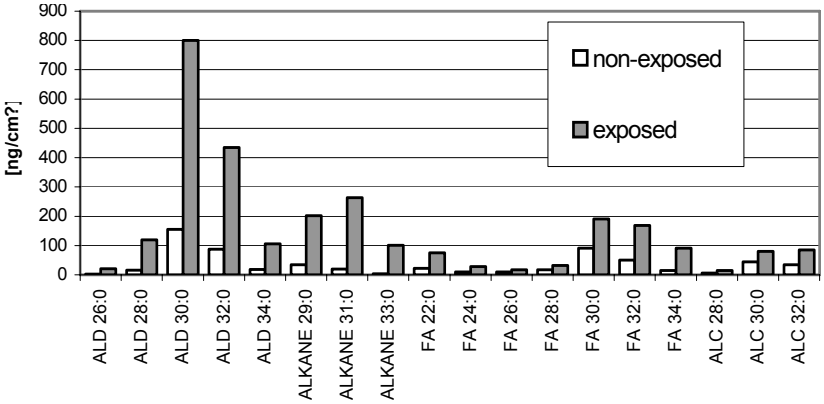

Figure 12.5. Comparison of individual major components of the wax layers of rush exposed to chlorobenzene and the corresponding controls (non-exposed)

The diagram in Figure 12.5 shows the range of components particularly affected by the pollutant exposure. Especially the amounts of aldehydes, alkanes, fatty acids, and alcohols with carbon numbers from C26–C32 are elevated dramatically in the exposed plants. The components with carbon numbers below C20 are hardly changed in their concentration.

Because of optimized analysis and data processing conditions large data sets were produced which allow a statistical evaluation. The classification of 34 rush samples (cases) with 78 components each (variables) by cluster analysis provided two groups of samples, exposed and non-exposed once. Only three outliners could not be related

correctly. The corresponding wax extracts arose from rush plants with the shortest pollutant exposure (first month), thus an influence to plants biosynthesis is hardly to recognize and consequently changes in their wax composition were still not developed. The statistic data interpretation fully confirmed the analytical results shown as example in Figure 12.5.

The mechanisms and consequences behind these results have to be studied and discussed in following experiments planed within the project.

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Chapter 13

Influence of Contaminant Stress on the Surface Lipids Composition of Some Helophytes

Shtemenko N.[1], Kuschk P.[2], Moeder M.[2],
Geyer W.[2], Haertig C.[2], Voevoda M.[2],
Shepelenko V.[1], and Alexeevskaya I.[1]
[1]*Dniepropetrovsk National University, Ukraine*
[2]*Helmholtz Centre for Environmental Research UFZ, Department of Analytical Chemistry, Leipzig D-04318, Germany*

Abstract. The aim of the investigation was to elucidate the extent and directions of possible alterations in the process of biosynthesis of surface lipid (SL) molecules in the presence of some toxicants and realize if some SL molecules could be stress markers. The emphasis is made on some water plants, representatives of *helophytes*. Some plants (contaminant exposed) grown on sewage ponds of Dniepropetrovs'k varnish-dye plant with complex contamination were investigated. Spectral (Fourier transform infrared, FTIR), thermogravimetric (TG) and gas chromatography – mass spectrometry (GC-MS) data were considered. FTIR-spectra of SL of contaminant non-exposed and contaminant exposed plant samples had some differences concerning absorbtion of some characteristic bands; TG and evolutional profiles of water, also carbon dioxide and carbon monoxide evolution had some differences in control and contaminant exposed plants. The results confirm their different molecular-dynamic characteristics, dependent from content and associative abilities. Changes in composition of SL took place under influence of contaminants. The content of fatty acids and hydrocarbons changed in SL of contaminant exposed plants in comparison with control.

Keywords. Surface lipids, helophytes, fatty acids, hydrocarbons, complex contamination

13.1. Introduction

For macrophytes the role of surface lipids (SL), (epicuticular waxes) as a layer of protective lipid molecules is well-recognized [1, 2, 5, 6, 10]. The most prominent functions of SL as represented by a hydrophobic microstructured plant surface are: the transport barrier (limitation of uncontrolled water loss or leaching from interior and foliar uptake), water repellency (control of surface water status), anti-adhesive, self-cleaning properties (reduction of contamination, pathogen attack and control of attachment and locomotion of insect), signaling (cues for host-pathogens/insect recognition and epidermal cell development), protection against harmful radiation, mechanical properties

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(resistance against mechanical stress and maintenance of physiological integrity) [1]. Cuticle – an extracellular, lipid covering, forming the interface between the plant and its environment is considered mostly to be non-living [1], the root uptake of toxicants and their transport to SL is negligible, therefore, there is little known about their influence on the process of the surface layer formation. Radiolabelling of epicuticular waxes and cutin of isolated tomato fruit cuticles were determined after fruit surface application of ^{3}H-phenyIalanine precursor and during fruit ripening, the precursor was incorporated in different phenolic components: the flavanone naringenin was found to be the major compound in the epicuticular waxes, while the amount of the labelled flavonoid in the cutin matrix was progressively increased throughout fruit ripening [7]. These experiments have broken the common agreement about non-vital surface of plants and made possible to propose that lipid components of the cuticle play an important role in the process of adaptation of plants to the influence of toxicants being transported from soil and water.

Some helophytes (synonyms emergent water plants, marsh plants, etc.) used for treatment of contaminated waters in constructed wetlands [3, 8, 12], are shown to have specific content of SL [11] and are absorbers of pollutants. For example, some selected helophytes took up and transformed more than 90% of 2,4,6-trinitrotoluene (TNT) under in vitro conditions during 10 days of planting them in TNT solutions [9]. Up to 8% of the TNT and its metabolites were found in aerial parts of the plants. We consider these species as good models for the elucidation of the upper underlined question. Thus the aim of the investigation was to analyze if SL of plants were changing under the influence of water pollutants, and to determine the scale and directions of these changes.

13.2. Materials and Methods

SL were obtained from leaves of healthy and well developed plants *Phragmites australis* Trin. and *Typha latifolia* L. grown in the Dniepropetrovsk botanical garden (control) and in the sewage ponds of Dniepropetrovsk varnish-dye plant (contaminant exposed) with complex contaminants such as organics and heavy metals during 1 month. SL were extracted by hot chloroform and dried as described in [10]. Fourier transform infrared (FTIR) spectroscopy of SL in the h-ATR and transmission (KBr disks) was imployed [3]. Molecular-dynamic characteristics of SL were studied by thermogravimetric analysis coupling with FTIR in air and in a nitrogen atmosphere (TG-FTIR) using TGA 209 (Netzsch**-Gerätebau GmbH)**)/Vector 22 (Bruker Optik GmbH) analysis with GRAMS/32 (Galactic Industries Corp. with temperatur programm: 30–820°C (10 K/min). SL were derivatized with methanolic HCl (Supelco) to form methyl esters of fatty acids. The derivatized mixtures were analyzed by GC-MS using an Agilent 6890 gas chromatograph coupled to a 5,973 mass selective detector (Agilent Technologies, Waldbronn, Germany). For GC separation a 30 m long HP5 MS capillary column was used (0.25 mm i.d., 0.25 μm film thickness) with following oven temperature program: 50°C – 1 min – 50 K/min – 170°C – 4 K/min – 300°C – 4 min. One microliter of each derivatized extract with methylated carboxylic function was injected

splitless (pulsed splitless) at an injector temperature of 300°C. The ion source of the mass spectrometer operated in electron impact mode at a temperature of 230°C. Full scan mass analyses were performed to get comprehensive information on the components contained in the extracts. Data were processed automatically using a database containing 178 GC retention times and mass spectral characteristics for identification of long chain alcohols, aldehydes and methylesters of fatty acids. In order to guarantee reproducible retention times the GC-analysis was locked on hexadecanoic acid methylester as a time reference point [4].

13.3. Results and Discussion

The FTIR spectra of SL from investigated plants were dominated by four groups of absorption bands in the wave number range from 3,000 to 670 cm^{-1} (Figure 13.1).

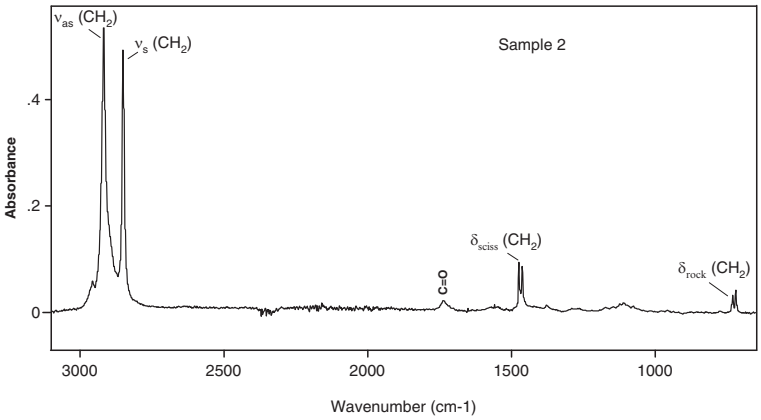

Figure 13.1. FTIR spectrum of SL from *P. australis* contaminant exposed (2), accomplished in h-ATR mode

The bands are indicative of IR absorption by the CH_2-group making up most of the aliphatic fraction of SL. The strong bands at 2,927–2,916 and at 2,856–2,848 cm^{-1} were assigned to valent asymmetric (ν_{as}) and symmetric (ν_s) CH_2 stretching, respectively and are typical for plant SL [3]. Deformational scissoring (σ_{sciss}) and rocking (σ_{rock}) CH_2 modes lead to the band doublets of intermediate intensity at 1,473–1,471 and 730–720 cm^{-1}, respectively. Carbonyl absorption was present at 1,720–1,745 cm^{-1} in all samples. The area from 400 to 1,900 cm^{-1} differs very much in control species and may be called a "specific area".

Data obtained from IR KBr – modes are qualitative once as equal quantities of SL of different examples were weighted, packed with KBr and analyzed. The differences between contaminant exposed and control plants were found in ratio of intensity of these characteristic bands during discussion of spectral data obtained in transmission technique (Table 13.1).

Table 13.1. Some characteristic absorbtion bands in IR spectra (transmission mode): assignment, peak frequence (ν, cm^{-1})/intensity (B-D) and their ratio

Plants	**δ_{sciss} CH_2**		**δ_{rock} CH_2**		**V C=O**		**B/D**	**B/C**	**C/D**
	ν, cm^{-1}	B	ν, cm^{-1}	C	ν, cm^{-1}	D			
T. latifolia (control)	1,473	5.19	720	1.95	1,734	2.63	1.97	2/66	0/74
T. latifolia (contaminant exposed)	1,472	7.28	720	2.94	1,736	3.37	2/16	2.48	0.87
P. australis (control)	1,464	6.72	720	2.55	1,736	10.87	0.64	2.58	0.25
P. australis (contaminant exposed)	1,472	7.01	721	2.72	1,737	10.88	0.62	2.64	0.23

This indicates that biosynthesis of the main components was close to natural and slightly changed under influence of toxicants, that may serve as indicators of the contaminants impact.

In Figure 13.2 it can be seen the curve of difference e − c has positive meanings in the specific area from 400 to 1,900 cm^{-1}. This area to our mind is to be also the matter of concern in the search of disturbances of the SL formation.

Comparing the SL spectral data of control and experimental plants of two species, clearer differences especially concerns absorption in the area of carbonyl group were found in experiments with *P. australis*, that shows species typical response on the same contaminants.

In Tables 13.2 and 13.3 the data of fatty acid and hydrocarbons composition of SL of the investigated plants are presented. The dominating fatty acids in SL of both control species (*Phragmites and Typha*) were fatty acids of the C_{16} and C_{18} groups; among hydrocarbons, odd numbered C_{25}–C_{29} components prevailed which are all typical for emergent water plants.

Changes in SL composition took place under the influence of contaminants exposure. In both species the content of fatty acids increased in SL of the contaminant exposed plants in comparison with control. The process of plant adaptation to toxicants of both investigated species differed in influence on biosynthesis of long-chained compounds in SL: in *Phragmites* we have found inhibition of elongation, resulting in a decrease of long-chained fatty acids and hydrocarbons; in *Typha* there was strong increase of fatty acids content with chain length of more than C_{20} (Table 13.4).

Thermograms – differential TG curves (DTG) and evolutional profiles of water, carbon dioxide and carbon monoxide of the SL (Figures 13.3 and 13.4) showed differences in the control and contaminant exposed that confirms their different molecular-dynamic characteristics, dependent on content and associative abilities.

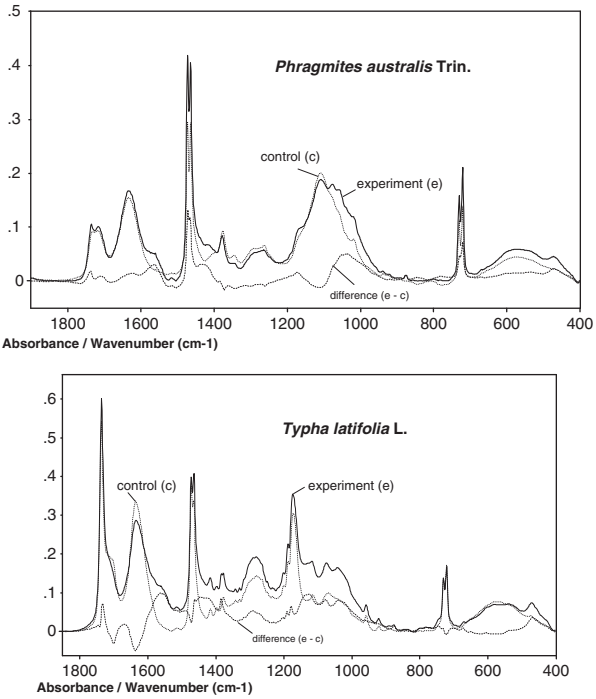

Figure 13.2. The IR-spectra of SL of *P. australis* and *T. latifolia* (control and contaminant exposed) compared in transmission technique

Table 13.2. Fatty acid composition (%) in SL of investigated plants

Fatty acid	*P. australis*		*T. latifolia*	
	Control	Contaminant exposed	Control	Contaminant exposed
$C_{10:0}$	2.83	–	–	–
$C_{12:0}$	11.47	3.49	3.46	1.75
dicarboxylic $C_{9:0}$	–	2.43	–	–
$C_{14:0}$	13.45	11.49	17.03	6.65
$C_{15:0}$	1.26	2.88	2.07	2.92
$C_{16:0,1}$	23.65	25.39	41.42	26.88
$C_{17:0}$	2.05	–	–	–
$C_{18:0,1,2}$	9.66	27.48	23.16	26.88
$C_{19:0}$	1.07	–	–	–
$C_{20:0}$	10.95	15.94	12.04	10.20
$C_{21:0}$	1.33	0.80	–	–
$C_{22:0}$	7.85	5.57	3.45	4.26
$C_{23:0}$	0.81	0.64	–	–
$C_{24:0}$	8.77	4.08	3.33	9.94
$C_{25:0}$	0.56	–	–	–
$C_{26:0}$	2.43	–	2.67	10.52
$C_{28:0}$	1.11	–	1.36	4.62
$C_{30:0}$	0.07	–	–	–
Total quantity (mg)	92.16	128.84	96.83	97.87

Table 13.3. Composition (%) of hydrocarbons in SL of investigated plants

Saturated n-hydrocarbons	*P. australis*		*T. latifolia*	
	Control	Contaminant exposed	Control	Contaminant exposed
C_{23}	–	20.77	20.34	19.50
C_{24}	–	36.05	5.78	–
C_{25}	–	43.18	29.11	9.83
C_{27}	6.88	–	22.73	14.66
C_{29}	38.68	–	22.03	57.01
C_{30}	3.80	–	–	–
C_{31}	44.20	–	–	–
C_{34}	6.43	–	–	–
Total quantity (mg)	11.04	6.14	10.03	13.26

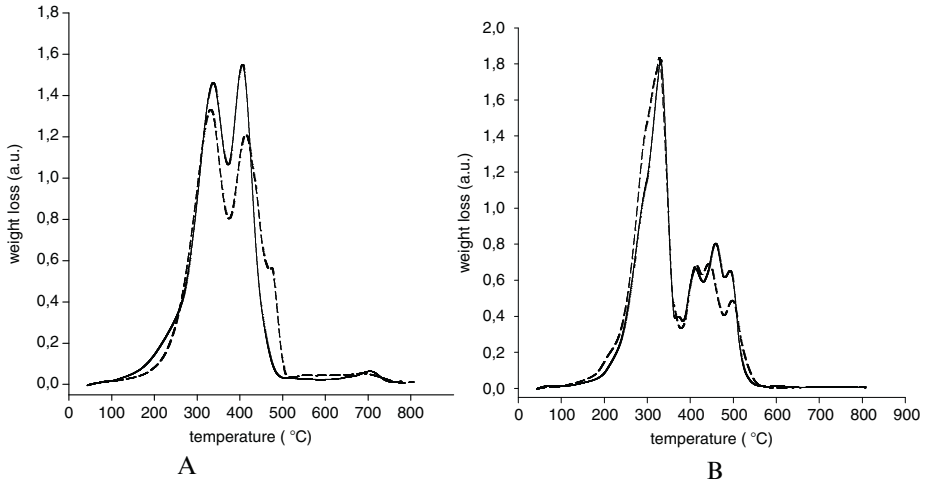

Figure 13.3. TG-FT-IR of SL of *Phragmites* grown in control (—) and contaminant exposed (- - -) conditions in nitrogen (**a**) and synthetic air (**b**) streams

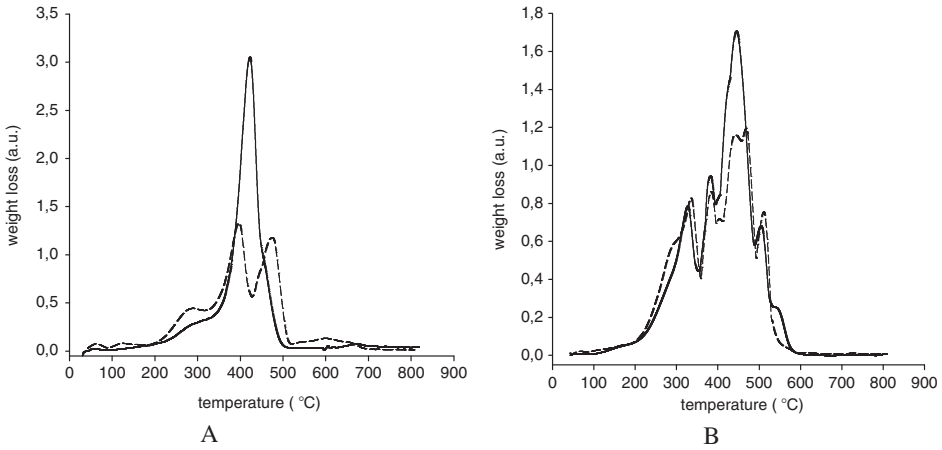

Figure 13.4. TG-FT-IR of SL of *Typha* grown in control (—) and contaminant exposed (- - -) conditions in nitrogen (**a**) and synthetic air (**b**) streams. Abscissa is first derivative of weight lost (u.a.)

Especially significant differences we may observe in the samples of *Typha* in nitrogen where the single peak in control is substituted by two in exposed samples. Here we can observe the shift to the high temperature area of decomposition of lipids molecule.

Table 13.4. Data of the TG-FT-IR analysis (temperature of the peak in ^{o}C/first derivative of weight lost (u.a.)/) of SL of the investigated plants

Kind of the profile	***Phragmites* control**	***Phragmites* (contaminant exposed)**	***Typha* control**	***Typha* (contaminant exposed)**
CO_2 profile in air	424 /2/, 464 /4/, 507 /11/	416 /1/, 480 /6/, 500 /14/	392 /2/ , 426 /3/, 510 /14/	392 /5/, 410 /4/, 450 /5/, 470 /6/, 512 /14/
CO_2 profile in N_2	395 /5/, 415 /6/, 428 /7/ , 508 /7/, 624 /9/, 652 /9/, 736 /4/, 760 /14/	408 /14/, 460 /7/, 500 /5/, 545 /4/, 632 /7/, 660 /7/, 692 /6/	452 /14/, 516 /8, 640 /6/ , 684 /4	440 /13/, 644 /9/, 710 /2/
CO profile in air	336 /14/, 360 /12/, 380 /10/, 424 /10/, 460 /9/, 504 /9/	340 /03/, 360 /12/, 388 /9/, 418 /9/, 432 /10/, 500 /11/	350 /14/, 372 /10/, 386 /9/, 404 /7/, 412 /12/, 504 /11/	370 /12/, 388 /9/, 398 /9/, 408 /10/, 450 /13/, 482 /14/, 509 /13/
CO profile in N_2	384 /4/, 412 /8/, 438 /11/, 452 /11/, 468 /9/, 532 /4/, 550 /5/, 592 /8/, 643 /13/	500 /13/, 520 /14/	460 /11/, 472 /13/, 484 /14/, 509 /12/, 565 /10/, 612 /9/, 628 /7/, 676 /4/, 710 /2/	332 /9/, 356 /10/, 370 /11/, 408 /12/, 428 /10/, 476 /6/, 500 /8/, 518 /9/, 534 /13/, 550 /12/, 610 /11, 700 /4/, 750 /4/
Hydrocarbons profile in air	328 /14/, 366 /7/, 423 /3/, 460 /4/	347 /14 , 383 /7/, 417 /6/, 460 /11/		
Water profile in air	338 /14/, 421 /8/, 460 /7/, 500 /5/	654 /13/, 385 /6/, 421 /7/, 476 /10/, 500 /8/		

The evolutional profiles of SL of this specie have also the tendency of shifting to more high temperature areas. This confirms that fact that in *Typha* the elongation process of the SL is intensified in the exposed plants and in a whole average molecular weight of SL components is higher.

In *Phragmites* we have the decrease of the average molecular weight of SL under influence of toxicants, but low molecular weight components, for example, fatty acids, have better associative abilities than high molecular weight fatty acids. That is why it is very difficult to make strict interpretation of the TG-data obtained and to find correlation between them and data of content. The only very important fact is obvious that under influence of toxicants take place changes of molecular-dynamic characteristics of SL molecules that may influence on their associative abilities.

13.4. Conclusions

The process of adaptation of plants to toxicantsis reflected in changes in SL composition of the investigated species. Particularly the influences of pollutants on the biosynthesis of long-chained compounds were considered. Partially an inhibition of elongation

resulted in decreasing contents of long-chained fatty acids and hydrocarbons. In other species the content of fatty acids with chain more than C_{20} were found to be strongly increased. On the present state of our knowledge we may conclude that contaminants influence the biosynthesis of SL components. The changes in SL composition concerned processes of elongation of fatty acids and their derivatives. In sum, a quite plant specific response to the contamination was observed. Probably on two ways the contaminants can influence the biosynthetic pathways of SL compounds: proper enzymes or enzymatic systems are inhibited or promoted, respectively.

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Chapter 14

The Plant Cuticle: A Complex Lipid Barrier Between the Plant and the Environment. An Overview

Heredia A.[1] and Dominguez E.[2]
[1]*Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, Spain*
[2]*E.E. La Mayora, CSIC, Spain*

Abstract. The cuticle is a translucent film of polymeric nature that covers the aerial parts of higher plants. It constitutes the interface between the plant and the atmosphere and therefore, plays several crucial roles. In this work we present a review on the morphology, structure, and main components (cutin and waxes) of the plant cuticle. Main physiological functions of the plant cuticle are described and special emphasis is given to its interaction with xenobiotics and air pollutants.

Keywords. Plant cuticle, cutin, waxes, chemical composition, xenobiotics, pollutants

14.1. The Plant Cuticle

Most epidermal cells of the aerial parts of higher plants such as leaves, fruits and non-woody stems are covered by a continuous extra-cellular membrane of soluble and polymerized lipids called the cuticle or cuticular membrane. Vascular plants have been protected by this complex mixture of molecules and biopolymers since they managed to establish themselves on dry land around 400 millions of years ago. The structure and composition of the cuticle varies largely among plants, organs, and growth stages [1] but it is basically composed by a cutin matrix with waxes embedded in (intracuticular) and deposited on (epicuticular) the surface of this matrix. In addition, remnants of the polysaccharide epidermal cell wall and trace amounts of amino acids may also be present in the plant cuticle [2]. Based on their constituents, the cuticle can be defined as a hydrophobic and non-reactive polyester with associated waxes.

Plant cuticular material occurs in large amounts in both natural and agricultural plant communities: between 180 and 1,500 kg per hectare [3], considering that the weight of an isolated cuticle ranges from 2,000 μg cm^{-2} (fruit cuticles) to 450–800 μg cm^{-2} (leaf cuticles). While the morphology and related nomenclature of the cuticle is still in dispute, most researchers agree that it is essentially a layered structure [1, 2]. In a cross section, the cuticle appears to blanket the outer epidermal cell wall. In some species, pectin material from the middle lamella is layered between the epidermal cell

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wall and the cuticular membrane. This layer can be chemically or enzimatically degraded to allow the isolation of cuticle samples. In terms of nomenclature, and from the innermost to the outermost layer, the cuticle consists of the secondary cuticle (cuticular or cutinized layer containing cell wall polysaccharides), the primary layered cuticle (cuticle proper or cuticularized layer) with embedded waxes, and the epicuticular wax layer. This model is showed in Figure 14.1. However, based on detailed morphological and microscopical descriptions, we can currently distinguish six different types of cuticles [1].

A special mixture of physical, chemical, mechanical and morphological properties give to the plant cuticle the characteristics of a unique and complex biopolymer. From a physiological point of view, the main function ascribed to the cuticle is to minimize water loss [4, 5]. However, from a more general point of view, this role in the regulation of plant water is accompanied by other important functions: the cuticle limits the loss of substances from plant internal tissues and also protects the plant against physical, chemical, and biological aggressions. In this sense, the cuticle has been well characterized for its role in gas exchange, as a lipophilic sorption compartment, and in protecting against mechanical, UV irradiation damage, and herbivore and pathogen attack [4, 6].

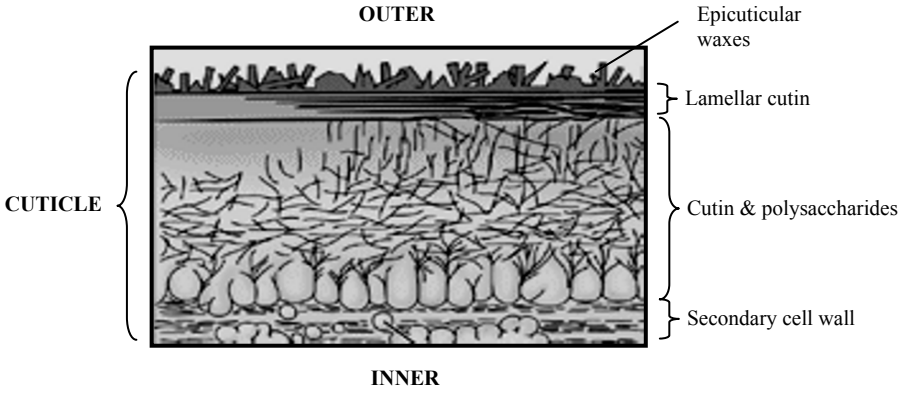

Figure 14.1. Diagram of a mature plant cuticle indicating the different cuticular components. Taken from [1]

14.2. Cuticular Waxes

Cuticular waxes is a general term that describes complex mixtures of homologue series of long chain aliphatics like alkanes, alcohols, alhehydes, fatty acids and esters, with the addition of varying proportions of cyclic compounds like triterpenoids and hydroxycinnamic acid derivatives [7, 8]. Table 14.1 summarizes the main different classes of waxes present in most plant cuticles, together with detailed information on their chemical structure.

The main function ascribed to waxes is to limit the diffusional flow of water and solutes across the cuticle. Composition, structure, and degree of crystallinity of epicuticular waxes determine the important property of the wettability of plant surfaces. On the other hand, cuticular waxes may also contribute to the attenuation of radiation, both photosynthetically active and ultraviolet radiations [9]. Understanding the role that cuticular waxes play in any of these functions needs a deep insight into their chemical composition and its relation with their physical structure. This is a critical aspect that deserves further research.

Table 14.1. Cuticular waxes components

Component	Chemical structure	Range	Main constituents	Species
Hydrocarbons	$CH_3(CH_2)_nCH_3$	*C_{21}–C_{35}*	*C_{29}, C_{31}*	*Almost all*
Ketones	R_1COR_2	C_{25}–C_{33}	C_{29}, C_{31}	*Brassica, Rosaceae, Leptochloa digitata*
Secondary alcohols	$R_1CH(OH)R_2$	*C_{29}–C_{33}*	C_{29}, C_{31}	*Pisum sativum, Brassica, Rosaceae, Malus*
β-diketones	$R_1COCH_2COR_2$	*C_{27}–C_{33}*	C_{29}, C_{31}, C_{33}	*Eucaliptus, Poa colenasia*
Monoesters	R_1COOR_2	C_{30}–C_{60}	C_{44}, C_{46}, C_{48}, C_{50}	Almost all
Poliesters		*M_r 800–1500*		Gimnosperms
Primary alcohols	RCH_2OH	*C_{12}–C_{36}*	C_{26}, C_{28}	*Almost all*
Aldehydes	RCHO	C_{14}–C_{34}	C_{24}, C_{26}, C_{28}	Vitis, Malus
Carboxylic acids	RCOOH	C_{12}–C_{36}	C_{24}, C_{26}, C_{28}	*Almost all*
Terpenes and steroids			Ursolic and oleanolic acid, betuline	*Vitis, Solanum*

14.3. Cutin

Cutin is the major constituent (between 40–80% of dry weight) of the cuticle and, from a chemical point of view, is defined as a polymeric network of oxygenated C_{16} and C_{18} fatty acids cross-linked by ester bonds [7, 10]. Cutin can be depolymerized by cleavage of these ester bonds by alkaline hydrolysis, transesterification and other methods [7, 11]. Any of these chemical methods yield monomers and/or their derivatives depending on the reagent employed. The 9- or the 10,16-dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acid are the major components of the C_{16} cutins. Only in some cases 16-hydroxy-10-oxo-C_{16} acid and 16-oxo-9 or 10-hydroxy C_{16} acid are main monomers. Major components of the C_{18} family of monomers are 18-hydroxy-9,10-epoxyoctadecaonoic acid and 9,10,18-trihydroxyoctadecanoic acid together with their monounsaturated homologues (Figure 14.2).

A suite of physical, chemical, and morphological properties gives the plant cutin the characteristics of a unique and complex biopolymer. These properties have been recently summarized and discussed in a comprehensive review [12]. Cutin is an amorphous and insoluble polymer; it shows very low water permeability, high specific heat and glass transition events within the range of physiological or ambient temperatures. Moreover, cutin shows interesting rheological properties: it can be considered as a viscoelastic polymer network. The interaction of these physical properties gives the plant cutin an interesting set of characteristics. Thus, isolated plant cuticles and cutins from several species showed significantly high specific heat [12]. This high value means that the cuticular material requires a greater amount of heat to raise its temperature one degree. Specific heat value of cutin is around 2–2.5 J K^{-1} g^{-1}, while cellulose, the main component of plant cell wall, has a specific heat of 1.5 J K^{-1} g^{-1}.

Family C_{16}

CH_3-$(CH_2)_{14}$-COOH

CH_2OH-$(CH_2)_{14}$-COOH

CH_2OH-$(CH_2)_x$-CHOH-$(CH_2)_y$-COOH

x, y = 6, 7; x + y = 13

Dicots

Family C_{18}

CH_3-$(CH_2)_{16}$-COOH

CH_2OH-$(CH_2)_{16}$-COOH

CH_2OH-$(CH_2)_7$-CH-CH-$(CH_2)_7$-COOH

CH_2OH-$(CH_2)_x$-CHOH-$(CH_2)_y$-COOH

x, y = 7; x + y = 15

Monocots and Gimnosperms

Figure 14.2. Chemical structure of the most common fatty acids present as building monomers in the plant biopolyester cutin

Although the cuticular material contributes only as a minor mass fraction to the whole leaves and fruits, it could play an important role as a thermoregulator in the course of the biophysical interaction between the plant and the environment. We are now starting to appreciate the complex physics, chemistry, and biology of this biopolymer that allows plants to minimize water loss while supplying them with a powerful, resistant yet flexible chemical barrier just at the interface with the atmosphere.

14.4. The Cuticle as an Interphase Between the Plant and the Atmosphere: The Partitioning of Xenobiotics

The plant cuticle can also be considered a sink for both lipophilic and water soluble materials. Xenobiotics, pollutants, or any other compound may arrive at the plant surface in the vapor phase, dissolved in droplets or in particulate form. If we consider that the surface area of the above ground parts of higher plants usually exceeds by far the area of the whole plant, it is particularly interesting to evaluate the amount of any compound of reference in the several compartments present in the plant surface.

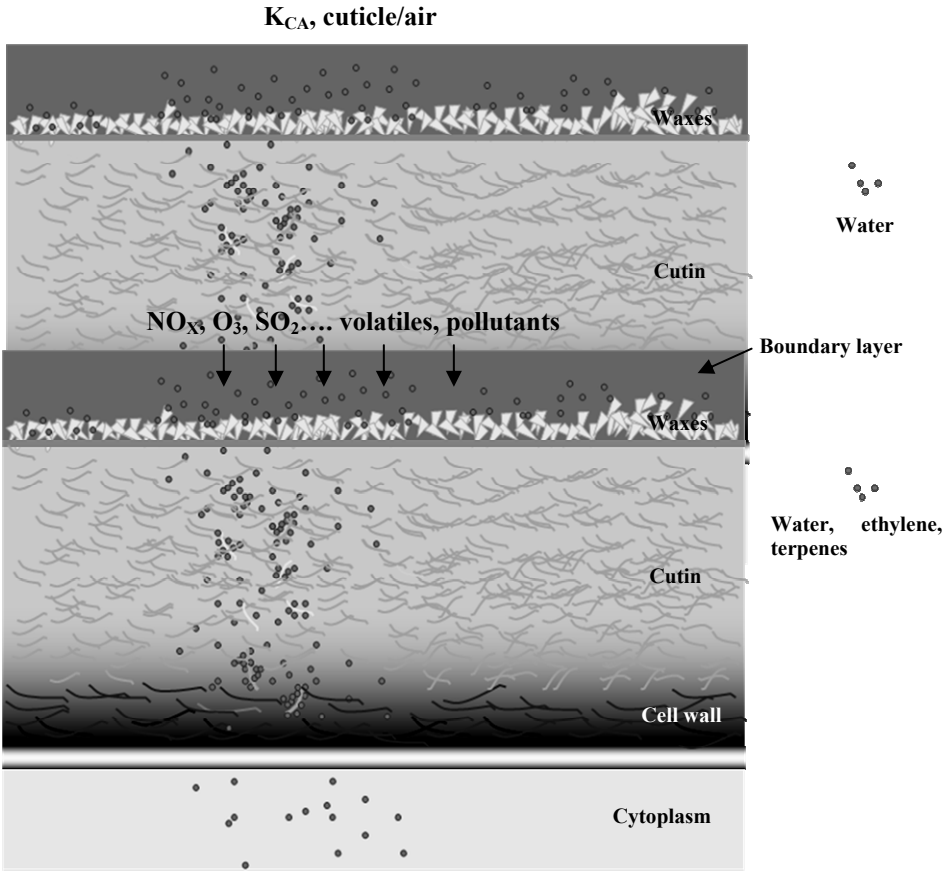

Figure 14.3. Cuticle diagram showing some compartments included in the equilibrium partitioning model for the atmosphere/leaf system (**a**) and its interaction with selected pollutants (**b**). For more details, see text

The partitioning of lipophilic organic material, the most abundant class of xenobiotics, between the atmospheric environment, the cuticle surface, and inside the leaf can be described using a model system of different compartments. A simplified model is represented in Figure 14.3a. In this scenario, equilibrium partitioning between the two different leaf compartments showed in this figure can be described using partition coefficients (K): K_{CW} denotes the distribution of any compound between the cuticle and water, whereas K_{CA} indicates its distribution between the cuticle and the air. Air can be considered as the atmosphere and water as the water solution present in the cell wall and cytoplasm. Partition coefficients at 25ºC for some reference compounds are given in Table 14.2. Data from this table indicate that the relative importance of the aqueous and the cuticle phase depends on the polarity of the compound; almost all the methanol can be found in the aqueous phase, while the more non-polar compounds are dissolved in the lipophilic compartments of the cuticle. The table clearly shows that the plant

cuticle is an important compartment that accumulates persistent lipophilic compounds from the environment. The values of K_{CA} follow the same trend, although it should not be forgotten the singular composition of the atmosphere and that these values have been obtained from the corresponding vapor-solid sorption isotherms. The K_{CA} values demonstrate that the cuticle is highly favored in the air/cuticle partitioning and thus can be expected to effectively scavenge lipophilic molecules for the surrounding air. Even polar compounds like methanol accumulate a hundred times more in the plant cuticle compared to the adjacent atmosphere [6, 13].

Table 14.2. Partition coefficients (25ºC) for the distribution of reference compounds between different plant compartments: the plant cuticle and water (KCW) and the cuticle and the atmosphere (KCA)

Compound	$\log K_{CW}$	$\log K_{CA}$
Methanol	−1.15	2.70
Phenol	1.51	5.93
2-Nitrophenol	1.84	5.13
2,4-Dinitrophenol	2.47	6.12
2,4,5-Trinitrophenol	3.13	8.76
Perylene	6.45	10.20

14.5. The Effects of Xenobiotics and Pollutants on Plant Cuticle Components

The cuticle plays a major role in the interaction of the plant with atmosphere pollutants, mostly of antropogenic nature [13]. These pollutants can degrade the cuticle and their components and, in doing so, alter its function as a protective barrier (Figure 14.3b). They can be classified in: antropogenic (SO_2, NO_x, CO_2, fluorine derivatives and O_3), biogenic (CO_2, water ethylene, isoprene …) and compounds derived from the biological reduction of pollutants (H_2S from SO_2, and NH_3 from NO_x).

Air contaminants and other gases are temporarily restricted to the interface between the plant and the environment. In this region three different phases coexist. First a liquid phase, due to water deposition, that wets the cuticle surface. This water can bind polar groups of the cutin or form molecular clusters inside the cuticle. This phase mainly acts facilitating the entrance of soluble pollutants, although chemical reactions can also happen. For example, NH_3 and SO_2-derived ammonia salts, strong mineral acids and reactive oxygen species such as hydrogen peroxide and hydroxile radicals can be generated. The second phase is of lipid nature, basically constituted by cuticular waxes and cutin. This lipid phase acts as a sink of the organic pollutants that are accumulated on the epicuticular waxes. Several chemical reactions have been described to happen. Sulfuric acid produced in the liquid phase can react with the hydroxile groups of long chain primary and secondary alcohols present in conifer waxes. Also, ozone can interact with parafins of epicuticular waxes, and NO_x have been described to accumulate in the cutin matrix of some species. Nitrogen oxides can react with waxes causing erosion of the crystalline structures and therefore modifying their water retention capability. Finally, the gaseous phase is localized *sensu stricto* in the interface between the

cuticle and the environment. Within this region the production of strong oxidants derived from the reaction between NO_x and atmospheric O_2 has been observed. It should not be forgotten the plant response leaching polar substances from the leaf to the cuticle surface as a consequence of acid precipitation, for example. These events can result in increase damage to the plant due to the loss of nutrients.

To study the interaction of pollutants with the plant cuticle will allow us to understand how these compounds enter the plant, which factors modify the rate of penetration (temperature, relative humidity) and on which stage of development plants are more prone to be affected by these pollutants. Moreover, cuticle degradation would not only facilitate the ulterior diffusion of other pollutants but will also assist in pathogen invasion and herbivore attack as well as modify pest dynamics. Once these pollutants have entered the cuticle they could modify the cellular metabolism [9].

Looking backwards, our current information on plant cuticle and cutin has been slowly obtained over the past 3 decades, thanks to the efforts of a limited number of research groups. We now have a model of plant cuticle that can partially explain the fascinating and complex properties (permeability, flexibility, and resistance to degradation) that are combined in this complex and composite barrier. Despite this, many questions remain unanswered, especially those regarding the link between cuticle composition, stage of plant development and how its modification (chemical, structural, etc.) can affect plant survival. The challenge today is to use the best of the new molecular and biophysical tools to find appropriate answers to a basic physiological question: what is the relationship between cutin composition, structure and cuticular functions?

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Chapter 15

New Approaches to Treatment of Poisoning by Soman

Tonkopii V.
Institute of Limnology, Russian Academy of Sciences, Russian Federation

Abstract. The acute toxicity of organophosphates (OPs) in mammals is primarily due to their irreversible inhibition of acetylcholinesterase in the nervous system which leads to increased synaptic acetylcholine levels. However, the toxic effect of some OPs is not limited to inhibition of cholinesterase: following the cholinergic crisis changes in non-cholinergic neurotoxic parameters, such as specific damage to cell membranes, are observed. One of the major problems in assessing the role of lipid peroxydation in any chemical toxicity is to resolve whether this pathogenic cascade is a cause or a consequence of damage. The present study was undertaken to elucidate the relations between lipid peroxidation, OPs toxicity and delayed, long lasting, non-cholinergic changes. We studied the influence of OP intoxication on lipid peroxidation in rat cerebral hemispheres. The level of lipid peroxidation was measured as the amount of common phospholipids, peroxidate lipids and malondialdehyde (MDA) in reaction with thiobarbituric acid. Results were compared to those with pre-treatment with atropine and reversible cholinesterase inhibitor – galanthamine alone or together with different antioxidants. OPs caused a rapid, dose-dependent increase of peroxidate lipids and MDA 15–30 days after intoxication. The level of lipid peroxidation correlated with the rate of conditioned reflex reaction. With paraoxon and sarin pre-treatment with atropine and galanthamine totally prevents the all symptoms of intoxication and changes in lipid peroxidation. Comparatively such type of prophylaxis in soman poisoned rats didn't normalize the biochemical and physiological parameters. The protective effect of antioxidants against soman – induced lipid peroxidation was shown. Therefore soman – associated lipid peroxidation is likely to arise mainly as a primary change which may, however, play a significant role in delayed neurotoxicity and conditioned reflex activity.

Keywords. Soman, antioxidants, lipid peroxidation

15.1. Introduction

Organophosphates (OPs) are widely used as pesticides and many have thus become environmental contaminants. In additional some OPs, like sarin, soman, Vx, tabun, are important warfare agents. Although their production and testing have been forbidden by international agreements, their manufacture still continues in some countries. In the treatment of OPs poisonings, the combination of muscarinic cholinoreceptors antagonists like atropine and some oximes (obidoxime, pralidoxime, HI-6 etc.) has been used [1]. The efficiency of oximes is not, however, satisfactory in the case of soman, cyclosarin and

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tabun poisonings, on account of the rapid dealkylation ("aging") of acetylcholinesterase (AChE). The resulting methylphosphonyl-AChE resistant against the nucleophilic attack of oximes [2, 3]. It was found that pretreatment with certain cholinesterase reversible inhibitors like physostigmine in conjunction with atropine and oximes, gave appreciable protection against poisonings by any OPs, including soman [4, 5]. Nevertheless the delayed neurotoxic effect was shown in animals poisoned by soman even after usage of different effective OPs antagonists.

The acute toxicity of organophosphates in mammals is primarily due to their irreversible inhibition of acetylcholinesterase in the nervous system which leads to increased synaptic acetylcholine levels. However, mechanism of toxic effect of some OPs is not limited to inhibition of cholinesterase: following the cholinergic crisis there are observed changes of non-cholinergic neurotoxic parameters like specific damage of cell's membranes [6]. One of the major problems in assessing the role of lipid peroxidation in any chemical toxicity is to resolve whether this pathogenetic cascade is a cause or a consequence of damage [7, 8].

Lipid peroxidation is the reaction of oxidative deterioration of polyunsaturated lipids. Peroxidation involves the direct reaction of oxygen and lipid to form free radicals. The latter, produced during lipid peroxidation, are similar to the chemically damaging radicals emanated by radiation. Tissues most susceptible to lipid – peroxidation appear to be those with low mitotic rates such as a brain. It has also been reported by different investigators that of the different tissues from the normal rat, the brain showed a considerably higher rate of lipid peroxidation than liver, kidney, spleen and heart homogenates [9].

15.2. Materials and Methods

Male albino rats weighting 150–200 g (60–90 days old) were used throughout this study. They received a pellet diet and water until the time sacrifice. We studied the influence of OPs intoxication (1.25–1.75 DL_{50}) by paraoxon, sarin and soman on lipid peroxidation in rat's cerebral hemispheres. The level of lipid peroxidation was measured as the amount of common phospholipids, peroxidate lipids and as amount of malondialdehyde (MDA) in reaction with thiobarbituric acid [10, 11]. Except the level of lipid peroxidation was also analysed by chemoluminescence method [12]. Results were compared to those with pre-treatment with atropine (20 mg/kg) and reversible cholinesterase inhibitor – galanthamine (8 mg/kg). The influence of different antioxidants (a-tocopherol and oxymetacyl) on lipid peroxidation and toxicity of OPs was estimated also. The rate of reaction of conditioned reflex of active avoidance was measured. As a conditional stimulus was switching of electric lamp, as nonconditional stimulus was the irritation of skin by the current.

15.3. Results

As shown in Table 15.1 MDA lipids contents in rat's cerebral hemisphere were markedly increased 1.5 h after poisoning of paraoxon, sarin and soman, as compared to normal values.

The results indicated a rapid, dose-dependent increase of peroxidate lipids and MDA and decrease of common phospholipids in rat's brain during 15–30 days after injection of soman (1 LD_{50}). The high degree of correlation between the level of MDA and common phospholipids (0.89) and between the MDA and the level of conditional reflex of active avoidance (0.93) in rats poisoned by soman was shown.

Pre-treatment of rats with acute poisonings of paraoxon and sarin by atropine and galanthamine totally prevents the all symptoms of intoxication and changes in lipid peroxidation and in conditioned reflex reaction. Comparatively such type of prophylaxis in soman poisoned rats didn't normalize the biochemical and physiological parameters. The protective effect of antioxidants (a-tocopherol and oxymetacyl) against soman – induced lipid peroxidation and conditioned reflex reaction was shown (Table 15.2). In experiments on rats, cats and dogs poisoned by soman it was found the increase of efficiency of prophylactic antidotes in the presence of different antioxidants. The antioxidant oxymetacyl as a component of antidote normalized the rate of lipid peroxidation in cat's cortex during 24 h after injection of soman (1.0–1.5 LD_{50}).

Table 15.1. Content of malondialdehyde (MDA) in rat's cerebral hemisphere after injection of OPs

Experiments	Dose (mg/kg)	MDA (nm/1g tissue)
Control	-	7.02±0.16
Paraoxon	0.75	8.2±0.15
Sarin	0.140	9.59±0.20
Soman	0.055	8.33±0.20
Soman	0.075	11.50±0.15
Soman	0.110	12.16±0.61

Table 15.2. The rate of reaction of active avoidance of conditioned reflex in rats after 5 days of poisoning by soman (1.75 DL_{50})

Experiments	Dose of drugs (mg/kg)	Quantity of stimulus combinations
Control	-	8.0±1.0
Soman Atropine + galanthamine	0.140 20.0 8.0	21.0±3.0
Soman α-tocopherol + atropine + galanthamine	0.140 15.0 20.0 8.0	5.1±1.0
Soman Oxymetacyl + atropine + galanthamine	0.140 10.0 20.0 8.0	7.5±2.0

15.4. Conclusions

Taken together, the present data show that the increase of lipid peroxidation in rat's brain poisoned by OPs made due with direct (soman) prooxidant action or non-direct (seasures, hypoxia) prooxidant action of OPs type of paraoxon and sarin. The protective

effect of antioxidants against soman – induced lipid peroxidation appears to result mainly as a primary change, which may play a significant role in delayed neurotoxicity and conditioned reflex activity.

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Chapter 16

Bioterrorism – Risk and Threat: The Misuse of Science

Ristanovic E.
Military Medical Academy, Serbia

Abstract. Bioterrorism represents a global threat caused by the use of microorganisms or their toxins with the purpose of causing death or a disease in humans, animals or plants. Compared to the very complex steps needed to produce some Weapons of Mass Effect, BW can be relatively easy to produce either on a very small scale or on a mass production scale. The technology and equipment can range from very simple to extremely complex. One of the difficulties in detecting and intercepting biological organisms and equipment needed to make biological WMD is the dual use aspect of biological commodities. The Centers for Disease Control and Prevention (CDC) has categorized bioterrorism threat agents in three classes based on their transmissibility, mortality, public health impact and response requirements. The rapid development of molecular biology and gene technology ensures development of new tools for the creation of genetically modified agents. The aim of this study is to address all aspects of biothreat agents and our concern with relevant examples. We will discuss bio-weapons proliferation related steps, identification, detection and diagnostic methods, biosafety measures, regulations, international laws and agreements in this field.

Keywords. Bioterrorism, microorganisms, gene-technology, dual-use

16.1. Biological Weapons and Bioterrorism: The Past, Present and Future

The main topic of this paper concerning the use of microorganisms as biological warfare agents and bioterrorism as global and serious threat.

Otherwise the various microorganisms we can see everyday in microbiological laboratories all over the world can be used for the improvement of diagnostics, vaccine development, in industry or misused as dangerous weapons in the hands of terrorists (Figure 16.1). So it is the dual use of microorganisms and the commodities used for their production, identification, detection and weaponization. Dual use is the problem that is considering seriously as a part of global joint bio-threat preparedness.

The term **biological weapons (BWs)** implies microorganisms or their products, regardless of their origin or production manner, that are not used for prophylactic, protective or any other peaceful purpose, as well the weapons, facilities or the dissemination pathways [1].

And what are the clues suggesting the biological agents release in some region? Clustering of morbidity and mortality in time and space, the appearance of unusual symptoms, unususal age distribution and occuring of disease outside typical seasone.

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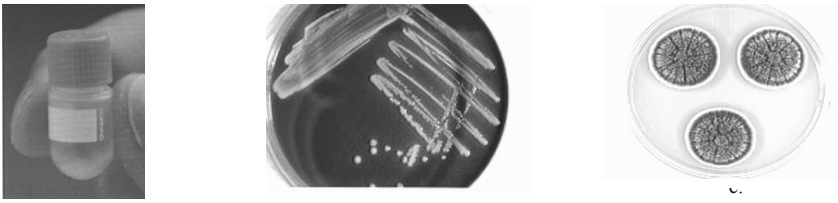

Figure 16.1. Various microorganisms -potential biological weapons (a-viruses, b-bacteria, c-fungi)

Bioterrorism presents the use of microorganisms or toxins derived from living organisms in order to cause death or disease in humans, animals or plants.

The history of BWs is as old as the human society. I suppose all of you had the opportunity to read about the spreading of the plague in the fourteenth century, the importation of variola virus in the Northern America with the infected blankets as the humanitarian aid, or about the Japanese Unit 731 and its horrible experiments during the Second World War [2]. The development of molecular biology and genetic engineering enabled the scientists to play a role of the God and provoked deep worries of the human beings.

The use of BWs presents great and real challenge because of:

1. Relatively easy and cheap production (the same effect-full destruction of all living beings at the 1 km^2 could be achieved also by conventional weapons and would cost about 2,000 USD, by chemical weapons costing about 800 USD, radiological weapons (cost only 600USD), and **BWs only -1 USD**) [1];
2. The high efficiency (equivalent to hydrogene bomb);
3. High specificity (for humans, animal and/or plants without destruction of infra-structure or other damages).

It is also worth mentioning the panic, feature, political damages and unstability, uncertainity provoked by BWs or bioterroristic actions as well the problems in the function of medical services connected to the having of well-trained first-responding teams, modern diagnostic and identification molecular tools (such as RAPID cycler PCR etc.) the existence of stockpiling or the strategies for the prevention, prophylaxis ant treatment of the victims/wounded or ill/and exposed persons?

The convention of Prohibition of BW was proclaimed in 1972 and ratified by the most countries [3]. But are all of them honest in their intentions? The UN resolution 1540 also concerns the prevention of proliferation of weapons of mass destruction including chemicals, biologicals, nuclears, and radiologicals.

The development of molecular biology, genetic engineering and biotechnology makes a new point of view considering the misuse of science in order to produce more efficient, hard-detectable and high dangerous biological weapons modifying the micro-organisms and making the detection and diagnostic procedures uneffective.

16.2. Classification of Potential BW Agents

The classification of potential BWs presents real problem because nowadays almost each microorganism can be used as bioweapon. Anyway, Centers for disease control

and prevention (CDC) from Atlanta, Geogia classify potential BW agents in three categories [4].

To the **Class A** belong the agents that provoke serious diseases causing high morbidity and mortality rates and subsequent problems in human population such as the causative agents of anthrax, plague, tularemia, variola, viral hemorrhagic fevers (Ebolla, Marburg, Lassa, Junin) etc.

To the **class B** of potential Biological weapons belong the so called agents of high-morbidity and low mortality rates such as the causative agents of brucellosis, glanders, Q-fever, typhus, many toxins as well the food and water-safety threats.

To **class C** of potential BW belong the agents that according to its characteristics in future could be used as BWs (Nipahvirus, hantaviruses, multiresistant *M. tyberculosis* etc.)

The last use of class A agent in bioterroristic actions was registered in 2001. It was the so called **anthrax campaign** (Figure 16.2). In the USA 22 persons got sick and among them were 5 with fatal outcome [5]. All bacterial strains isolated from various biological materials were identical; it was the Ames strain that was usually used for laboratory examinations in Fort Detrick, US and in Porton Down, UK [6]. Also, the powders used in bioterroristic acts were prepared professionally and contained even one trillion spores in the 1 g of the substance.

a. b.

Figure 16.2. Anthrax campaign in 2001. a- skin anthrax b- new way of transmission: letters fulfilled with powder

Tularemia is the zoonotic disease caused by the bacterium *Francisella tularensis.* The reservoirs of bacteria are the rodents and it can be transmitted by ticks. *F.tularensis* can be also used as potential BW. Disease is widely distributed in the northern hemisphere and endemic in the former Yugoslavia. Tularemia often appears during the wartimes (from Stalingrads battle to the crashes in the ex-Yugoslavia-Croatia, Bosnia and Herzegovina, southern Serbia and the province Kosovo and Metohija) that can be explained by bad hygienic conditions, although it was also confirmed the use of tularemia as bioweapon in the First and the Second World War [7].

Brucellosis is also zoonotic disease widely distributed in the southern-east Europe including our country and provoked by bacteria of the genus *Brucella*. It is worth mentioning that *Brucella suis* was the first weaponized microbiological agent.

And I will also mention some the examples showing the use of some class B agents in terrorist actions. So, in 1984 the members of the Rajneesh cult in Dales, Oregon contaminated the salad bars with *S. typhimurium*. The goal was to incapacitate the voters at the local elections. In 1993 in Milwaukee, USA-municipal water supply was contaminated with *Cryptosporidium parvum* that provoked gastroenteritis in 40,000 citizens.

From the C class I will only mention **hantaviruses**, causative agents of the hemorrhagic fever with the renal syndrome (HFRS) that are widely distributed in the former Yugoslavia, especially in Serbia with the provinces, Macedonia, Montenegro and Bosnia and Herzegovina where we registered great-outbreak in 1995. during the civil war, with 3,000 ill persons and 481 serologically confirmed cases. The new serotypes of the hantaviruses Belgrade and Dobrava were isolated in the area of the ex-Yugoslavia. The severe forms of disease are present in the Serbia, Montenegro and Macedonia, moderate forms in Slovenia and Croatia while in Bosnia and Herzegovina appears severe and moderate forms in the equal ratio. The last HFRS outbreak appeared 3 years ago in the southern Serbia [8].

So, the microorganisms classified as BWs are widely distributed around us, that makes the job of potential bioterrorists much easier: everything can looks like a natural outbreak.

16.3. Bioweapons and the Misuse of Science

In the past the use of BW were directed to the copying the nature. The development of science makes possible the change of the strategy in order to modify the nature through biotechnology and to overcome the problems and disadvantages in the use of bioweapons such as difficulties in managing and diffusing, boomerang effects and bad efficiency.

So new bioweapons including modified viruses, designed diseases, new agro-diseases, livestock raids or repro-blockers are easier to manage, easier to diffuse, would have low boomerang effect and higher efficiency in comparison to the simple microorganisms used earlier as BWs.

The progress in the molecular genetic makes possible the increasing the virulence of the pathogens as well the resistance to antibiotics (by simple inserting of the resistance genes in the genomes of *E. coli*, *Y. pestis* and the other microbes), vaccines and therapies, resistance to the environmental conditions (UV, heat, cold) or the alterations of the microorganisms in order to defeat identification, detection or diagnostic exchanging gene sequence [9, 10].

Sequencing of the genomes makes possible *in vitro* synthesis of all the pathogens including i.e. Ebola virus, smallpoxvirus, poliovirus [11]. So, we can ask the serious question is any disease on the planet eradicated? The scientists play the role of the God so made the terrible mixture of Ebola virus and smallpoxvirus and named this monster as Ebollapox. It is also worth mentioning the so called *gene-shuffling* process based on the destruction on the genomes by enzymes followed by stochastic recomposition [12].

In the next period we can expect that potential BWs will target the immune system so that benign diseases will become deadly, or even producing of the bioweapons specific for some races, nations etc. using the gene specifities.

So, while the *physics had main role in the development of WMD in the twentieth century, in the twenty-first century molecular biology will be the most important in this field!*

16.4. Instead of Conclusion

On the end, we can repeat the most important remarks of this paper: first of all, the new technologies lead to a better understanding of life that could lead to new biological weapons and serious global threat that must be avoided by co-ordinated actions at the international level that would include the education of the scientific communities and increased safety of databases, increased support to the BTW convention, increased participation to arms and trades control at all levels, increased intelligence and early warning, real response strategies and policies as well the closer collaboration between countries that is also one of the purposes of this workshops.

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Chapter 17

New Methods to Detect Sulfur Mustard (SM) and SM-Induced Skin Damage

Kehe K.[1,*], Müller H.[1], Balszuweit F.[1], Steinritz D.[1], Thiermann H.[1], Klaus S.M.[2], and Kreppel H.[3]
[1]*Bundeswehr Institute of Pharmacology and Toxicology, Germany*
[2]*Securetec Detektions-Systeme AG, Germany*
[3]*Bundeswehr Medical Office, Department of CBRN Medical Defence, Germany*

Abstract. Sulfur mustard (SM) is a strong vesicant and a bifunctional alkylating agent targeting mainly nuclear DNA. By introducing several modifications, mostly in guanine and adenine, it severely damages the DNA, and consequently the cell. Because of its bifunctionality, it also creates crosslinks between guanine bases of the same or a different strand (17% of total alkylation). The high capability of SM to alkylate DNA was on one hand utilized to develop a rapid handheld diagnostic kit to identify SM on exposed skin and on the other hand to biomonitor SM DNA damaging effects on skin cells. A sensitive small size immunochromatographic test-strip system (SM-Detector) was developed, together with Securetec AG, Munich, Germany. For detection, a capture oligonucleotide was fixed on the test strip, which reacts with free SM on skin surfaces. An anti-SM-DNA-adduct antibody (TNO, The Hague, Netherlands) was used for visualisation of the SM adduct. The SM-Detector was evaluated by the Bundeswehr Medical Service in laboratory experiments and under field conditions. The latter was conducted in military training scenarios during a NATO exercise in Canada. In order to test the SM-Detector for direct measurement SM (2–200 µmol/L, 50 µL) was spread on pig skin. Another scenario included a soldier exploring a cave exposed with SM vapour. The SM-Detector was fixed to a side pocket of the individual protective suit and showed a positive reaction due to SM vapour in the air. The Comet assay is a rapid in vitro test system to detect DNA damage on a single cell level. However, SM-induced DNA damage cannot be determined using the standard Comet assay, since the alkylating agent forms crosslinks between the DNA strands, preventing migration in the electrical field. In order to improve the detection of SM-induced DNA damage, we utilized repair enzymes, for example formamido-pyrimidine-gly-cosylase (FPG), endoglycosylase III (ENDO III) and 3-methyladenine-glycosylase (AAG). These enzymes specifically recognize modifications of the DNA induced by SM and create additional strand breaks, which in turn can be detected by the Comet assay. The lowest detectable SM concentration using the modified Comet assay is 30 nmol/L when using FPG, 10 nmol/L in case of ENDO III and 30 nmol/L after AAG treatment. In summary, the handheld SM detector (SMD) is able to detect the agent, both as a vapour and on skin surfaces. The Comet assay modified by a precursory incubation with repair enzymes significantly raises the sensitivity of the detection of SM induced DNA damage in skin cells, thus allowing a biomonitoring of SM effects in skin cells below vesicating SM concentrations.

* Corresponding Author: Dr. Kai Kehe, Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstr. 11, 80937 Munich, Germany, E-mail: kai.kehe@lrz.uni-muenchen.de

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Keywords. Sulfur mustard, detection, test strip, Comet assay, biomonitoring, DNA damage

17.1. Introduction

Sulfur mustard is a strongly vesicating chemical warfare agent. It was first synthesized in the nineteenth century by Despretz. Nearly 100 years later it was extensively used during World War I, and then during the Iraq–Iran-War (1980–1988). Large quantities of SM are still stockpiled, sea-dumped and found as abounded chemical ammunition posing a continuous threat to populations [1]. Additionally, international terror organisations show activities to acquire chemical warfare agents. Thus, deployed troops are a possible target to this threat.

Exposure to SM can firstly be recognized by a more or less characteristic garlic or mustard like odor. Physical signs of intoxication typically occur several hours after contact to the agent. The most susceptible organ systems towards SM poisoning are the eyes, lungs and skin. Eye symptoms are the earliest sign of contact with SM vapour and include conjunctivitis, photophobia and blepharospasm. Pulmonary symptoms correlate with the amount of inhaled SM vapour. A symptomless period of several hours is typical after SM inhalation. Thus, an early onset of respiratory symptoms, e.g. lacrimation, rhinorrhea, loss of smell and taste, within the first hours is a critical sign and indicator. Severe exposure often results in pulmonary complications later on [2]. Skin symptoms, e.g. erythema and blistering, typically occur several hours after SM exposure. The clinical impact of cutaneous SM exposure mainly depends on three factors: skin temperature, moistness, and anatomical location. Thus, moist body areas with a thin epidermal layer (e.g. scrotum, axillae, anal region) appear to be highly sensitive to SM vapour. Eighty percent of applied SM evaporates from skin surfaces and only 20% is absorbed [3].

Of all possible targets within the epidermis the cell nucleus is regarded as the most SM sensitive cell component. Several reactions affect the DNA by forming mono- and bifunctional SM adducts. Sixty-one percent of all alkylations refer to N7 of guanine forming 7-(2-hydroxyethylthioethyl) guanine (7-HETE-G). SM concentrations of 2.3 µmol/L produce one 7-HETE-G molecule per one million nucleotides [4, 5].

The typical reaction of SM with DNA prompted our research for methods to detect SM vapour and SM-DNA adducts. A new sensitive small size immunochromatographic test-strip system (SM-Detector) based on an anti-SM-DNA adduct antibody was developed to detect SM vapour on skin surfaces, together with Securetec AG, Munich, Germany. For further analysis of SM exposed skin a Comet assay was used improved by introducing DNA repair enzymes to uncover SM-induced DNA damage.

17.2. Materials and Methods

17.2.1. Chemicals

SM and the anti-SM-DNAadduct antibody (2F8) were obtained from TNO, Rijswijk, The Netherlands. All other chemicals used were reagent-grade products obtained from Sigma (Deisenhofen, Germany).

17.2.2. Lateral Flow Technique

The test strip has an adhesive plastic backbone where different fleeces and a membrane are attached on. All materials are in capillary contact in order to allow chromatographic flow from one to the other end. At the front end (starting zone) a wicking pad is mounted on the plastic card which absorbs the chromatography buffer and constantly releases it to the rest of the test strip materials. The wicking pad is followed by the release pad which contains specific antibodies labelled with gold particles and DNA for the *in situ* reaction with SM. The release pad is in contact with the sampling device which either provides SM-DNA adducts or solely SM. The test signal is visualized by specific antibodies that are immobilized as test line on a nitrocellulose membrane.

17.2.3. Cell Culture

The HaCaT cells are spontaneously immortalized human keratinocytes and were kindly provided from Prof. Fusenig (DKFZ, Heidelberg, Germany). The cells were cultured at 37°C under a 5% CO_2 humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen life technologies) containing 5% FCS, 4,500 mg/L glucose, glutamine and sodium pyruvate [6].

17.2.5. Treatment with Sulfur Mustard

SM was dissolved in ethanol before preparing the final dilutions in DMEM. Cells were exposed to SM for 30 min. After removal of SM the cells were rinsed with PBS and subjected to further analysis.

17.2.6. Comet Assay

All experiments were done in dimmed light to prevent UV-damage during the procedure. A repeated determination was carried out for each tested SM concentration. The cells were exposed to 1 µmol/L SM and then embedded in low melting agarose and dropped on an agarose-coated slide. After cooling another layer of low melting agarose was added. The slides with the solid coats were placed in a lysis buffer at 4°C for 60 min. Afterwards, slides were rinsed three times with 1X FLARE Buffer (Trevigen, USA). Following this step, the cells were treated with a suitable dilution of the respective repair enzyme. In preliminary tests we found 2 Units FPG, 2 Units ENDO III and 0.8 Units AAG per slide, respectively, appropriate. The samples were incubated in a humidity chamber for 60 min at 37°C (90 min when treated with AAG). To unwind the DNA, the slides were immersed in an alkali solution for 30 min. Then they were put in an electrophoresis chamber and exposed to 1 V/cm for 30 min in an alkali buffer (pH > 13). After staining with ethidium bromide, cells were measured by a Axiovert fluorescence microscope (Zeiss, Jena, Germany) and "Komet 5.5" software (Kinetic Imaging Ltd., London, UK). 50 cells per slide were analyzed, receiving 100 data sets for each concentration (double determination). As seen in preliminary tests, tail length and Olive tail moment are most sensitive to show DNA damage, so these two parameters were used for quantification.

17.2.7. Statistics

Each experiment was repeated three times (n = 300 per concentration). All data were expressed as mean ± standard deviation and analyzed using two-way ANOVA. Differences were considered to be significant for $p < 0.05$.

17.3. Results

17.3.1. Antigen Generation on Test Strip

The used monoclonal antibody (2F8) only detects SM adducts at the N7-guanine. Therefore, different guanine-rich oligonucleotides were used to test the efficacy of adduct formation and subsequent adduct detection on the test strip (data not shown). One sequence was identified to be most efficient and therefore was used for the subsequent development of a SM detector (SMD) prototype. These adducts were visualized using the lateral flow technique to form a clearly visible line (Figure 17.1). The SMD was able to detect SM vapour released from a 20 µmol/L solution and from pig skin exposed with 2 µmol/L SM (50 µL), both diluted in phosphate buffered saline.

During the NATO CBRN Defense Live-Agent exercise in 2006, it was tested whether the SMD detects evaporated SM in a contaminated area. A SMD attached to the individual protective equipment of a soldier, who entered a cave contaminated with SM vapor, showed a clear positive result. It was shown that the prototype SMD can detect SM on skin and in the environment.

As the vesicating concentration of SM is estimated about 100 µmol/L and the concentrations detected were one to two orders of magnitude below that level, the SMD can provide an early warning.

17.3.2. Comet Assay with Repair Enzymes

In Figure 17.2 the effect of SM-treatment (30 min) on DNA-migration in the comet assay is demonstrated. SM-treated HaCaT cells show an increase of tail length after incubation with each of the tested repair enzymes. Treatment of the Comet slides with AAG or Endo III increased the tail lengths from 30.7 ± 6.5 to 43.2 ± 7.5 µm and 29.8 ± 4.5 to 39.8 ± 4.5 µm, respectively. In FPG treated Comet slides the tail length was increased from 18.3 ± 7.5 to 57.0 ± 22.0 µm. Preliminary experiments showed that the lowest detectable SM concentrations (volume 50 µL, each) were 30 nmol/L when using FPG, 10 nmol/L in case of ENDO III and 30 nmol/L after AAG when using the modified Comet assay.

Sham treated HaCaT cells show a tail length of 9 µm. Treatment of the Comet slides with AAG and Endo III did not significantly alter tail length. In contrast, FPG treated slides showed a significant increase from 9.0 ± 2.0 to 25.0 ± 17.0 µmol/L.

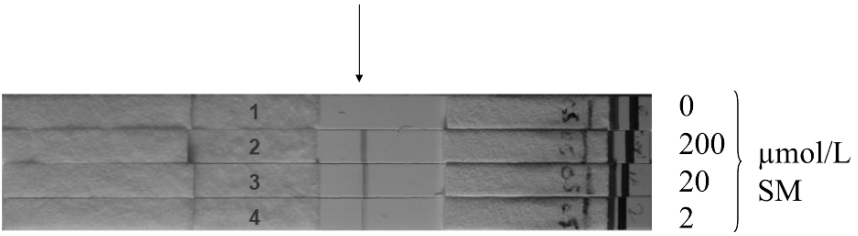

Figure 17.1. Positive result of SM on lateral flow test strip. Lowest limit of detection was 2 µmol/L (320 ng/mL) SM. Fifty microliters of sample was applied on the sample pad, which corresponds to a total amount of 0.1 nmol SM

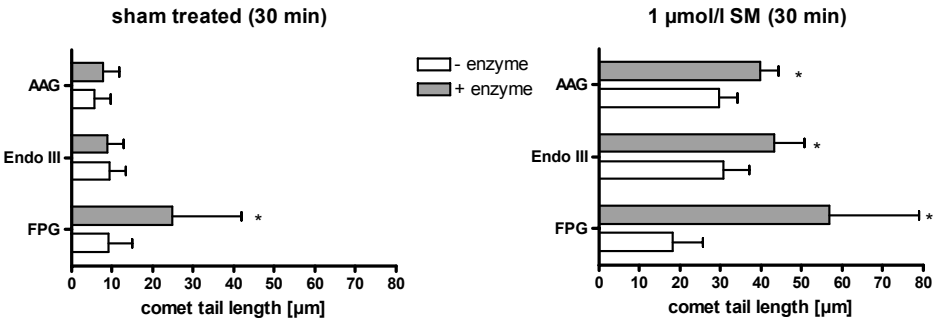

Figure 17.2. Effect of FPG, ENDO III and AAG on comet tail length in HaCaT cells treated with 1 µmol/L SM (30 min). All data were expressed as mean + standard deviation and analyzed using two-way ANOVA. Differences were considered to be significant for $p < 0.05$ (*)

17.4. Discussion

SM is one of the oldest chemical warfare agents. The last military use dates back 20 years. However, the risk of terrorist use does exist and appears to increase. Beside intentional use, accidental exposure to SM due to old ammunition, sea-dumped shells and old production facilities continues to be a threat. Medical personnel which is confronted with SM contaminated patients has the difficulty to confirm diagnosis and to estimate the severity of the poisoning. The aim of this study was to establish new methods to overcome those deficiencies.

Using a test strip format we developed a simple and rapid handheld prototype to confirm SM exposure on skin. The detection limit was 2 µmol/L SM (applied volume: 50 µL) which corresponds to 320 ng/mL SM. The vesicating concentration is reported to be greater than 100 µmol/L [7]. Thus, the SMD's detection limit is 50 times below the vesicating concentration of SM.

Matrix effects (biological samples, blood, plasma, e.g.) and cross reactivity with other chemical agents as well as nitrogen mustard might impair the use of the device. Those points however, have to be addressed in order to increase the test's robustness and usability in the field and are thus investigated at present. Furthermore, the prototype will be improved by optimisation of fleece materials, fleece pre-treatment and running buffer composition as well as sample collection and running buffer application (e.g. integrated buffer ampoule).

SM causes DNA crosslinks and monofunctional adducts. As SM crosslinks are mainly observed at concentrations greater than 10 µmol/L [8], a modified Comet assay for detection of SM concentrations below the crosslinking threshold was used. SM induced DNA damage could be detected via introduction of the DNA repair enzymes FPG, AAG and Endo III. These enzymes have been described to excise SM monoadducts from the DNA and thereby introduce DNA strand breaks which can be detected by the standard Comet assay technique [5, 9, 10]. The modified Comet assay was successful in determining the biological effects of less than 1 µmol/L SM (applied volume 50 µL). At 1 µmol/L SM, the transient cell cycle is interrupted, without showing cytotoxic effects [11]. Moreover, DNA damage following 1 µmol/L SM is reported to be repaired soon [12]. Thus, the modified Comet assay is able to detect SM induced DNA damage at concentrations that are two magnitudes lower than vesicating SM concentrations.

Oxidative damage may produce similar effects which also would be detected by use of FPG and ENDO III. In contrast, AAG does not recognize oxidative damage and seems to be most suitable in this modified Comet assay. AAG, however, detects DNA alkylation independently of the causing agent. Thus, for verification of SM exposure an additional method is needed.

17.5. Conclusion

The hand-held SM detector prototype is highly specific and sensitive for sulfur mustard, detecting vapour and contaminations on skin surfaces. Use of an improved device for the measurement of sulfur mustard in wounds is desired. Future work should enable the device to detect N-mustard and other alkylating chemicals as well.

The modified Comet assay using repair enzymes significantly raises the sensitivity of the detection of SM induced DNA damage in skin cells, allowing biomonitoring of effects from SM that are caused at concentrations far below vesicating SM concentrations.

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Chapter 18

Infections Diseases in the Context of Terrorist Threat

Andreychyn M. and Kopcha V.
I. Ya. Gorbachevsky Ternopil State Medical University, Ukraine

Abstract. From the great number of pathogenic and conventionally pathogenic for human beings microorganisms, only some of them can be used for terroristic purposes, since they should meet a lot of the relevant properties. Today priority is given to those biological agents, which have undergone successful tests, and are characterized by high index of morbidity, pathogenicity, low lethal doses, high contagiousness with short incubation period, and which result in high social and economic costs.

Keywords. Infections disease, terrorist threat

Among the great variety of microbes inhabiting our planet and coexisting with higher organisms, only some of pathogens can be used for terrorist purpose, since they should comply with certain requirements conditioned by biological, technical and economic criteria.

In accord with CDC classification [1], all potential biological factors of mass destruction are conventionally divided into three groups: A – pathogens of the highest priority (causative agents of smallpox, hemorrhagic fevers, anthrax, plague, tularemia, botulinum toxin); B – factors of medium significance (causative agents of brucellosis, equinia, Q fever, toxin *Clostridium perfringens*, staphylococcal enterotoxin, ricin); C – pathogens, value of which it is usually difficult to determine (Hanta, Nipah viruses, viruses of tick-borne encephalitis, yellow fever, poly-resistant microbacteria of tuberculosis).

18.1. Natural Smallpox

In seventeenth to eighteenth centuries pandemic of natural smallpox raged throughout Europe. At that time, about ten millions of people became ill every year, and by the end of eighteenth century at least 150 millions of people died.

After the year 1980, due to liquidation of causative agent in the whole human population, previous obligatory vaccination against this quarantine infection was stopped. In accord with WHO data [2], now virions of natural smallpox are stored only at State Research Center of Virology and Biotechnology "Vector" in Koltsovo (Russia) and Center for Disease Control and Prevention in Atlanta (the USA). All other institutions having in possession the natural smallpox virus were to eliminate the said causative agent stock in full. But no reliable information about such elimination is available.

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Besides, there is high probability of natural smallpox virus reanimation from corpses of people died from this disease in the past. It is well known that virions can survive in dry material (pustule, crust) for a long time.

For urgent preventive measures in case of bioterrorist attack, WHO has the required vaccine supplies in Switzerland. It was also reported that Israel has formed its own stock of vaccine as based on all population of the country.

Natural smallpox virus belongs to causative agents of fatal diseases, which can be used by terrorists for biological attacks. It is preconditioned by factors below:

1. High epidemic potential of causative agent. Mechanism of its transfer is very effective, and it allows to cover maximum quantity of people during one attack. It is known that when the Indian painter in 1960 has brought natural smallpox in Moscow, about 2 weeks and 25,000 medical teams were needed for total anti-smallpox vaccination, even with strong system of anti-infection protection and flash-like response. Nevertheless, 60 persons fell ill.
2. Virus is very stable in the environment.
3. People vaccinated previously have lost their immunity, and those born after 1980 have no immunity at all.
4. According to Academician L. Sandakhchiev, Director of the State Research Center of Virology and Biotechnology "Vector", the modern science knows about natural smallpox virus less than about AIDS virus, or Ebola fever. Research were terminated, vaccines are out of date, and new ones are neither developed nor investigated.
5. The old vaccine is not suitable for people, suffering from immuno-deficient diseases, and number of such persons for the last 20 years has grown considerably. Besides, it is unlikely that terrorists would use wild strains of smallpox. It's more probable they would choose genetically modified variants with much stronger harmful action, in the absence of effective immune response.
6. It is known that virus of monkey pox can be transferred from animal to human being and cause disease similar to mild case of natural smallpox, but it can pass from human to human by air as well. It was confirmed by outbreak in Zaire in 1996, when about 800 persons were ill. It is not impossible that, due to genetic recombination, much more lethal variant of smallpox occupying «vacant place» [3] will be formed.

It was calculated that 20 secondary sources at minimum (for natural smallpox) coefficient of causative agent transfer equal to 50 (real figure for present-day non-immune population is 100) could infect 1,000 persons. It will be the first wave of epidemic only. Assuming that the disease is diagnosed too late (it is proved that present-day doctors fail to diagnose natural smallpox, and real capacities of laboratories do not allow to confirm this diagnosis), the other wave will cover 20,000 infected persons, the third one – 400,000, the next – eight million persons etc. Taking into account high speeds of transport (real target for bioterrorists can be the international airport), epidemic can quickly build up into pandemic. The majority of diseased persons will die [4].

In case of using particularly pathogenic smallpox virus (for example, «India-1»), speed of its transfer and mortality rate may exceed the mentioned figures considerably [5].

Let's imagine the situation when the group of terrorists obtains samples of natural smallpox virus and organizes production of biological weapon in aerosol form. Figure 18.1 represents graphs of development of hypothetic outbreak of natural smallpox after

bioterrorist act in one-million city where 800,000 persons are not protected from causative agent, provided the terrorist act is revealed in 10 days, fact of disease is ascertained in 90% of citizens, immunity formed at 16th day after vaccination, vaccination efficiency is 75%, and vaccination covers 100,000 persons.

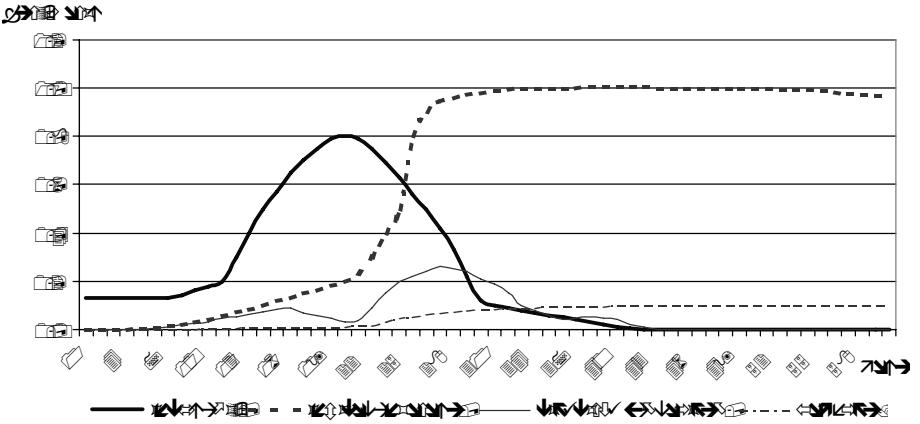

Figure 18.1. Dynamics of expected development of natural smallpox outbreak after bioterrorist act [6]

With the help of computer modeling, it was established that outbreak of smallpox would be quickly liquidated by means of anti-epidemic measures (in the most favorable conditions, when anti-epidemic team starts to act immediately upon detection of bioterrorist act itself, obtains full information, uses the most advanced diagnostic means and organizes adequate treatment of diseased people), however, peak number of infected persons would be equal to 740, and 88 people would die.

18.2. Viral Hemorrhagic Fevers

These are natural focal infections fixed to certain locality by their biological links – area of spreading of the live source and carrier. However, increasing speeds of transport and conveyance of people are accompanied by growing threat of the said diseases' spreading.

In accord with epidemiologic peculiarities, hemorrhagic fevers are divided into three groups: contagious (Lassa, Marburg, Ebola, Korean, Argentine, Bolivia) hemorrhagic fevers, tick-borne (Crimean-Congo, Omsk fever, Kayasanur forest disease), mosquito (yellow fever, Dengue, Chikungunya) fevers. All of their agents, except Dengue virus, are capable to infect people through respiratory passages or digestive tract, and can be used as a biological weapon.

Immediately after discovery of hemorrhagic fever viruses, the work on creation of military strains on the basis of these agents started. In the USSR there was a huge concern "Bio-preparat", which plants and research institutions were engaged in development of military viruses Marburg and Ebola for almost 20 years. It happened that in April 1988 microbiologist Ustinov, working on this problem, died, and highly pathogenic causative

agent of Marburg fever – virus-mutant named U – was isolated from his tissues. In 1990 this virus, together with Ebola virus, was adopted by Soviet Army. Since no effective remedies from these diseases existed, it was planned to spray Marburg and Ebola viruses over remote hostile territory with the use of ballistic missiles.

It is expected that during deliberate use of the said viruses as a biological weapon, mass incidence of disease with unnatural epidemic processes, hemorrhagic manifestations and too high morbidity rate would be observed.

Medical workers and hospitals, as a rule, are not ready to diagnose these diseases and to treat them. Consequently, inevitable companions of such biological attack would be mass panic and psychosis. In case of Dengue fever, the largest number of cases of temporary working/fighting capacity loss by people and the least number of fatal cases are expected. Le Renar, the writer describing bacteriological war before the World War II, passed an opinion that usage of this disease as a weapon would be "more humane" as compared with other diseases. During 2–4 days the diseased person suffers from severe ache in bones and joints, and then during several months, in the period of recovery, he/she will suffer from neuralgia, disorder and depression [7].

18.3. Viral Encephalitis

Disease of the central nervous system (tick-borne encephalitis and Japanese encephalitis, Western and Eastern equine encephalitis, encephalomyelitis of Western Nile, St. Louis encephalitis etc.) can be caused by viruses belonging to various species, families and taxonomic classes. The most probable bioterrorist agents can be arboviruses. In natural conditions they are spread by mosquitoes and ticks. A number of laboratories investigated these causative agents due to their relatively good ability of replication in laboratory conditions and rather simple conditions of virus storage. So, these viruses became etiological factors of many infections of laboratory personnel.

Possibility of infection spread by spraying and relatively low cost of production of a great number of viruses can be precondition of their usage for biological attack. In case of using virus aerosol, possible consequences are short incubation period, variety of diseases untypical for the given region, which are accompanied by prevalence of encephalitis or meningocephalitis phenomena in clinical presentation [7].

18.4. Severe Acute Respiratory Syndrome

"Atypical pneumonia", or Severe Acute Respiratory Syndrome – SARS, is a disease discovered not long ago. It occurred firstly in Chinese province Guangdong in the middle of November 2002. Total number of diseased reached 8,500 persons, 808 of which died. This disease is registered in 32 countries; the largest quantity thereof in China, Singapore, Canada. No SARS diseased persons are revealed in Ukraine.

"Asian positioning", circumstances and method of SARS spreading cause certain alertness, since the picture of outbreak is similar to bioterrorism model. On the other hand, infection can be spread also to initiators of this wildcat venture, and certain time is required for creation of efficient vaccine; these factors restrict bioterrorist actions.

In accord with one of versions, the causative agent has artificial origin, and it "escaped": from the laboratory where it was developed. Lessons of SARS showed that deliberate epidemic of the like nature should have serious economic, political, social and psychological impact on the society. Measures taken by epidemiologic service may conflict with adopted legal norms, or fall beyond medical or military-medical jurisdiction [8].

18.5. Q Fever

In the second half of the past century, numerous natural diseases were diagnosed in soldiers being on service in endemic regions, in particular, during the war. For example, Q fever broke out among American soldiers in 1944–1945 in Italy.

Q fever belongs to widespread zoonoses. Its causative agent is stable to natural and chemical impact, and it is easily transferred in aerosol. Minimum quantity of bacteria required for infection of human is one to ten microorganisms only. According to WHO estimates, the powder containing *C. burneti*, may cause the same rate of diseases as tularemia or anthrax. Q fever is a severe disease, but not lethal one; it makes the patient dependant on medical care for a long time, and it would have negative impact on working capacity and battle readiness of people in military environment. All that substantiates feasibility of using the microbe for bioterrorist and military purposes [9].

18.6. Anthrax

High stability of anthrax causative agent spores in the environment, possibility of aerogenic infection, high lethality in case of pulmonary form of anthrax, make this bacteria very promising factor of biological weapon.

Britain investigators during World War II conducted field tests of anthrax on Gruinard Island, near Scotland. After that, the territory of island was disinfected many times, but live spores of anthrax are found there till now. In Africa such spores were revealed in bones of animals which lived 250 years ago. Viable spores were found also in ash after burning of diseased animals' skins. In accord with official figures, occasional emission of 1–2 g of prepared powder containing anthrax bacteria from Sverdlovsk plant of biological weapons turned to be fatal for 68 persons of 79 infected ones. But according to unofficial sources, there were much more victims. Spreading of powder with anthrax bacteria by means of correspondence resulted in 22 cases of disease of Americans in autumn 2001; in 10 cases aerogenic infection took place, and 5 diseased persons died. In accord with WHO estimates, spraying of 50 kg of anthrax bacteria over five-million city would cause 2,500,000 infections, including 100,000 fatal ones. All antibiotic supplies would be consumed during 1 week, and citizens could not use such preparations. Assuming the model developed by CDC, attack with the use of anthrax bacteria would result in complete deprivation of medical care, and termination of increase in population in future years because of death of people of reproductive age [10].

However, production of such efficient anthrax powder is not a simple process, and it may be inaccessible to terrorists not equipped with advanced technologies. Infamous sect Aum Senrike sprayed anthrax bacteria in outskirts of Tokio many times without

any result. Spores themselves tend to gluing together with formation of conglomerates of such size which can not get into lungs during breathing. They can only settle down on clothes and skin of people and cause cutaneous form of disease. Should the said spores be mixed with neutral powder and sprayed in crowd or put into ventilation ducts or air conditioners, development of pulmonary form is guaranteed. Infective dose should contain at least 8,000 spores. In case of inhalation of 10,000 spores, 80% of infected persons will die. Shallow breathing, usage of respirator (or anti-dust mask) prevent from getting of such doses into respiratory passages.

It is known that taking into consideration "postal" spreading of anthrax occurred in the USA, the American government allocated $1 billion for equipping of post offices with special devices for mail disinfection by the method of radioactive irradiation.

18.7. Plague

According to historical sources, an attempt of using plague agent for mass extermination of enemy was successfully made by Tartar Mongols in 1346, during siege of Cafa (now Feodosia), the fortress on the Crimea peninsula, which was defended by Greeks. During World War II, special Japanese detachment No. 731 dropped bombs filled with plague agent on Chinese towns in Manchuria. Ishi Shiro, commander of detachment No. 731, gained the most success by using of human flea *Pulex irritans*, which survived after transportation by air, naturally fell on people, but could infect rats as well, causing continuation of epidemic. Calculations showed that it is enough to inhale 100–500 bacteria of plague for causing pulmonary form of this disease (for reference: inhalation of 1,000–10,000 anthrax spores is required for development of pulmonary anthrax).

Scientists of the USSR and the USA worked on creation of effective biological weapon of plague bacilli. Americans have not achieved considerable success; instead, Soviet developers were capable to produce large amounts of microorganisms prepared for stuffing of delivery vehicles. In this direction, over ten research institutions worked. According to K. Alibek, 1970th of the past century witnessed beginning of production of genetically modified plague bacillus, multi-resistant to all antibiotics known at that time.

In 1995 the American microbiologist-amateur *Larry Wayne Harris* purchased plague bacteria by post, having demonstrated easy access to cultures of microorganisms stored in laboratories of the USA. After this incident, the USA Congress introduced new anti-terrorist law.

It is necessary to mention results of training scenario *Topoff* in Denver Center of Arts (Colorado, the USA), developed by the management of health service in May 2001. "Terrorists" sprayed aerosol supposedly containing plague bacilli. In 2 days the medical specialists started to record "infected persons". By the end of the third day, when the epidemic of plague was "officially" confirmed, 783 patients with pulmonary form of disease were registered, and admitted to 22 city hospitals. 126 persons of them "died" during that time. On the next day, quantity of "diseased" increased to 1,871 persons, and in 1 day more – up to 3,060 persons. In accord with WHO data, spreading of 50 kg of *Yersinia pestis* over five-million city would cause 150,000 of disease cases, including 36,000 fatal cases.

According to WHO data, during the last 50 years about 1,700 cases of plague in people were recorded per year, including 84% – in bubonic form, 13% – in septic form, and 2% – in pulmonary form. These data, of course, are underestimated, since some countries inform only about plague diagnoses confirmed in laboratories (microbiologically or serologically), but it makes as little as one third of all suspected cases. A number of countries dominate in the continent's statistics. For example, in two countries only – in Madagascar and Tanzania – 62.5% of all cases in Africa, in Peru and Brasilia – 82.9% of all disease cases on American continents, and in Mongolia and Vietnam – 78.5% of Asian cases, were recorded [11].

18.8. Tularemia

No doubt, tularemia was used in the past as a biological weapon. In 1932–1945 its causative agent was investigated by Japanese department No. 731 and used in Manchuria. Ken Alibek states that epidemic of tularemia among tens of thousands of Soviet and German soldiers before and during Stalingrad battle was the result of directed usage of this microorganism on the part of the USSR. Firstly, it was runaway success: marshal Paulus, failing to reach Volga, was forced to make pause in his decisive rushing to Stalingrad. But Russian military leaders could not use this success in proper way, because of disease spread through battlefront and quick infection of military personnel of warring parties [12].

In 1950–1960th of the past century, the USA scientists developed the weapon capable to spray tularemia aerosol, and at the same time conducted approbation of vaccine and treatment schedules. Some investigations of aerosol were carried out on volunteers, predominantly, Seventh Day Adventists, avoiding military service in such a way, through their religious convictions. Live attenuated vaccine providing for partial protection prom pulmonary form of tularemia was elaborated. According to K. Alibek, in 1980th the Soviet scientists churned out the production and filled the missile warheads with bacteria. Tularemia was investigated at Bio-preparation Plant in Omutninsk (Kirov region, Russia). Development of the relevant program was continued up to nineties of the last century; it resulted in obtaining of the agent resistant to antibiotics and vaccines. The scientists succeeded in preparing transformed plasmids which upon mounting in tularemia bacteria provided their resistance to tetracycline and chloromycetin. Besides, both in the USSR and in the USA the virulent biovar of bacteria, resistant to streptomycin [13], was selected.

During bioterrorist attack, bacteria of tularemia can be used for contamination of foodstuffs. In such case, clinical implications differ from typical clinical forms and are similar to ori-nasal form. Suspicion about deliberate contamination of food can occur in absence of anamnestic data concerning consumption of fowl not subjected to veterinary examination.

But the most effective method of bioterrorist attack might be bacterial aerosol. Its spraying over densely populated area would cause a number of unusual acute diseases accompanied by fever in 3–5 day after the incident. Pneumonia, often with exudative pleuritis, will develop in many diseased persons later on. Epidemic, in its initial phase, cannot be distinguished from natural growth of incidence of influenza, other acute

respiratory viral infection, and atypical pneumonia. Suspicion might occur in case of sudden occurrence of a great number of severe diseases, quick deterioration of general condition of patients in connection with development of serious pleuro-pneumonias, including those among young people without pre-morbid background [14].

In accord with WHO estimates (1969), spreading of 50 kg of highly virulent strain *F. tularensis* over the territory with population of about five million persons might cause 250,000 cases of disease accompanied by loss of fighting/working efficiency, and result in 19,000 lethal cases. Material damage from such attack might be equal to $5.4 billions/100,000 infected persons.

18.9. Brucellosis

Brucellosis belongs to zoogenous diseases. Spreading of disease among agricultural animals may result in considerable economic costs. Therefore, this agent is of interest from eco-terrorist and military considerations. Lethality for people does not exceed 2–4%, but this disease tends to become chronic one, it causes long-term disablement and loss of working capacity, involving high costs for medical and social assistance. In the experts' opinion, the most probable form of people infection is aerosol form of *B. melitensis* i *B. suis*, being the most pathogenic one. Infective dose does not exceed 100 bacteria.

Taking into account long-term incubation period (up to 2 months) and asymptomatic disease course in the majority of patients, importance of brucellae for tactical weapons (i.e. weapons acting accurately and immediately) is minimum. They rather have strategic value (delayed effect, and hardly assessed consequences), in particular, if such attack is hidden one, because aggressor tries to avoid direct responsibility to public opinion and response strike.

Approbation of brucellae as a biological weapon in the USA started in 1942 p.; scientific investigations in this regard continued till 1969, when Americans withdrew from offensive program with the use of these bacteria. Undoubtedly, brucellae are kept in arsenals of certain countries and can be used at suitable time [7, 9].

18.10. Equnia and Pseudo-cholera

Agents of equinia and pseudo-cholera are not demanding to conditions of growing; they are easily transferred by aerogenic, alimentary and non-percutaneous channels; they get into organism of humans and animals through mucous membrane of mouth, nasal pharynx, conjunctiva and damaged skin. Their infective dose is very small (for example, aerogenic inoculation of one to ten microorganisms is lethal for hamster). Till now, no efficient vaccine from these diseases exists, and treatment with antibiotics will take a long time. So, the above diseases are considered as an important factor of biological weapons. During World War I, saboteurs used equinia for extermination of draft animals of enemy troops – horses and mules. It is considered that equinia epidemics among draft animals of Red Army at the Eastern front were connected with deliberate infection of horses by German agents. They also succeeded in infection of animals exported from

the USA. Japanese people intentionally infected the horses, civil persons and prisoners with equinia at Pinfang Institute during World War II. The USA carried out investigations of equinia causative agent in 1943–1944, but they did not include it in the arsenal of biological weapons. After World War II, the Soviet Union and the USA were suspected in carrying out works with equinia for military purpose.

Cases of pseudo-cholera were registered in French army, carrying on the war in South-East Asia in seventies of the past century, and among American soldiers in Vietnam [7, 9].

18.11. Botulism

This disease is caused by botulinum toxin belonging to the strongest biological poisons. In accord with reported data, lethal dose for human with body weight of 60 kg is 0.05 mg/kg of crystalline toxin of A type [15]. During World War II, infamous Japanese detachment No. 731 approbated the cultures of botulism bacilli on war prisoners, causing their death. There is known fact of production of botulinum toxin by American military industry during the said war. The Soviet Union tested botulinum toxin on Vozrozhdenie (Renaissance) Island in Aral Sea. According to American sources, until recently botulinum toxin for bioterrorist purposes might be used by four countries – Iraq, Iran, Northern Korea, and Syria.

With a view to reaching bioterrorist or military purposes, botulinum toxin can be sprayed. But particularly effective method is contamination of water supply sources of large cities. The main difficulty for medical services consists in timely differentiation between using of botulism as a weapon and ordinary food poisoning. A great number of diseased and unusual epidemiology of poisoning will indicate usage of botulinum toxin for terrorist purpose [16].

Terrorists are aware of huge potential of this toxin as a biological weapon. Aum Senrike Group tried to spread botulinum toxin in Tokyo and other cities of Japan, as well as at American military bases, over and over again. However, these attempts were unsuccessful, by unknown cause.

18.12. Clostridial Necro-Toxicosis

Requirements to biological weapon are met in full by endotoxins of gaseous gangrene aren't, because obtaining and cultivation of this microbe present no difficulties, and its toxins can be delivered in aerosol to the object of attack. Evidently, they can be previously introduced into foodstuffs and water by sabotage action. Aerosol use of such poison will result in disease of many people; they would suffer from severe poisoning accompanied by high lethality.

The less probable method is direct usage of vegetative form and spores of gaseous gangrene agent for bioterrorist or military purpose. But any war or man-induced catastrophe is, first of all, "traumatic epidemics". Wounds and traumas always represent potential threat of gaseous gangrene development. Such complication without immediate

intensive treatment shall cause death. Consequently, the violent act resulting in contaminated bodily injuries, creates preconditions for occurrence of the said severe complication, which might be planned beforehand.

Toxins of gaseous gangrene bacteria can be obviously used in aerosol form both independently and in combination with various kinds of biological weapons, for achieving maximum degree of enemy defeat [7].

18.13. Staphylococcal Toxicosis

Staphylococci produce up to 30 various extra-cellular substances with toxic properties. In pathogenesis of staphylococcal alimentary toxicosis, enterotoxins play the main role. Today six serovars of these enterotoxins (A, B, C, D, E, F) are known, but their quantity is supposedly larger. For bioterrorist or military purposes, enterotoxin B should be, evidently, noted, since it possesses the most thermal stability and retains its activity at temperature of +60°C during 16 h.

Staphylococcal enterotoxin used as a biological weapon is capable to cause disease of people which lasts about 1–2 weeks. Large quantities of patients demanding medical assistance can exceed capacities of medical departments.

Center for Disease Control (CDC, the USA) included staphylococcal enterotoxin to category B of hazardous biological factors [7].

18.14. Mycotoxicosis

Mycotoxins are composed of nearly 40 various proteins which are produced by fungi from Fusarium family, mainly, *F. graminearum* and *F. culmorum*. In natural conditions, these fungi affect cereals, preferably, during prolonged harvesting operations in conditions of cold rainy weather. In purified form, mycotoxins are oily substances belonging to strong inhibitors of protein synthesis, which restrain DNA formation and affect the cell wall and mitochondria. Besides, these toxins have mutagenic, teratogenic and estrogenic properties. Consumption of infected cereals poses a serious threat to health of people and animals. Clothes contaminated with mycotoxins can be the source of poisoning both for patient and for medical personnel.

Accessibility and simplicity of application of mycotoxins can promote their use for bioterrorist and military purposes. Both people and agricultural animals can become the objects of attack.

In 1930th in Kazakhstan and Siberia, and during World War II there were numerous outbreaks of alimentary-toxic aleukia, connected with consumption of bread made of crops wintered in field conditions. Similar diseases were known earlier in the countries of Western Europe and America. In 1988 in Malaysia 45 children fell ill during festival, and 13 children of them died. All of these children consumed macaroni contaminated with mycotoxins [17]. There are known cases of using the biological weapon in Cambodia, Laos and Afghanistan (so called “yellow rain”), active components of which were, probably, mycotoxins.

18.15. Ricin Poisoning

Possibility of using ricin (castor poison) as a biological weapon arises out of its general accessibility, ease of obtaining, and low cost. About 1 million tons of seeds *Ricinus communis* are processed in the world every year. Five percent of ricin remains in production waste after processing. This toxin is rather stable and very toxic for humans. It can be used for poisoning of foodstuffs, water and air (through alimentary and inhalation ways of poisoning).

This substance was many times used by terrorists [18]. The most known political crime was the murder of Bulgarian dissident Georgiy Markov in London in 1978. The killer has made ricin injection to this well-known politician with the use of needle mounted in umbrella tip. Death was instantaneous.

After aerogenic penetration of sub-lethal dose of ricin, such symptoms as fever, chest pain, cough, shortness of breath, nausea, arthralgia, and diaphoresis will appear in 4–8 h and grow sweepingly. Besides, pulmonary and heart insufficiency will develop. Lethal dose of ricin is 0.0001 mg/kg of body weight. Accordingly, the lethal dose for the child is contained in one to two seeds of ricin plant, and for adults – in ten seeds [7, 9].

As it is shown by the above facts, historic data about biological weapons and bioterrorism throw back to the past. Along with scientific and technical progress, the active factors, methods of usage and delivery were improved. Today priority is given to those biological intermediates which have successfully passed the tests, are characterized by high index of disease incidence, pathogenicity, low fatal doses, high infectivity with short incubation period, and those agents which result in high social and economic costs.

For terrorist purpose, attackers may use viruses, Coxielas, bacteria, toxins, both independently and in combination with each other, and with chemical poisons. The above biological intermediates should meet certain parameters, coupled with numerous clinical and epidemiological peculiarities of diseases, medical and preventive achievements of medicine, technological, technical and economic indices.

In our opinion, for essential improvement of the system of medical counteraction to bioterrorism, below listed high-priority measures should be taken in Ukraine:

1. Improvement of legislative base regulating introduction and effective functioning of bio-safety system, adaptation of regulatory acts to standards of EU countries.
2. Providing for periodic trainings of epidemiologists, infectiologists, workers of specialized laboratories and other medical institutions in case of attack.
3. Introduction of computer research and information systems for continuous monitoring of the environment, collective immunity of population, and selling of medical preparations through pharmacy system in all regions of Ukraine.
4. Beginning of genetic monitoring of pathogenic viruses of animals for assessment of possibility to “switch” these agents to humans, and organization of tracing the evolution of viruses pathogenic for humans.
5. Drawing up of the governmental scientific program dealing with the problem of prevention and counteraction to bioterrorism, with specification of medical aspects of such activity.

6. Inclusion of medical aspects of anti-bioterrorist protection in curriculums of higher medical educational establishments of all levels of accreditation.

7. Improvement of cooperation of Ukraine with international organizations acting against bioterrorism and usage of biological weapons in any form.

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Chapter 19

Disposal and Destruction Processes of Ammunition, Missiless and Explosives, Which Constitute Danger When Storing

Shyman L. and Ustimenko Y.
SE RIC "Pavlograd Chemical Plant", Ukraine

Abstract. In order to reduce the risk associated with the storage of abundant quantities of ammunition, missiles and explosive materials, complete technologies of their disposal and destruction have been developed at SE "RIC "PCP". These processes concern resource-recovery technologies which allow saving and returning various material resources, such as plastics, chemicals, etc., to the Ukrainian economy. NCTE, CTE, GME, HTP, RCEM processes implemented at the enterprise are characterized by more safety and minimum environmental discharge, hence they are an effective alternative to such destruction methods of ammunition, missiles and explosive materials as open burning (OB) and open detonation (OD).

Keywords. Destruction, ammunition, missiles, processes

Accumulation and long storage of ammunition, missiles and explosive materials (EM) in arsenals and military bases of Ministry of Defence (MOD) of Ukraine, which are no longer suitable for military use, is an urgent problem for Ukraine. At present according to specialists' estimates more than 1 million tons of ammunition, missiles and EM are non-demanded by MOD of Ukraine or are excessive and constitute a risk of emergency situations to be followed by drastic consequences for neighboring populated areas and industrial centres, as well as they represent danger of environment pollution.

It should be noted that accumulation and storage of great quantities of ammunition, missiles and EM in bases and arsenals also constitute a risk of unauthorized theft and provoke terrorism.

Thereby at least 50,000 t of different types of ammunition, missiles and EM, which require immediate disposal or destruction, are annually assigned unserviceable for military purposes (see Figure 19.1).

For the purpose of removal of non-demanded and excess quantity of ammunition, missiles and EM from military bases and arsenals, the works on their disposal and destruction are performed in Ukraine in the framework of Government programs, and also with the participation of International organizations.

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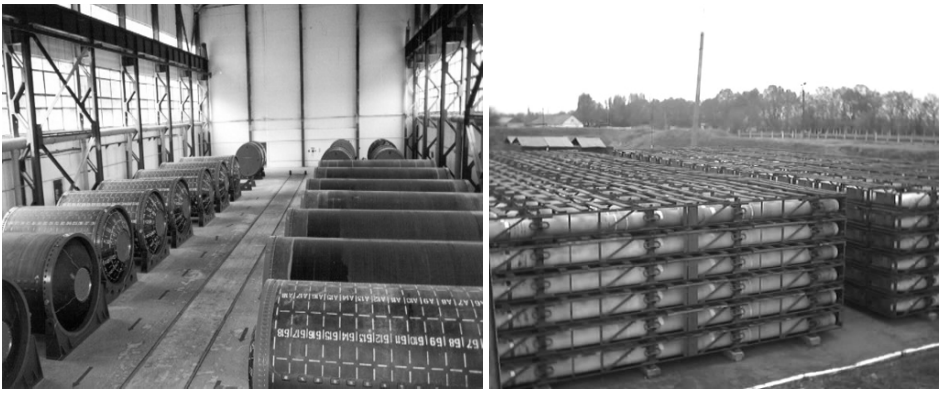

Figure 19.1. Unusable Rocket motors (**a**) and Ammunition (**b**)

SE "RIC "Pavlograd Chemical Plant" (SE RIC PCP) is one of the leading enterprises in Ukraine, which carries out the works on disposal and destruction of ammunition and anti-personnel mines, missiles and propellant, as well as different explosive materials for military purposes. Technical, research and production capacity of the enterprise has allowed developing and introducing a number (several) of complex processes for destruction and disposal, practically, of the whole assortment of ammunition, missiles, solid propellant and explosive materials.

These processes concern resource-recovery technologies, which allow saving or returning different material resources such as metals, plastics, chemicals etc. to Ukraine economics In addition these processes feature more safety and minimum environmental discharge and, accordingly, represent an effective alternative to destruction methods such as open burning (OB) and open detonation (OD) of ammunition, missiles and explosive materials. The enterprise's production facilities, supplied with resource-recovery technologies and processes, allow disposal and destruction of at least 30,000 t of ammunition and missiles loaded with different types of explosives and propellants.

SE "RIC "PCP" has put into effect the following processes: noncontact thermal extraction of EM from ammunition (NCTE process); contact thermal extraction of EM from ammunition and their elements (CTE process); high-temperature processing of ammunition, their elements and EM (HTP process); hydromechanical extraction of EM from ammunition, missiles and their elements (GME process); manufacturing of industrial explosives with using reprocessi products of EM, extracted from ammunition and missiles (RCEM process). Figure 19.2 shows the processes diagram, where the reprocessing of EM extracted from ammunition and missiles into industrial explosives for mining industry and construction (RCEM), and reprocessing of metal scrap, plastics waste, soluble chemicals for recycling in civilian products (RCP) is final stage.

NCTE process, which is shown in Figure 19.3, allows destruction of ammunition and components of missiles, torpedoes, aircraft bombs, loaded with fusible explosive materials such as TNT or its mixtures with other explosive materials. Explosive materials being extracted are reprocessed into powdery or granulate industrial explosives with using of RCEM-1 process or into industrial detonator blocks (busters) for mining operations and construction with using RCEM-2 process.

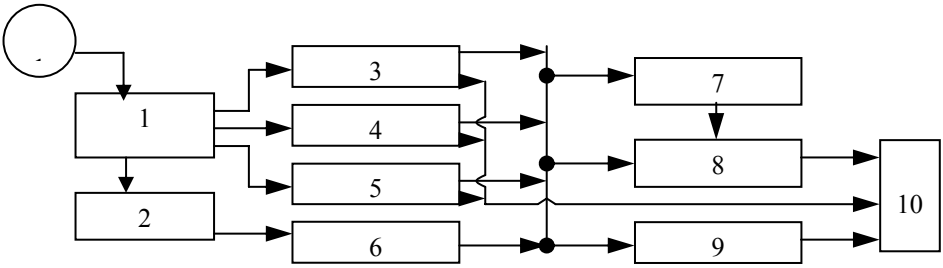

Figure 19.2. Complex processes scheme for destruction and disposal of ammunition, missiles, solid propellants and explosive materials
1 – Processes of preparation of ammunition and missiles to destruction; 2 – preparation of components of ammunition, missiles and small caliber ammunition to the destruction; 3 – NCTE processes; 4 – CTE processes; 5 – GME processes; 6 – HTP processes; 7 – off-gas cleaning; 8 – liquid waste treatment; 9 – solid waste and scrap treatment; 10 – recycling of material resources (RCP and RCEM); 11 – army bases and arsenals

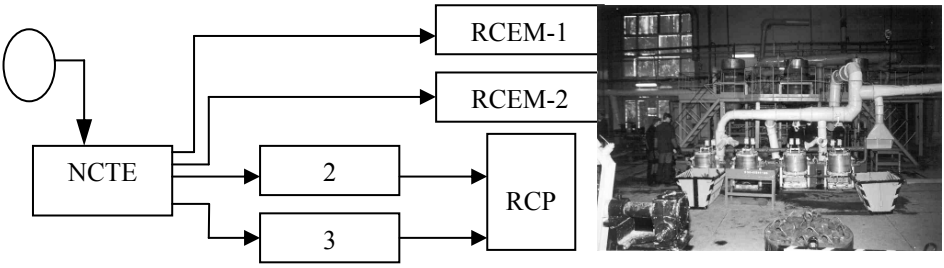

Figure 19.3. Scheme of process of destruction of ammunition and its components by non-contact thermal extraction of explosive materials (NCTE)
1 – Processes of preparation of ammunition and missiles to destruction; 2 – neutralization and treatment of liquid waste; 3 – treatment of solid waste and scrap

Process of CTE, which scheme is shown on Figure 19.4, allows destruction of ammunition and the components of missiles, torpedoes and aircraft bombs which are loaded with nonfusible heterogeneous mixtures of explosive materials, physical resistance of which are destructed from thermomechanical and jet impact, for example, RDX, HMX or their mixtures with other explosive materials. Herein, the extracted explosive materials undergo processing into granulated industrial explosives with the use of process RCEM-1 or they are processed into industrial detonator blocks (busters) and filling materials for non-electric initiating system such as NONEL for mining operations and construction works with the use of process RCEM-2.

During process of CTE when using paraffin or water-steam mixtures as process liquids for thermomechanical impact, their treatment for reuse over one cycle is required.

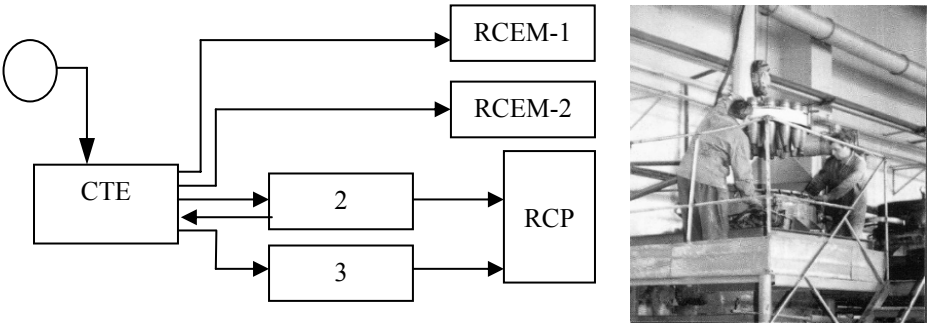

Figure 19.4. Scheme of process of destruction of ammunition and its components by contact thermal extraction of explosive materials (CTE)
1 – Processes of preparation of ammunition and missiles to disposal; 2 – neutralization and treatment of liquid waste and process liquids; 3 – treatment of solid waste and scrap

Process of HTP, which scheme is shown on Figure 19.5, allows destruction of small caliber and small-arms ammunition, land and antipersonnel mines (including PFM mines), grenades, as well as caps, fuses, tracers, detonators and other components of ammunition, missiles, torpedoes, mines and aircraft bombs which are loaded with explosive materials of different types including pyrotechnic mixtures.

Process of HTP despite its generality with regard to destruction of ammunition, missiles and their components which are loaded with nearly any type of explosive material and solid propellant, has meaningful constraints for critical mass of explosives or solid propellant contained in ammunition, missiles or their components, sent for destruction with the help of HTP. As a rule, critical mass of explosives or solid propellant for processes of HTP is limited to 2 ÷ 4 kg depending on the type of explosive material and structure of ammunition or their components.

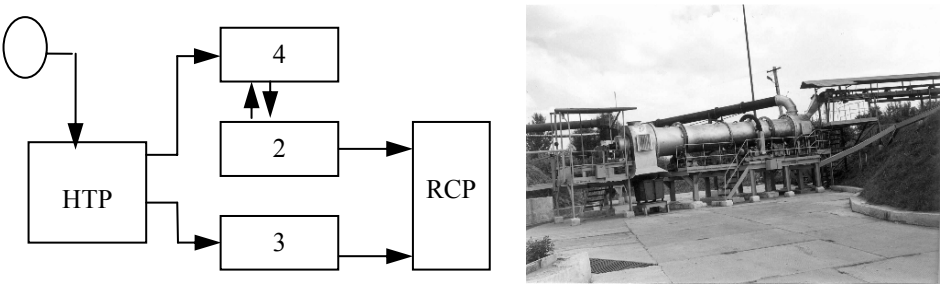

Figure 19.5. Scheme of process of destruction of ammunition, missiles and their components by their high-temperature processing (HTP)
1 – Processes of preparation of ammunition components, missiles and small caliber ammunition to disposal; 2 – neutralization and treatment of liquid waste; 3 – treatment of solid waste and scrap; 4 – treatment of gas emissions

Process of GME, which scheme is shown on Figure 19.6, permits to destruct medium and large caliber ammunition, aircraft bombs, Navy mines, rocket motors, warheads of missiles and torpedoes which are loaded with homogeneous and heterogeneous explosive materials and are unsuitable or dangerous for processes of NCTE, CTE, HTP (Figure 19.7).

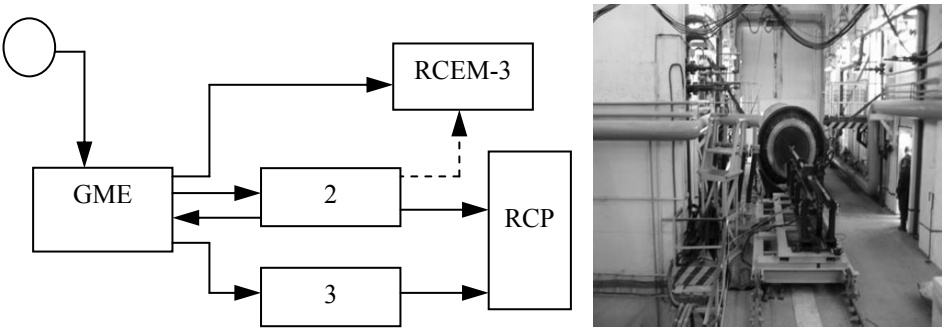

Figure 19.6. Scheme of process of destruction of ammunition, missiles and their components by hydromechanical extraction BM (GME)
1 – Processes of preparation of ammunition and missiles to disposal; 2 – neutralization and treatment of liquid waste; 3 – treatment of solid waste and scrap

Figure 19.7. Results of processes of destruction of ammunition and missiles
1 – Scrap metal for processing (RCP); 2 – mechanized loading of boreholes with emulsion explosives (RCEM)

During process of GME extracted explosive materials undergo processing into emulsion and gel water-containing industrial explosives using the processes of RCEM-3. [1, 2] Such processes allow production of emulsion and gel explosives not only as cartriged ones but also as bulk systems for mechanized borehole loading during mining and construction operations [3]. Herein, such industrial explosives feature more safety while using and smaller toxicity of the explosion products in comparison with nitroglycerine and TNT-containing explosives traditionally used during blasting works.

It is important to note that all processes (NCTE, CTE, HTP, GME, RCEM), which are implemented at the enterprise, are organized so that to minimize to the uppermost the emissions of gaseous, liquid and solid waste to the environment. To put it into practice, closed cycles of water circulation and such technologies of waste processing which allow using the waste, for example, from NCTE, CTE, HTP and GME processes as basic materials for RCEM processes, have been created.

Implementation and functioning of mentioned processes during disposal and destruction of ammunition, missiles and EM enables to organize nearly complete cycle of their destruction beginning from transportation from military bases and stockpiles till output of reprocessing products for civil use.

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Part 3

DIAGNOSIS OF EXPOSURE TO CHEMICAL AND BIOLOGICAL AGENTS

Chapter 20

Biomarkers of Nerve Agents Exposure

Dishovsky Ch.[1,*], Popov T.[1], Petrova I.[1],
Kanev K.[1], Hubenova A.[2], and Samnaliev I.[a\]
[1]*Military Medical Academy, Sofia, Bulgaria*
[2]*Clinic of Toxicology, MHATEM "N.I. Pirogov", Sofia, Bulgaria*

Abstract. Nerve agents (OPC) could be used in terrorist acts. Investigation of longer-lived biomarkers for verification of exposure to toxic compounds is important for diagnosis and prognosis of there intoxications. We study the effects of doses of toxic agent and the times expire after intoxications on the potential biomarkers. In our studies soman and tabun applied at different single doses −1.0 LD_{50}, 0.5 LD_{50} and 0.1 LD_{50} were able to cause significant changes of BuChE and AChE in erythrocytes, brain and liver for a period of 10 days after the challenge. Intoxications with different doses of soman and tabun proved that brain and erythrocyte AChE are most sensitive regardless of the dose used. In the same conditions we implement a procedure for analysis of phosphylated BuChE in plasma or serum samples, which is based on reactivation of the phosphylated enzyme with fluoride ions (developed from [2]). The method of fluoride reactivation with further treatment and analysis by gas chromatography represented positive results for soman and tabun poisoning up to the fifth day after exposure, despite the implemented dose. We implement the method of fluoride reactivation in clinical practice. Two incidents with acute poisoning with chlorpyrifos and mixture of chlorpyrifos and diazinon have been investigated. Our studies showed that the method for fluoride reactivation is applicable in clinical practice to cases of intoxications with organophosphorus pesticides with capability to detect reactivated fluorophosphates longer than the original intoxicant and its metabolites could be detected in biological samples. The combination of routine biochemical and analytical methods with fluoride reactivation will improve the diagnosis and prognosis of intoxication with nerve (OPC) agents in case of terrorist acts with these compounds.

Keywords. Biomarkers, chemical warfare agents, diagnosis, nerve agents, fluoride reactivation

20.1. Introduction

Organophosphorus compounds (OPC) are widely used like insecticides. They were developed also as warfare nerve agents. The recent use of sarin in the terrorist acts in Matsumoto city and Tokyo underground was removed any doubts about the possibility of using chemical weapons by terrorists.

* Corresponding Author: Dr. Christophor Dishovsky, Military Medical Academy, 3, St. G. Sofiiski Str., Sofia 1606, Bulgaria; E-mail: *christophord@yahoo.com*

C. Dishovsky and A. Pivovarovi (eds.), *Counteraction to Chemical and Biological Terrorism in East European Countries*,

The main problem connected with the chemical terrorism is that in addition to chemical weapons, terrorists can use different toxic chemicals from chemical industry, agriculture or products released from terrorist acts on industrial facilities.

Widely used methods to diagnose and biomonitor exposure to OPC, e.g., nerve agents are measurement of enzyme activity of acetylcholinesterase (AChE) and butyryl-cholinesterase (BuChE) in blood and analysis of the intact poison or its degradation products in blood and/or urine.

Measurement of cholinesterase inhibition in blood does not identify the anticholinesterase and does not provide reliable evidence for exposure at inhibition levels less than 20%.

The intact poison and its degradation products can only be measured shortly after exposure. Moreover, the degradation products of pesticides may enter the body as such upon ingestion of food products containing these products [1].

Polhuijs et al. [2] developed a procedure for analysis of phosphylated BuChE in plasma or serum samples, which is based on reactivation of the phosphylated enzyme with fluoride ions: this converts the organophosphate moiety completely into the corresponding phosphofluoridate, which is subsequently isolated and quantitated. It can be calculated that inhibition levels ≥0.1% of inactivated BuChE (i.e., trace level exposure) should be quantifiable. Application of this method to serum samples of the victims from the Tokyo subway attack and of the Matsumoto incident yielded sarin concentrations in the range of 0.2–4.1 ng/mL serum [1].

The aim of this study was to find out conditions for implementation of the fluoride reactivation in relation to intoxications of white rats with soman and tabun and to implement this method in clinical practice in cases with OPC pesticides intoxications.

20.2. Materials and Methods

20.2.1. Experiments on Rats

Animals. Male albino rats “Wistar”, weighing between 180 and 220 g were used for the experiments.

Biochemical investigations. Biochemical investigations were carried out 1, 5 and 10 days after soman and tabune poisoning. Intoxications were caused by three doses of OPC – 1.0 LD_{50}, 0.5 LD_{50} and 0.1 LD_{50}, injected s.c. in a volume of 0.1 mL/100 g. body weight. For each dose of OPC used rats were divided into three groups (n = 8 in group). Control group (ten rats) was treated with 0.9% NaCl at the same experimental conditions. Biochemical observations were made 24 h, 5 and 10 days after the challenge by using Screen Master (Hospitex Diagnostics, Italia).

Biochemical parameters. The following biochemical parameters were determined: Butyryl Cholinesterase (ChE, EC 3.1.1.8.) (Kinetic method, U/L); Acetylcholinasterase (AChE, EC 3.1.1.7.) in erythrocytes, brain and liver (Ellman method, mkmol/mL/min);

Fluoride reactivation. Chemicals and materials. The native agents Soman (GD, 96.2%) and Tabun (GA, 86.7%) used for inhibition were obtained from the stocks of the Laboratory of Experimental Toxicology. SepPak C_{18} cartridges (type Plus Long, 820 mg, 1.6 mL) were produced from Waters Associates (Millipore Corporation, Milford, MA). Strata-X cartridges (type 8B-S100-TAK 30 mg, 1.0 mL) were purchased from Phenomenex (California, USA). Nexus cartridges (200 mg) were obtained from Varian (Middelburg, The Netherlands).

Rat blood serum. The blood was collected separately on 24th h, 5 and 10 days after intoxication with the organophosphorus compound. Six additional animals without administration of nerve agent were used to prepare control samples. Between one and five samples from each group were processed further to analyze the reactivated soman or fluorotabun. Serum samples from intoxications with tabun were kept frozen between 6 and 8 months.

Fluoride reactivation of soman. Rat blood serum samples (200 μL) were diluted with three parts of acetate buffer (0.189M acetic acid and 10.8M sodium acetate, pH 3.5) and 40 μL potassium fluoride (5.25M) were added, leading to a final concentration of 0.25M KF. The mixture was homogenized on Vortex and incubated for 30 min at 25°C [3, 4].
An important condition for obtaining correct results is distinguishing between intact soman in blood serum and reactivated soman as a result of fluoride reactivation. For that purpose was applied procedure with simultaneous processing of aliquot amounts from the same serum blood sample under identical conditions with the only difference of adding 40 μL deionized water instead of potassium fluoride solution for releasing of bounded organophosphate moiety.

Fluoride reactivation of tabun. Rat blood serum samples (250 μL) were diluted with three parts of acetate buffer (0.189M acetic acid and 10.8M sodium acetate, pH 3.5) and 50 μL potassium fluoride (5.25M) were added, leading to a final concentration of 0.25M KF. The mixture was homogenized on Vortex and incubated for 30 min at 25°C.

Solid-phase extraction of soman. Prior to extraction, SepPak cartridges were rinsed once with ethyl acetate (4 mL) and twice with water (2 x 5 mL) without leaving the sorbent to go dry. Next diluted serum (0.840 mL) was applied and the cartridge was rinsed with 1 mL water. Finally 2 mL of ethyl acetate were used to elute the yielded soman in centrifuge tube. The tubes were frozen in liquefied nitrogen and the upper layer (ethylacetate with the component of interest) was transferred into sample vial (1.5 mL) for analysis.

Solid-phase extraction of fluorotabun. Prior to extraction, strata-X cartridges were conditioned ethyl acetate (1 mL) and equilibrated with water (1 mL). Next diluted serum (1.050 mL) was applied along with internal standard diisopropyl fluorophosphate and the cartridge was rinsed with 1 mL water. Finally 1 mL of ethyl acetate was used to elute the compounds of interest in centrifuge tube. The tubes were frozen in liquefied nitrogen and the upper layer (ethyl acetate extract) was transferred into sample vial (1.5 mL) for analysis.

Gas chromatography. Analyses were performed on Agilent 6890N GC, equipped with nitrogen-phosphorus detector (NPD). The capillary column was J & W Scientific HP-5, length 30 m, 0.32 mm i.d. and film thickness 0.25 μm.

20.2.2. Investigations of Clinical Cases

Human blood serum and plasma. BD Vacutainer® (BD Plymouth, UK) SST and heparine tubes were used for collection of blood samples.

Cholinesterase activity. Activity of ChE (cholinesterase) in blood serum was measured with spectrophotometer "Hospitex Diagnostics" – Screen Master with diagnostic kits from "Giesse Diagnostics".

Fluoride reactivation of organophosphorus pesticides. Human blood serum and plasma samples (1 mL) were diluted with three parts of acetate buffer (0.189M acetic acid and 10.8M sodium acetate, pH 3.5) and 200 μL potassium fluoride (5.25M) were added, leading to a final concentration of 0.25M KF. The mixture was homogenized on Vortex and incubated for 30 min at 25°C.

Solid-phase extraction of organophosphorus pesticides. Prior to extraction, Nexus cartridges were rinsed once with n-hexane (4 mL), once with ethyl acetate (4 mL) and twice with water (2 x 5 mL). Next diluted serum (4,200 mL) was applied and the cartridge was rinsed with 2 mL of water. Finally 2 mL of ethyl acetate were used to elute the yielded fluorophosphates in centrifuge tube. The tubes were frozen in liquefied nitrogen and the upper layer (ethyl acetate with the component of interest) was transferred into GC sample vial for analysis.

Gas chromatography. Analyses were performed on Agilent 6890N GC, equipped with nitrogen-phosphorus detector (NPD). The capillary column was J & W Scientific HP-5, length 30 m, 0.32 mm i.d. and film thickness 0.25 μm.

20.3. Results and Discussion

The toxic signs of poisoning were most demonstrative after exposure to 1.0 LD_{50} and moderate after 0.5 LD_{50} of soman and tabun. The highest dose caused subconvulsions, convulsions and in some cases death. After poisoning with 0.5 LD_{50} some animals showed tremor, fasciculations and salivation without subconvulsions or convulsions. There were no symptoms in case of intoxication with 0.1 LD_{50}. In this group rats were completely free from toxic signs. On this background all doses of soman and tabun used caused inhibition of butyrylcholinesterase, erythrocyte and brain acetylcholinesterase (Tables 20.1 and 20.2). Inhibition of butyrylcholinesterase was most pronounced 24 h and 5 days after the challenge. Ten days later there was a tendency to recovery of enzyme activity Erythrocyte acetylcholinesterase demonstrated much more susceptibility to the toxic effects of soman and tabun especially applied at doses of 1.0LD_{50} and 0.5 LD_{50}. The inhibition of the enzyme activity 24 h and 5 days after poisoning showed high extent of statistically significance. The lowest dose of soman caused inhibition as

well although at a less extent. In comparison to the control group there was no restoration of erythrocyte acetylcholinesterase activity to the end of experiments. The results obtained from estimation of brain acetylcholinesterase reviewed the most profound inhibition for the whole period of observation regardless of the dose of soman and tabun used. At the same time the liver AChE showed insignificant deviation in comparison with control group. Even 1.0 LD_{50} of soman and tabun did not caused inhibition of this type of AChE.

In accordance with the rate of inhibition the susceptibility of cholinesterase to tabun irrespective of the dose used are arranged as follow: brain AChE > erythrocyte AChE > butyrylcholineterase > liver AChE.

Table 20.1. Activity of esterases after soman intoxication

(**a**) Activity of esterases (UI/L; μmol/mL/min) in rats after acute intoxication with soman 1.0 LD_{50}

Parameters	Control	Day 1	Day 5	Day 10
BuChE serum	403.9 ± 30.22	314.35 ± 22.40***	337.5 ± 96.44	375.1 ± 33.5
AChE RBC	4.37 ± 0.7	1.84 ± 0.95***	2.73 ± 0.53***	3.61 ± 0.83
AChE brain	0.127 ± 0.031	0.022 ± 0.0046***	0.036 ± 0.0048***	0.049 ± 0.0098***
AChE liver	1,148.0 ± 90.75	1,052.2 ± 117.25	1,119.3 ± 256.7	1,205.4 ± 330.4

(**b**) Activity of esterases (UI/L; μmol/mL/min) in rats after acute intoxication with soman 0.5 LD_{50}

Parameters	Control	Day 1	Day 5	Day 10
BuChE serum	403.9 ± 30.2	279.3 ± 44.5***	276.8 ± 29.5***	374.7 ± 38.3
AChE RBC	4.37 ± 0.70	1.61 ± 0.26***	2.79 ± 0.68**	3.17 ± 0.83**
AChE brain	0.127 ± 0.031	0.025 ± 0.001***	0.038 ± 0.001***	0.040 ± 0.009***
AChE liver	1,148.0 ± 90.7	1,399.1 ± 365.8	1,435.0 ± 298.7	1,040.4 ± 199.3

(**c**) Activity of esterases (UI/L; μmol/mL/min) in rats after acute intoxication with soman 0.1 LD_{50}

Parameters	Control	Day 1	Day 5	Day 10
BuChE serum	403.9 ± 30.2	344.9 ± 61.2*	310.8 ± 51.8	370.1 ± 69.8
AChE RBC	4.37 ± 0.70	3.35 ± 0.59	4.82 ± 0.97	3.61 ± 0.32
AChE brain	0.127 ± 0.031	0.064 ± 0.024***	0.069 ± 0.026***	0.047 ± 0.005***
AChE liver	1148.0 ± 90.7	1273.7 ± 265.0	1327.4 ± 273.9	1183.4 ± 284.4

In comparison to the control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 20.2. Activity of esterases after tabun intoxication

(**a**) Activity of esterases (UI/L; μmol/mL/min) in rats after acute intoxication with tabun 1.0 LD_{50}

Parameters	Control	Day 1	Day 5	Day 10
BuChE serum	403.9 ± 30.22	359.03 ± 51.38	364.06 ± 49.16	369.4 ± 83.02
AChE RBC	4.37 ± 0.7	2.773 ± 0.33***	3.590 ± 0.23*	3.994 ± 0.42
AChE brain	0.127 ± 0.031	0.036 ± 0.007***	0.039 ± 0.011***	0.082 ± 0.013
AChE liver	1,148.0 ± 90.75	859.83 ± 129.7**	975.8 ± 276.02	1,148.0 ± 101.46

(**b**) Activity of esterases (UI/L; μmol/mL/min) in rats after acute intoxication with tabun 0.5 LD_{50}

Parameters	Control	Day 1	Day 5	Day 10
BuChE serum	403.9 ± 30.2	338.9 ± 54.8*	329.3 ± 41.2*	327.7 ± 59.3*
AChE RBC	4.37 ± 0.70	2.80 ± 0.36***	4.33 ± 0.83	3.93 ± 0.88
AChE brain	0.127 ± 0.031	0.037 ± 0.008***	0.049 ± 0.012***	0.063 ± 0.029**
AChE liver	1,148.0 ± 90.7	663.7 ± 131.5***	963.6 ± 136.6	1,189.1 ± 271.8

(**c**) Activity of esterases (UI/L; μmol/mL/min) in rats after acute intoxication with tabun 0.1 LD_{50}

Parameters	Control	Day 1	Day 5	Day 10
BuChE serum	403.9 ± 30.2	361.62 ± 34.5*	308.16 ± 36.4***	375.4 ± 41.6
AChE RBC	4.37 ± 0.70	3.16 ± 0.74***	3.24 ± 0.44**	4.49 ± 0.75
AChE brain	0.127 ± 0.031	0.029 ± 0.005***	0.050 ± 0.006***	0.093 ± 0.030
AChE liver	1,148.0 ± 90.7	861.0 ± 219.2*	753.4 ± 148.5***	1,250.5 ± 481.0

In comparison to the control group: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Presence of intact soman and tabun for all developed samples was not discovered. The results of analyses of extracted samples with yielded soman and fluorotabun after performed fluoride reactivation are presented in Figures 20.1 and 20.2 respectively.

The results in showed that implementation of the method for fluoride reactivation on in vivo intoxicated with soman and tabun in rats allow detection of the yielded compound up to 5 days after intoxication.

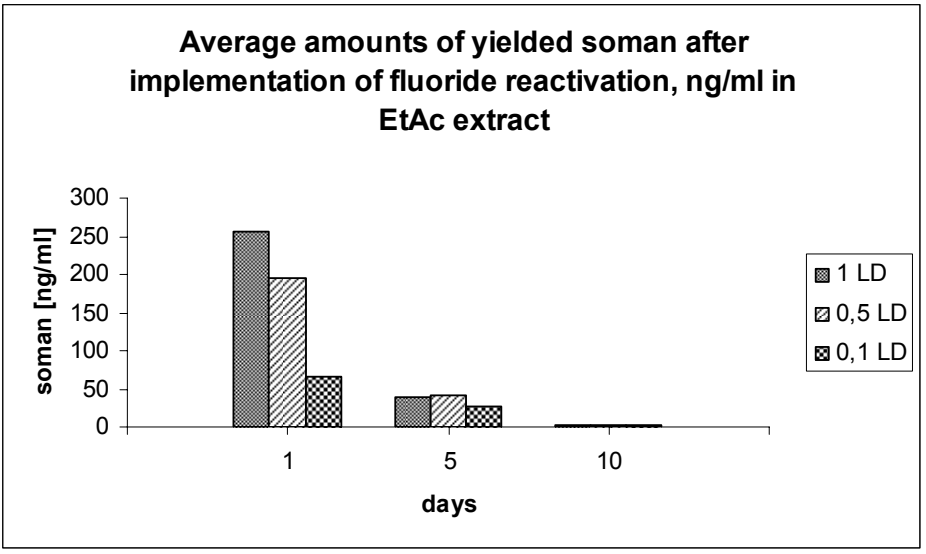

Figure 20.1. Acute intoxication with tabun-yielded soman after fluoride reactivation

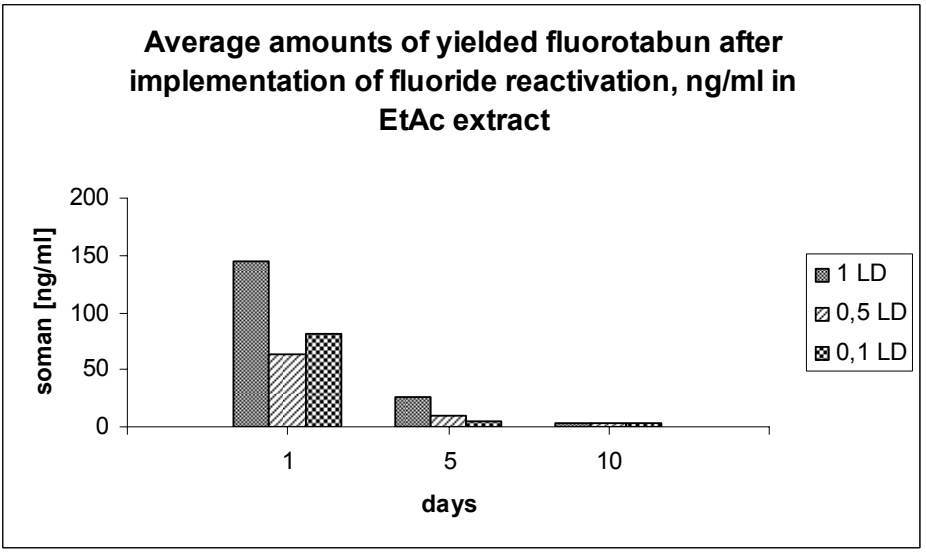

Figure 20.2. Acute intoxication with tabun-yielded fluorotabun after fluoride reactivation

20.4. Clinical Investigations

20.4.1. Case 1

A 41 year old woman attempted suicide by ingesting 30–50 mL of commercial formulation of chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate). The patient was hospitalized and received intensive medical treatment including Toxogonin, Atropine Sulphate and Diazepam. Despite that the first urine and blood samples, collected on the 27th day after intoxication were sent for analysis in the laboratories of Military Medical Academy, both metabolites of chlorpyrifos are detected in concentrations higher than normal levels in unexposed persons.

The blood sample was collected in gel-barrier tube and the separated serum was incubated with potassium fluoride. Simultaneously a sample of pooled blank serum from unexposed persons was processed too, following the same procedure.

On the first view it can be seen that two new peaks, eluting at 5.460 and 6.126 min are presented in the chromatogram, which might be a result of fluoride reactivation. In order to recognize the compounds, sample was concentrated 20-times and analyzed on GC-MS in the Laboratory of Clinical Toxicology, Military Medical Academy.

20.4.2. Case 2

A middle-aged man ingested by mistake mixture containing of organophsophorus pesticides, containing chlorpyrifos and diazinon (O,O-diethyl-O-(2-isopropyl-6-methyl-pyrimidine-4-yl)phosphorothioate). The patient was hospitalized in Military Medical Academy, Clinic for Toxicology and this helped us to study the case more comprehensive.

The blood samples this time were collected in heparine tubes to obtain blood plasma, rather than blood serum. The first sample, taken a few hours after the intoxication confirms the result from the first case with same two eluting peaks at the same retention time with better response (which has its reasonable explanation). An aliquot of the same blood plasma was processed with addition of water instead of potassium fluoride solution. In the analyzed extract those peaks were missing.

The obtained spectra from GC-MS analysis (both SCAN and SIM modes) recognized the first peak as diethyl fluorophosphate (DEFP) with probability higher than 95% in comparison with the library. The second peak was interpreted as O,O-diethyl fluorothiophosphate (DEFTP).

During the treatment of the patient in the hospital, nine samples of blood plasma up to 28th day after intoxication were analyzed. Since DEFP and DEFTP were not available in our laboratory, the obtained results for peak area were compared to those of diisopropyl fluorophosphate. Change in concentration of yielded DEFP and DEFTP in dependence on time of collection of blood samples is presented bellow (Figures 20.3 and 20.4).

The curve represented on Figure 20.3 has one apex on the second point (3 days after exposure), while the curve for DEFP has two apexes, for the samples taken on the third and ninth day after intoxication and hospitalization (Figure 20.4). Concentrations of DEFP are in one order lower than concentrations for DEFTP, depending on the rate of desulfuration of the parent compound.

Results for ChE activity after intoxications with chlorpyrifos and diazinon are presented in Table 20.3. In both cases ChE activity of the obtained blood serum or plasma was depressed in comparison to normal values (4,200–10,800 U/L for women and 5,600–11,200 U/L for men) in all time of investigation.

20.5. Conclusions

Intoxications with different doses of soman and tabun proved that brain and erythrocyte AChE are most sensitive regardless of the dose used. The enzyme inhibition was still observed 10 days after the challenge. Results from implementation of the method of fluoride reactivation in rats intoxicated with soman and tabun, were obtained up to 5 days following administration of the agent. Despite storage of the serum samples from tabun intoxicated animals for 6 and 8 months at −28°C, fluoride ions were still capable to release the organophosphyl moiety. Until 10 days after poisoning phosphyl moiety could be regenerated by fluoride.

The method for fluoride reactivation is applicable to cases of intoxications in clinical practice with organophosphorus pesticides with capability to detect reactivated fluorophosphates 49 days after exposure, which is longer than the original intoxicant and its metabolites could be detected in biological samples.

Discrepancy of BuChE activity and clinical health state was observed for both cases as well: The BuChE activity after 49 days medical treatment of Chlorpyrifos-ethyl intoxicated patient (case 1) was reactivated near to the normal value, while in case 2, the patient was discharged from hospital 1 month after intoxication in a good clinical health state but still not reactivated BuChE.

Table 20.3. BuChE activity of the blood serum after intoxications with oganophosphorus pesticides, UI/L

Case 1 (41 year old woman)		**Case 2** (middle-aged man)	
Days	ChE activity (UI/L)	Days	ChE activity (UI/L)
0	322	1	160
1	311	1	180
3	270	2	130
6	245	2	160
11	193	2	140
17	231	6	140
20	255	6	200
24	313	6	160
31	673	9	110
38	1,165	13	120
44	2,020	17	150
		24	180
		27	260

Normal values of BuChE: 4,200–10,800 U/l for women and 5,600–11,200 U/l for men.

The good correspondence between the level of exposure (manifested in the clinical picture of intoxication) and the level of reactivated diethyl fluorophosphate (DEFP) and reactivated O, O-diethyl fluorothiophosphate (DEFTP) confirms that they are convenient biomarkers for indication of exposure with OP pesticides Chlorpyrifos and Diazinon.

The method of fluoride reactivation is applicable in clinical practice for demonstrating intoxications with organophosphorus pesticides and nerve agents. It appeared that the method gives positive results despite of using standard therapy of poisoning, including oximes.

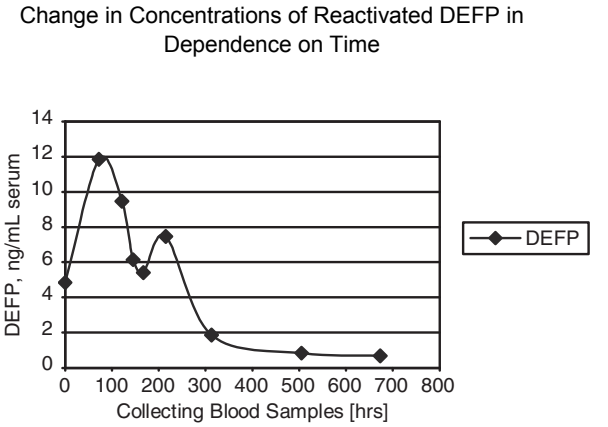

Figure 20.3. Concentrations of reactivated diethyl fluorophosphate (DEFP)

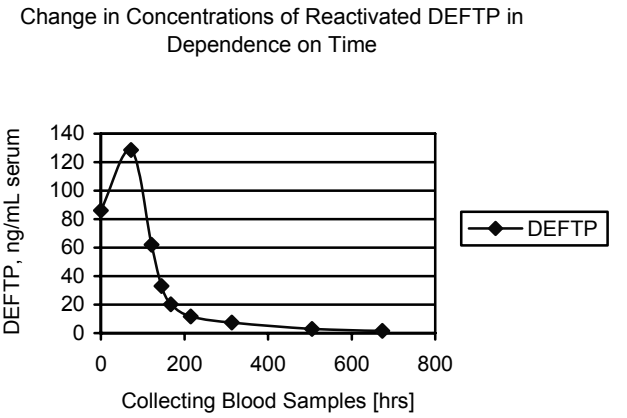

Figure 20.4. Concentrations of reactivated O,O-diethyl fluorothiophosphate (DEFTP)

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Chapter 21

The Dietary Grape Polyphenol Concentrate "ENOANT" Enables Protection Against Biological Agents

Zagorouiko V.[1], Mizin V.[1], Bogadelnikov I.[2], and Ogay U.[1]
[1]*National Institute for Vine and Wine "Magarach", Ukraine*
[2]*Crimean National Medical University, Ukraine*

Abstract. Alcohol-free grape products have a wide variety of beneficial effects successfully used in prevention, treatment and rehabilitation of many diseases. The dietary concentrate "Enoant" contains high levels of grape polyphenols (18–20 g/L total polyphenols). Flavonoid monomers and nonflavonoid polyphenols and their derivatives, including malvidin, cyanidin, delphinidin, peonidin, petunidin, quercetin, rutin, (+) catechin, (−) epicatechin, (−) epicatechin-gallate, gallic acid, syringic acid, caffeic acid, protocatechic acid, chlorogenic acid and trans-resveratrol have been identified in the concentrate by HPLC. "Enoant" is considerably richer in some flavonoid monomers compared to relevant Cabernet-type dry wines. *In vitro* studies have shown antibacterial effects of "Enoant" at 1.25 mg/mL (a strong one on *St. aureus* and none on *E. coli* and *C. albicans*) and no toxic effects at the same concentration (chemoluminescence test). Clinical studies in children with pneumonia have shown that "Enoant" restores bacterial microflora in the intestines after damage by antibiotic therapy. *In vivo* data from experiments in rats indicates stress-protective, hepatoprotective and antiatherogenic effects of "Enoant" at 0.3 mL/kg (human body weight) and reduced frequency of chromosome aberrations in the course of spontaneous and thyroxin-induced mutagenesis. Clinical studies have shown that "Enoant" reduces injuries of normal tissues by Cisplatinum, Carboplatinum and radiation therapy (hemotoxicity and nephrotoxicity) in patients with oncopathology. *In vivo* experiments in rats and clinical studies demonstrate that "Enoant" reduces injuries of normal tissues by alcohol intoxication (hepatoxicity and nephrotoxicity) and also alarm level and depression in treatment of the alcohol dependence syndrome. Benefits from administration of "Enoant" consisting of considerable improvement of functional status of the liver and a smooth recovery after the operation have been registered in patients with B and C hepatitis operated for cholelithiasis. *In vivo* experiments in rats and clinical studies have shown immunocorrective and anti-inflammatory effects of "Enoant" on periodontal tissues in gingivitis, periodontitis and in traumatic fracture of the low jaw.

Keywords. Grape polyphenols, prevention, treatment, rehabilitation

Modern medical technologies rely, for better results, on increasing use of novel natural factors with curative effects. Natural antioxidants that have recently come to be made use of at health resorts of the Crimea enter as such compounds [1, 2]. Bioantioxidants, i.e. antioxidants which naturally engage in metabolism, are of special value for medical purposes. Effects related to bioantioxidant deficiency can be generated by

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accumulation of products arising from oxidation induced by free radicals, lipid peroxides for the major part. This entails a considerable reduction in resistance of the human organism to factors which activate radical reactions, i.e. stress, a relatively increased oxygen concentration, ionizing radiation, etc.

With the aim to correct antioxidant status of the human organism, dietary factors conducive to greater risk of antioxidant deficiency have first to be determined. These may be (1) low bioantioxidant levels of the diet, (2) diets providing oxidants, including alcohol or substances which impair function of enzymatic and non-enzymatic elements of the antioxidant system of the human organism (such as nitrites; nitrates; products arising from oxidation of lipids; toxic chemicals; lead, mercury and cadmium compounds), and (3) imbalances of nutrients and bioantioxidants in the diet (such as increased caloric value and excessive intake of fat, especially of refined vegetable oil and products high in cholesterol and vitamins A and D) which exert direct or indirect pro-oxidant effects.

An increased bioantioxidant need may also emerge under specific conditions, such as (1) stress, (2) exposure to harmful man-made factors of the environment, (3) senescence, (4) too much or too little excessive, (5) diseases accompanied by activated processes of oxidation induced by free radicals, (6) smoking, (7) alcoholism and administration of drug preparations whose mechanism of action involves a disturbed bioantioxidant absorption in the gastrointestinal tract, and (8) administration of xenobiotics, including a number of pharmaceuticals (such as cytostatics; antibiotics; narcotic and non-narcotic analgesics; non-steroid anti-inflammatory preparations) which activate processes of oxidation induced by free radicals.

Polyphenols are the most active natural bioantioxidants and may also be considered contenders for the role of protectors against side affects of pharmaceuticals.

Polyphenols, including flavonoids, are of vital importance for the human organism. Nevertheless, they are not synthesized in humans via natural metabolic pathways and may only be administered as part of vegetable foods. Grape berries are the richest source of vegetable polyphenols and flavonoids. Unfortunately, water solubility of polyphenols is restricted, which entails poor biological availability of these substances in humans. Very small amounts of polyphenols are assimilated by the human organism via direct administration of grape berries. When employed by medical technologies, they can be made biologically available and administered at substantially high doses as part of grape wines and food-grade concentrates [3].

It is already known that grape polyphenols have reliable curative and preventive effects, consisting of reduced oxidation of low density lipids, decreased platelet aggregation, lower development of increased tonus of artery smooth muscles, reduced coronary blood flow, diminished frequency of stenocardia attacks, myocardial infarction, oncogenesis and bacterial infections of the gastrointestinal tract, including those induced by *H. pylori* [4–9]. Polyphenols (proanthocyanidins in particular) are fixed by proline of collagen and elastin found in arteries, which improves their resistance to blood pressure and restores normal NO synthesis in the epithelia, and this synthesis, in turn, regulates vascular relaxation [10]. Grape polyphenols have been found to prevent expression of human immunodeficiency virus by reducing levels of peroxide radicals in cultured blood cells [11].

Pre-clinical studies indicate that grape polyphenols, including quercetin, kaempherol and resveratrol, are capable to inhibit ribonucleotide reductase and to suppress DNA synthesis in mammals by direct blocking proliferation of tumor cells while alcohol, on the contrary, promotes this process [12–14].

Thus, it may be concluded that grape polyphenols possess a broad range of biological activity. Actually, all individual polyphenol and flavonoid species exert antioxidant effects though the best result is provided by the total polyphenol extract from grape skins and seeds.

The alcohol-free dietary concentrate "Enoant" developed by the National Institute for Vine and Wine "Magarach" enters as a practical approach to the use of the grape Cabernet Sauvignon grown in the Crimea as an element of medical technologies. It contains 18–20 g/L total polyphenols, including 3–5 g/L of coloring substances.

Various species of polyphenols were identified in "Enoant" by HPLC: flavonoid anthocyanin monomers and their derivatives, including malvidin (101.5 mg/dm^3), cyanidin (4.1 mg/dm^3), delphinidin (13.9 mg/dm^3), peonidin (13.2 mg/dm^3) and petunidin (2.9 mg/dm^3), other flavonoid monomers and flavonols, including quercetin (136.6 mg/dm^3), rutin (22.0 mg/dm^3), (+)catechin (553.0 mg/dm^3), (−)epicatechin (137.8 mg/dm^3) and (−)epicatechin-gallate (127.6 mg/dm^3), and non-flavonoid monomers, including gallic acid (925.7 mg/dm^3), syringic acid (133.9 mg/dm^3), caffeic acid (8.3 mg/dm^3), protocatechic acid (2.5 mg/dm^3), chlorogenic acid (2.7 mg/dm^3) and *trans*- resveratrol (2.6 mg/dm^3).

"Enoant" is much more superior to Cabernet wines in flavonoid monomers from grape skins and seeds. This is especially true for (+)catechin which has the highest degree of reduction of all grape polyphenols and is the most potent antioxidant [3].

Antioxidant activity of "Enoant" was assessed analytically from data on air oxidation kinetics of the reduced form of 2,6-dichlorophenol indophenol found in the concentrate, yielding the antioxidant activity index A of 2.2 10^{-1} min^{-1} dm^3 ·mL^{-1}, which is considerably high, being 15 times as much as that of ascorbic acid and in three orders of magnitude higher than normal antioxidant activity of human plasma.

"Enoant" as a source of polyphenols enables ready assimilation by the human organism of a complex of biologically important grape polyphenols and flavonoids in water-soluble form, the absence of alcohol being another advantage.

The National Crimea Medical University and the National Institute for Vine and Wine "Magarach" coordinate experiments and clinical studies of effects exerted by "Enoant" when employed in complex treatment of patients with different pathologies.

In vitro studies done at the National Crimea Medical University demonstrated an antibacterial effect of "Enoant" at a minimum concentration of 1.25 g/L, on *St. aureus*; at 0.52 mg/kg body weight, and the concentrate was found effective in treatment of biocenosis disorders of the intestines in children; at the same concentration, in rat, the concentrate had cytoprotective, hepatoprotective, nephroprotective, anti-ulcerous and antitoxic effects [15–18].

Experiments in rats done at the Institute of Hygiene and Medical Ecology of the Academy of Medical Sciences of Ukraine showed that "Enoant" reduced biological age as indicated by lower frequency of chromosome aberrations in the course of spontaneous and thyroxin-induced mutagenesis. "Enoant" exerted a curative effect on radiation

injures, which enables it to be made part of supporting therapy in treatment of oncology by using radiotherapy though no radio protective effect has been revealed [19, 20].

Studies in rats done in the National Pharmaceutical University (Kharkov) indicate that the concentrate at a dose corresponding, for humans, to 0.3 mL per kg body weight displays stress-protective, hepatoprotective and antiatherogenic activities [2].

Effects of "Enoant" on recidivating and chronic bronchitis and biocenosis disorders of the intestines as accompaniment in children were studied at the National Crimea Medical University and the Institute of Pediatrics, Obstetrics and Gynecology of the Academy of Medical Sciences of Ukraine [22, 23]. Considerably lower injures of steric structural organization of blood proteins were observed in patients treated with "Enoant" relative to those treated with quercetin. Due to its strong antioxidant properties, "Enoant" considerably reduced concentration of active forms of oxygen, enhanced activity of the antioxidant system of the human organism and improved the balance between the latter and the system of non-enzymatic protection, which, in turn, led to a decreased flow of compounds having radical groups and activation of energy-exchange processes. Phenomena of biocenosis disorders of the intestines were also considerably diminished, especially those induced by antibiotic therapy.

Putative effectiveness per oral ("per os") administration of "Enoant" at 10–15 mL in the form of baths for the oral cavity three times a day was studied at the Bogomolets National Medical University (Kiev) in complex treatment of fractures of the low jaw which account for 60–90% of all fractures of the visceral cranium and are often complicated with traumatic osteomielitis. By day 7, microbial indices were normalized, immunity improved (including increased levels of thymus-dependent blood lymphocytes), hygienic condition of the oral cavity also considerably improved, gingivitis severity decreased and accelerated positive dynamics of main clinical symptoms was achieved (by 2–3 days).

Effects of "Enoant" on the dynamics of the condition of patients operated for cholelithiasis were studied at the Surgery Department of the Training and Research Medical Center of the Administration of the President of Russia Federation. The concentrate was administered at 30 mL per day during for days before the operation and at 15 mL per day during for days after the operation. By the date of discharge, the condition of the "Enoant"-treated patents was better relative to the control group, indicated by a considerably enhanced feeling of well-being, improved blood biochemical indices (reduced cholesterol, AST- and ALT-transaminases and a lower atherogenic index), a faster normalization of bilirubin level and of functional status of the cardiovascular system. Administration of "Enoant" resulted in a considerable improvement of functional status of the liver and the cardiovascular system in patients with cholelithiasis accompanied by B and C hepatitis, which, in turn, led to a smooth recovery after the operation [24].

Clinical studies of the use of "Enoant" in complex treatment of patients with oncopathology of different organs and systems of the organism were done at the Kavetski Institute of Experimental Pathology, Oncology and Radiology of the National Academy of Sciences of Ukraine and in the Kiev oncology hospital. Results from these studies indicate that the concentrate displays hemoprotective properties and considerably promotes erythropoiesis [25]. Anemia is a well-known life-threatening side reaction to cytostatic therapy observed in 60% of patents. Patients with anemia after the first course of cytostatic therapy who had hemoglobin level of 85.5 ± 0.8 g/L on the average across

the group received "Enoant" at a daily dose of 15 mL (5 mL three times a day) for 17 days totaling 250 mL before the beginning of the second course. This enabled an increase in hemoglobin of up to 104.4 ± 5.5 g/L, and the second course of cytostatic therapy was safely applied. Administration of the concentrate had no effect on erythrocyte and platelet levels, which is a positive factor conducive to success of anti-tumor therapy. "Enoant" acted as a safe and effective promoter of erythropoiesis and produced a good response in the patients [27].

The use of "Enoant" as part of rehabilitation of patients with mediastinal cancer whose treatment followed the conventional classical scheme was studied at the Open International University of Human Development "Ukraine" (Melitopol) and at the Melitopol Interregional oncodispensory. The conventional special treatment consisted of one to three courses of cytostatic therapy (Cyclophosphan 1,000–2,000 mg, Vinblastin 10 mg, 5-Ftoruracil 500–700 mg and Adriamicyn) and two stages of gamma-therapy (of up to 60 Gray). As addition, all patients received "Enoant" during the indicated treatment and over the following 7 days. Administration of the concentrate at average (up to 0.45 mL/kg body weight per day) and especially increased (up to 0.85 mL/kg body weight per day) doses considerably dampened the toxic effects of the therapy and led to significant improvement of the test patients' quality of life and life-span: life-spans of "Enoant"-treated patients and those of the control group were 28.2 ± 0.05 months and 9.5 ± 0.02 months, respectively [27].

Effects of "Enoant" during treatment and prevention of the alcohol dependence syndrome were studied at the Center of Narcology and Psychosomatic Medicine "Medissa" (Simferopol). Increased alarm level and depression after consumption of alcohol has been prohibited constitute the major difficulty in treatment of this pathology. Alarm level and depression decreased in "Enoant"-treated patients by 12% (on the HAM-A scale) and 15% (on the BDI scale), respectively, which, in turn, increased effectiveness of the therapy.

"Enoant" was made part of complex treatment of patients with generalized periodontitis accompanied by dramatic changes in immune homeostasis indices at the Kharkov Medical Academy of Post-Graduate Education. In addition to the classical therapy, the patients received "Enoant" both per os at a daily dose of 0.52 mL/kg body weight and in the form of applications and instillations into the periodontal pockets for 15 days. Restoration of normal immunity indices was the most remarkable effect of the concentrate, i.e. relative and absolute contents of T-cells ($CD3^+$) and the ratio of regulatory $CD4^+$ and $CD8^+$ subpopulations of T-cells. Against the restored balance of the ratio of these types of cells, the indices of B-lymphocytes and natural killers also improved and approached those of the control group of patients. IgA and IgG concentrations and that of phagocyte segmented neutrophils also decreased and reached normal levels.

Clinical effects of "Enoant" were studied during complex treatment of patients with hypertensive disease, chronic cardial ischemia and chronic bronchitis at the National Crimea Medical University. The concentrate had pronounced positive effects on patients with hypertensive disease in relation to parameters of functions of the cardio-respiratory system and other systems of the organism, including improvement of the auscultative respiration pattern, increase in respiratory volume, decreases in minute blood volume and heart capacity, a reduction in heart rate frequency and respiration rate, a decrease in β-lipoprotein content and an increase in catalase activity of blood

serum, and also an increase in the color index. Parameters of functions of the cardiorespiratory system and other systems of the organism were also beneficially affected in patients with chronic cardial ischemia, including reduced complaints of fatigue, a reduction in labored respiration, a reduction in heart pain, and, in general, in all kind of complains. Positive effects of the concentrate were also indicated by a reduction in pulmonary rales, lower diastolic arterial pressure, a reduction in heart rate frequency, lower β-lipoprotein content and decreased total blood bilirubin. Increases in catalase activity of blood serum, color index and tolerance to physical exercise were also registered as reliable positive effects.

Many effects of "Enoant" are common both for chronic bronchitis and obstructive chronic bronchitis, such as activation of the antioxidant system of the organism (which also holds true in patients with hypertensive disease and chronic cardial ischemia) and tendency for reduction in arterial pressure (which also holds true in patients with chronic cardial ischemia). Nevertheless, a number of effects exerted by "Enoant" are different in the two forms. A reduction in labored respiration and cough, an increased value of the Hench test, an increased erythrocyte number in parallel with increasing erythron capacity, a decreased number of band neutrophils and reduced stress level judging by the Garkavi reactions, normalized minute respiratory volume, increased respiratory volume and a reduction in heart rate frequency, an increase in maximum volumetric expiratory flow rate at a level of 75% and 50% of forced vital volume, and a lower indexes of Robinson and Cerdo were benefits from administration of "Enoant" in patients with chronic bronchitis. Positive effects of "Enoant" in patients with chronic obstructive bronchitis consisted of reduced complaints of heart intermissions, a reduction in pulmonary rales and wetness of the skin, and reduced pathological changes in the expectoration.

Complex therapy received by "Enoant"-treated patients with hypertensive disease, chronic cardial ischemia and chronic bronchitis in spa treatment entailed a desire to reduce consumption of wine and strong alcohol beverages. By the end of the course, a reduction in willingness to consume wine was registered in 57% of patients with hypertensive disease, in 37% of patients with chronic cardial ischemia and in 16% of patients with chronic bronchitis. The desire to reduce alcohol consumption was more pronounced in "Enoant"-treated patients in relation to wine than to strong alcohol beverages.

The above data shows that effects of "Enoant" as part of medical technologies are related to successful achievement of a number of clinical goals, such as (1) reduction in action of risk factors, (2) reduction in stress and elimination of distress, (3) correction of function of the vegetative nervous system, (4) reduction in the intensity of inflammatory processes in the lungs, in the bronchi and in the upper respiratory tracts, (5) improvement in patency and evacuation function of the bronchi; (6) normalization of function of external respiration; (7) normalization of parameters of oxygen-transport function of blood; (8) normalization of arterial pressure and hemodynamics; (9) improvement in effectiveness of function of the cardiorespiratory system and increase in its functional reserves; (10) normalization of lipid exchange; (11) increase in detoxification reserves and antioxidant potential; (12) increase in tolerance to physical exercise, (13) enhancement of feeling of well-being as a patient's own integral judgement on the current status of his or her health [1, 2].

21.1. Conclusions

Experimental and clinical studies have shown curative and preventive benefits of the dietary grape polyphenol concentrate "Enoant" as part of therapeutic and surgical technologies. These may contribute to the achievement, with better effectiveness, of clinical goals in complex treatment and rehabilitation, including reduction in risk factors, reduction in clinical manifestations and negative consequences for the human organism of impaired functions of the cardiorespiratory system, erythron, the antioxidant system and lipid exchange, and dampening of toxic side effects of pharmacotherapy.

The effects of "Enoant" demonstrated by the reported research completely agree with effects of polyphenols and flavonoids from clinical studies and experiments done elsewhere.

Data from clinical studies and experiments on effects exerted by "Enoant" indicates that the criterion for optimization of medical technologies should be based on both a sufficient daily dose of the concentrate (0.25–0.5 mL/kg body weight) and on the absence of individual negative reactions in the patients.

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Chapter 22

Blood Esterases as a Complex Biomarker for Exposure to Organophosphorus Compounds

Makhaeva G.[1,*], Rudakova E.[1], Boltneva N.[1],
Sigolaeva L.[2], Eremenko A.[2], Kurochkin I.[2],
and Richardson R.[3]

[1]*Institute of Physiologically Active Compounds, Russian Academy of Sciences, Moscow Region 142432, Chernogolovka, Russia*

[2]*Chemical Department, M.V. Lomonosov Moscow State University, Moscow 119992, Russia*

[3]*Department of Environmental Health Sciences, School of Public Health, The University of Michigan, Ann Arbor, MI 48109, USA*

Abstract. The growing threat of international terrorism brings with it new scenarios for disaster. For example, in the case of toxic organophosphorus compounds (OPs), it possible for terrorists to use known agents or inadvertently to produce highly toxic OPs of unknown structure as the result of attacks on chemical plants or stockpiles of pesticides and other chemicals. Defending against such agents requires rapid, sensitive, and specific detection of them and their biological effects. Thus, the development of biomarkers of human exposures to OPs is a vital component of the system of prediction and early diagnosis of induced diseases. The phosphylating properties of OPs lead to their differential interactions with various serine esterases. These enzymes include primary targets, e.g., acetylcholinesterase (AChE, acute toxicity) and neuropathy target esterase (NTE, delayed neuropathy, OPIDN); as well as secondary targets, e.g., butyrylcholinesterase (BChE) and carboxylesterase (CaE), which act as scavengers of OPs. The set of activities of these esterases as well as that of paraoxonase (PON1), which can hydrolyze and detoxify OPs, constitutes the "esterase status" of an organism that largely determines indi-vidual sensitivity to OPs and that may be used as a complex biomarker of exposure. This complex biomarker is more effective and informative than the standard determination of erythrocyte AChE and total blood cholinesterases. In particular, it assists with distinguishing between acute and delayed neurotoxicity induced by OPs, as we showed in experiments on acute exposure of hens to a neuropathic compound, *O,O*-dipropyl-*O*-dichlorovinyl phosphate. In addition, measuring decreased activities of BChE and CaE, which are often more sensitive biomarkers of OP exposure, allows us to reveal exposure to low doses, as demonstrated by treating mice with low doses of phosphorylated oximes. The aim of the ISTC Project summarized here is to develop a smart biosensor system for simultaneous analysis of a set of blood esterases including AChE, BChE, NTE, CaE, and PON1. The speed, sensitivity, and integrated approach of the method will allow hazards to be assessed and appropriate interventions to be recommended before overt toxic damage has occurred.

* Laboratory of Molecular Toxicology, Institute of Physiologically Active Compounds Russian Academy of Sciences, Chernogolovka, Moscow Region 142432, Russia, E-mail: gmakh@ipac.ac.ru boxylesterase, neuropathy target esterase, organophosphorus compounds (OPs)

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22.1. Introduction

The growing threat of international terrorism brings with it new scenarios for disaster. For example, in the case of toxic organophosphorus compounds (OPs), it possible for terrorists to use known agents or inadvertently to produce highly toxic OPs of unknown structure as the result of attacks on chemical plants or stockpiles of pesticides and other chemicals. Defending against such agents requires rapid, sensitive, and specific detection of them and their biological effects. Thus, the development of biomarkers of human exposures to OPs is a vital component of the system of prediction and early diagnosis of induced diseases.

The term *biomarker* is used to mean biological, biochemical, and molecular markers that can be measured by chemical, biochemical, or molecular techniques [1]. In humans, biomarkers must be present in easily and ethically obtainable tissues, one of which is blood. Biomarkers are usually divided into three categories: biomarkers of exposure, effect, and susceptibility [2].

The phosphylating properties of OPs containing pentavalent phosphorus lead to their differential interactions with various serine esterases. These enzymes include primary targets, e.g., acetylcholinesterase (AChE, acute toxicity) [3] and neuropathy target esterase (NTE, delayed neuropathy, OPIDN) [4], as well as secondary ones, e.g., butyrylcholinesterase (BChE) and carboxylesterase (CaE), which act as scavengers of OPs [5–7]. Recently, some other proteins possessing esterase activity have been identified as secondary targets for OPs: acylpeptide hydrolase, fatty acid amide hydrolase, arylformamidase, and albumin [7–10]. The set of activities of four blood serine esterases: AChE, NTE, BChE, and CaE, as well as serum paraoxonase (PON1), which can hydrolyze and detoxify OPs [11], is denoted by the term, "esterase status" of an organism. The esterase status incorporates aspects of susceptibility and exposure; i.e., it largely determines an individual's sensitivity to OPs, and it may be used as a complex biomarker of exposure to these compounds. This complex biomarker can be more effective and informative than standard determination of BChE activity in plasma, AChE activity in erythrocytes (red blood cells, RBCs), and NTE in lymphocytes [12]. It will allow us to accomplish several important goals: (1) assess an exposure as such and to confirm the nonexposure of individuals suspected to have been exposed; (2) determine if the exposure was to agents expected to produce acute and/or delayed neurotoxicity; and (3) perform dosimetry of the exposure, which provides valuable information for medical treatment.

Below we shall consider the enzymes constituting the esterase status, delineate their roles as biomarkers of exposure and susceptibility, and describe our approach to the development of a new multimodal biosensor for simultaneous determination of the activity of these blood esterases.

22.2. AChE: Role in OP Acute Toxicity and Use as a Biomarker

An immediate hazard associated with nerve agent OPs is acute cholinergic toxicity and death, arising from their inhibition of acetylcholinesterase (AChE) at cholinergic synapses of the central and peripheral nervous systems [13, 14]. The resultant cholinergic syndrome appears at approximately 50% inhibition of AChE throughout the nervous system, and >90% inhibition can result in death if no adequate treatment is provided [14, 15].

Cholinergic toxicity from OPs can be elicited solely by inhibition of AChE [3]. OPs inhibit the enzyme by phosphylation (e.g., phosphorylation, phosphonylation, or phosphinylation) of a serine hydroxyl group within the active site. The phosphylated enzyme regenerates extremely slowly, unless the reactivation is accelerated by particular nucleophiles such as oximes or fluoride. The AChE-OP conjugate can undergo loss of an OP ligand ("aging") to yield a negatively charged phosphyl adduct on the active site serine, but while this reaction has practical implications for therapy, it does not change the qualitative nature of the toxicity [16] (Figure 22.1).

The knowledge of structural and pharmacodynamic similarities between brain and RBC AChE within a given species has provided a rational basis for using RBC AChE inhibition by anti-AChE OPs as a surrogate measure of brain AChE inhibition by these compounds [3]. AChE activity in blood often corresponds to that in the target organs, and it can be considered as an appropriate parameter for biological monitoring of exposure to nerve gases and other anticholinesterases [15].

22.3. BChE and CaE: Role in OP Acute Toxicity and Use as Biomarkers

In a manner similar to their reaction with AChE, OPs can also react with BChE and CaE, but inactivation of these non-target enzymes does not contribute directly to the toxic effect. Like AChE, BChE (EC 3.1.1.8) and CaE (EC 3.1.1.1) possess a nucleophilic serine in their active sites and decrease the toxicity of OPs by acting as scavengers, i.e., alternative phosphylation sites, thereby decreasing the concentration of the OP available for interaction with AChE or other target sites [5–7, 18].

Because many OPs react with these non-target esterases *in vitro* more efficiently than with AChE, and given that plasma esterases would be the first binding sites encountered by OPs following their absorption into the blood, they tend to be more sensitive biomarkers than RBC AChE is, and can therefore detect exposure to lower doses of OPs. BChE activity measurements in either plasma (or serum) or whole blood are generally used as a sensitive biomonitor of the exposure to OPs [3, 17, 19]. In general, AChE and BChE, which have half-lives of 5–16 days, provide excellent biomarkers of exposure to OPs [15].

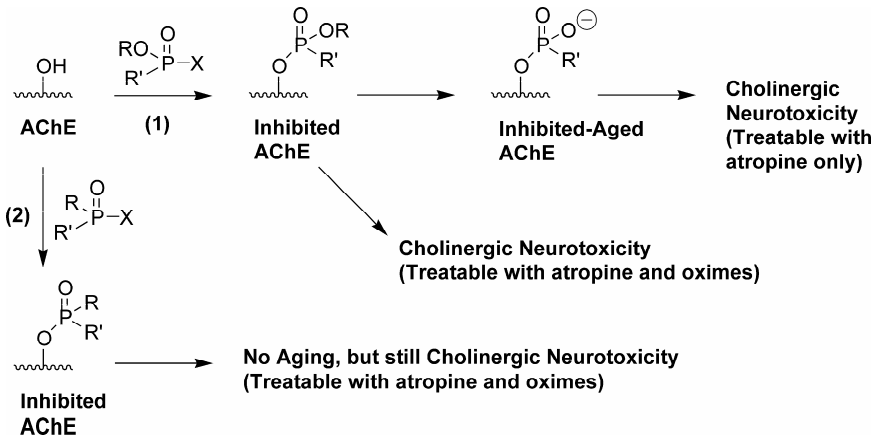

Figure 22.1. Reaction of AChE with OPs. Pathway (1) shows inhibition by a phosphonate, which inhibits the enzyme and can undergo loss of a ligand (aging) to yield a negatively charged phosphonyl group attached to the active site serine. Pathway (2) shows inhibition by a phosphinate, which inhibits the enzyme but does not undergo aging. In either case, cholinergic toxicity ensues, but AChE inhibited by the nonaging compound can be reactivated by powerful nucleophiles such as oximes. Reproduced from [4]. By permission

Determining decreases in BChE and CaE activities allows us to reveal exposure to low doses. This was demonstrated in our experiments on i.p. treatment of mice with low doses of two phosphorylated oximes, diethylphosphates (DEPs), possessing different leaving groups: $(EtO)_2P(O)ON{=}CXCl$, X = CH_2Cl (I) and $CHCl_2$ (II). In the kinetic experiments *in vitro*, DEPs were shown to be irreversible inhibitors of AChE, BChE, and CaE [20]. The values of the bimolecular rate constants of inhibition (k_i) are presented in Table 22.1. The data show that the DEP inhibitor activity toward AChE is lower than that against the secondary targets, BChE and CaE, and the introduction of Cl atoms into the leaving group resulted in a greater increase in the inhibitory potency of DEPs to BChE and CaE, than to the primary target, AChE.

Table 22.1. Inhibitor activity of DEP to AChE, BChE and CaE determined *in vitro* [20]

	k_i ($M^{-1}min^{-1}$)		
DEP	**AChE**	**BChE**	**CaE**
I (X = CH_2Cl)	6.76×10^4	1.74×10^6	5.37×10^6
II (X = $CHCl_2$)	1.26×10^5	9.77×10^6	7.42×10^7

It was found that i.p. injection of DEP (I) and (II) to mice at doses equal to 0.15 LD_{50} resulted in fast and substantial plasma BChE and CaE inhibition. Moreover, the compound that was more active *in vitro* (II) inhibited both enzymes to a greater extent (Figure 22.2a, b); the difference was significant at each time point (*t*-test, $p < 0.05$). RBC AChE was much less inhibited by both DEPs, and brain AChE was not inhibited. (Figure 22.2c, d). The results suggest a possible protective role of blood BChE and CaE against the exposure to DEP (I) and particularly (II), and a greater sensitivity of BChE and CaE compared to that of AChE as biomarkers of the exposure to DEPs. For both BChE and CaE, the efficiency of the esterase as a biomarker for the given compound

corresponded to the antiesterase activity of this compound *in vitro*; the higher the k_i (BChE) *in vitro*, the more intensive the inhibition of plasma BChE *in vivo*. The same relationship was found for CaE.

According to recent data, humans have a negligible level of CaE protein and CaE activity in blood [9, 21].Therefore, plasma CaE can be a scavenger of OP compounds and a biomarker in mice and rats, but not in humans. To check these data, we carried out measurements of the activity of AChE, BChE, and CaE in plasma and whole blood of rats and humans. Two substrates were used for CaE assay: 1-naphthyl acetate and phenyl acetate. Our results confirmed that rat plasma contains a high CaE activity, whereas in human plasma CaE activity is negligible (Figure 22.3). Furthermore, Figure 22.4 displays the activity of AChE, BChE and CaE measured in whole blood of rats and humans. Like rat plasma, rat blood also contains high CaE activity. Although CaE activity in human blood is low, it is a bit higher than that in plasma, which could be attributed to CaE activity in monocytes [22]. Inspection of these results indicates that rat and human blood have different esterase status.

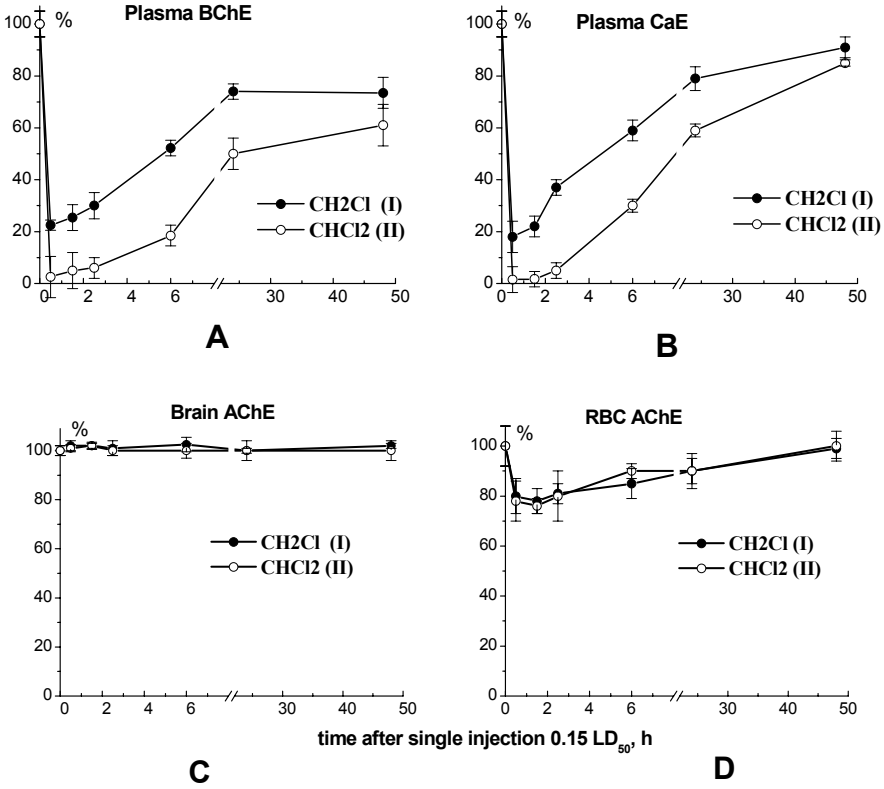

Figure 22.2. Time dependence of plasma BChE (**a**) and CaE (**b**), as well as brain (**c**) and erythrocyte (**d**) AChE activities of mice injected with DEP (I) and (II) in doses equal to 0.15 LD_{50}. Results are % control value for each tissue expressed as mean ± SEM, n = 3: plasma BChE 1.12 ± 0.04 μmol/(min × mL), plasma CaE 4.29 ± 0.14 μmol/(min × mL), brain AChE 8.23 ± 0.31 μmol/(min × g tissue), RBC AChE 0.398 ± 0.041 μmol/(min × mL erythrocyte)

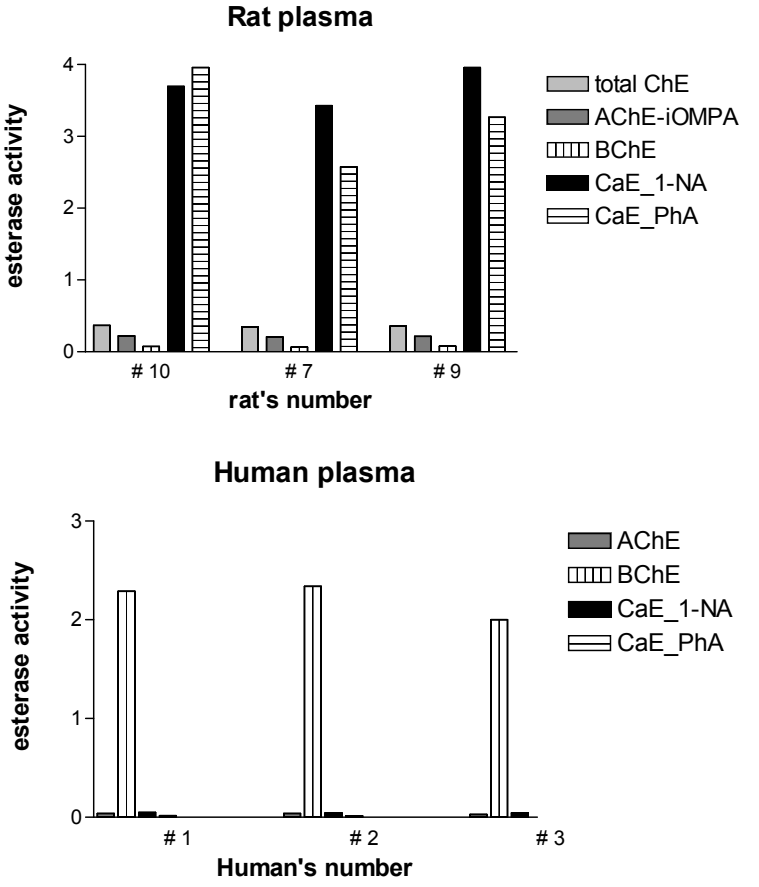

Figure 22.3. Activity of AChE, BChE, and CaE in plasma of individual Wistar rats and humans, μmol substrate/min/mL plasma. AChE activity was determined with the colorimetric method of Ellman [23], using acetylthiocholine (ATCh) as the substrate, and measuring the absorbance of the chromophore at 412 nm. Two specific inhibitors of BChE, tetraisopropylpyrophosphoramide (iso-OMPA) or Ethopropazine, were used to eliminate BChE activity. BChE activity was determined with the same method as AChE, using butyrylthiocholine as the substrate. "Total ChE" is the sum of soluble AChE and BChE determined using ATCh as the substrate. CaE activity was determined spectrophotometrically with a standard substrate, 1-naphthyl acetate (1-NA) [24]. The reaction was followed by monitoring the appearance of 1-naphthol at 322 nm (ε_{322} = 2,200 M^{-1} min^{-1}). To discriminate CaE activity, inhibitors of PON1/arylesterase (EDTA) and cholinesterases (physostigmine) were used. Phenyl acetate (PhA), which is used in our experiments for the biosensor esterases analysis, was also studied as a substrate for spectrophotometric CaE assay. The reaction was followed by monitoring the appearance of phenol at 270 nm (ε_{270} = 1,310 M^{-1} min^{-1})

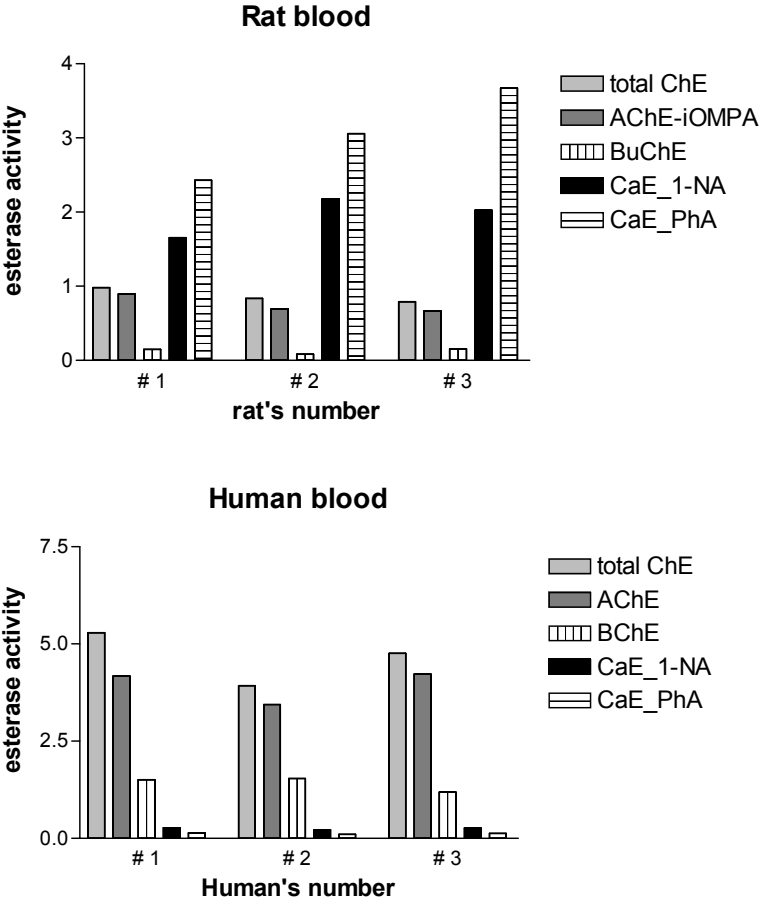

Figure 22.4. Activity of AChE, BChE, and CaE in whole blood of Wistar rats and humans, μmol substrate/min/mL blood. AChE activity was determined with the modified colorimetric method of Ellman [23], using acetylthiocholine (ATCh) as the substrate, and measuring the absorbance of the chromophore at 436 nm to diminish interference by hemoglobin absorption [25]. Two specific inhibitors of BChE, tetraisopropylpyrophosphoramide (iso-OMPA) or Ethopropazine, were used to eliminate BChE activity. BChE activity was determined with the same method as AChE, using butyrylthiocholine as the substrate. "Total ChE" is the sum of blood AChE and BChE determined using ATCh as the substrate. CaE activity was determined spectrophotometrically with a standard substrate, 1-naphthyl acetate (1-NA) [24]. The reaction was followed by monitoring the appearance of 1-naphthol at 322 nm (ε_{322} = 2,200 M^{-1} min^{-1}). To discriminate CaE activity, inhibitors of PON1/arylesterase (EDTA) and cholinesterases (physostigmine) were used. Phenyl acetate (PhA), which is used in our experiments for the biosensor esterases analysis, was also studied as a substrate for spectrophotometric CaE assay. The reaction was followed by monitoring the appearance of phenol at 270 nm (ε_{270} = 1,310 M^{-1} min^{-1}).

22.4. Neuropathy Target Esterase (NTE): Role in OP Delayed Neurotoxicity and Use as a Biomarker

Certain OPs can produce permanent neurological dysfunction, e.g., sensory deficits and paralysis, associated with OP-induced delayed neurotoxicity (OPIDN) [4, 26, 27]. Such compounds inactivate another serine hydrolase (NTE) in preference to AChE [28]. Because of this selectivity, neuropathic OPs may elicit little or no warning signs of acute cholinergic toxicity, so that victims of neuropathic OPs might not know they have been exposed until OPIDN develops 1–4 weeks later.

Neuropathic OP compounds have not heretofore been used in warfare or terrorist acts. However, from the viewpoint of their synthetic simplicity, absence of initial signs or symptoms of exposure, and lack of prophylactic or therapeutic measures, it is possible that rogue nations or terrorist groups will consider delayed neuropathic agents attractive as weapons of permanent incapacitation against military or civilian populations. In addition, neuropathic OPs of unknown structure might be produced from chemical reactions during terrorist acts at chemical plants or stockpiles of pesticides and other chemicals. Therefore, part of an effective chemical defense strategy is to develop methods for detecting delayed neuropathic agents via sensitive and selective biomarkers and biosensors [16, 29, 30].

A considerable body of evidence points to a neuronal serine hydrolase, NTE (EC 3.1.1.5), as the primary target molecule in OPIDN. OPIDN is initiated by the concerted inhibition and aging of a threshold level (>70%) of NTE in the central and peripheral nervous systems [26, 31–33]. As with other serine esterases, the inhibition of NTE is thought to occur by a nucleophilic attack of the active site serine (Ser^{966}) at the phosphorus of the OP, with displacement of a primary leaving group. Aging of the phosphylated enzyme, which is a relatively fast process (typical half-life of about 10 min or less), involves loss of a substituent from the inhibitor, leaving a negatively charged phosphyl moiety covalently attached to the active site serine (Figure 22.5).

Therefore, if an OP compound is expected to be a delayed neuropathic agent, it must be capable of inhibiting NTE; moreover, it appears that the resulting NTE-OP conjugate must also be capable of undergoing aging [4, 34].

While the physiological and pathogenic roles of NTE are being deciphered, the fact that an excellent correlation exists between the inhibition/aging of NTE within hours of exposure and the subsequent induction of OPIDN is sufficient for using this information for the development of biomarkers and biosensors for neuropathic OPs (i.e., compounds capable of producing OPIDN) [26, 29, 35, 36].

NTE inhibition has proved to be an excellent endpoint for *in vitro* assessment of the neuropathic potential of ageable OP compounds, e.g., phosphates, phosphonates, and phosphoramidates [4]. Moreover, the relative potency of an OP compound or its active metabolite to inhibit NTE versus AChE has been shown to correlate with the ratio of the neuropathic dose to the LD_{50} [26]. Values of the ratio k_i(NTE)/k_i(AChE) >1 indicate that the dose required to produce OPIDN is less than the LD_{50}, whereas values <1 correspond to doses higher than the LD_{50} being required to produce OPIDN [26, 34, 37]. Furthermore, according to Johnson (1982), the compounds for which the ratio

k_i(NTE)/k_i(AChE) is ≥0.05 should be subjected to careful toxicological study, because neuropathies caused by intoxication with such compounds may develop after successful therapy for acute cholinergic poisoning.

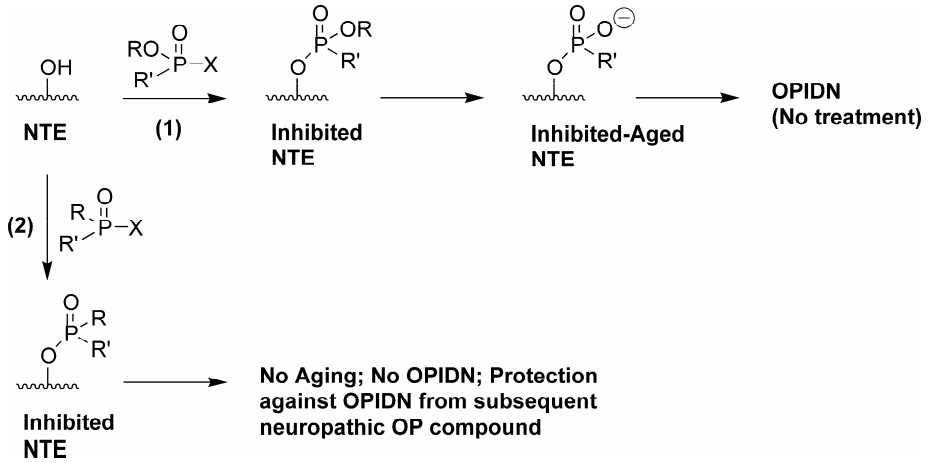

Figure 22.5. Reaction of NTE with a phosphonate in pathway (1) yields a phosphonylated (inhibited) enzyme, which can undergo net loss of an R-group to yield an inhibited-aged enzyme. Inhibition alone does not produce OP-induced delayed neurotoxicity (OPIDN); both inhibition and aging are required, and there is no treatment for the neuropathy. Reaction of NTE with a phosphinate in pathway (2) yields a phosphinylated (inhibited) enzyme, but because of the stability of the C-P bonds linking the R- and R′-groups to phosphorus, the phosphinyl moiety on NTE does not undergo aging, so that OPIDN does not occur. Inhibition of NTE with a nonaging inhibitor is not biological inert; it protects against subsequent exposures to neuropathic (age-able) NTE inhibitors. R, R′ = substituted or unsubstituted alkyl or aryl groups, which can be different or equivalent. X = primary leaving group, e.g., fluoride. Reproduced from [4] by permission.

The discovery of NTE in circulating lymphocytes and platelets [38–41] enabled it to be used as a biomarker of animal and human exposure to neuropathic OPs [41–45]. In addition, lymphocyte NTE inhibition has been suggested as a predictor of OPIDN or an adjunct for its early diagnosis [41, 46].

The development of an electrochemical method for NTE assay enabled measuring NTE activity using small volumes (about 100 μL) of whole blood [29, 47]. Such an approach used tyrosinase-based biosensors. The high sensitivity of the tyrosinase carbon-paste biosensors for the phenol produced by hydrolysis of the substrate, phenyl valerate, allowed NTE activity to be measured in diluted samples of whole blood; it cannot be done using the standard colorimetric assay [29, 47–49]. The NTE activity in human blood determined using the tyrosinase biosensor was (mean ± SEM, n = 3) 47.4 ± 2.5 nmol phenol/(min × mL blood) [47].

The developed biosensor was used to establish correlations of NTE inhibitions in blood with that in lymphocytes and brain 24 h after dosing hens with a neuropathic OP, *O,O*-dipropyl-*O*-dichlorvinylphosphate, PrDChVP [29].

The experimental data are presented in Figure 22.6. Brain, lymphocyte, and blood NTE were inhibited in a dose-responsive manner (linear trend, $p < 0.0001$), and each NTE compartment showed a similar pattern and degree of inhibition. Thus, there were strong correlations of NTE inhibition between brain, lymphocytes, and blood [29]. These results support the use of whole blood NTE inhibition measured by the biosensor method as a biochemical marker for exposure to neuropathic OPs. Furthermore, these results are in agreement with previous work using lymphocyte NTE as a biomarker within 24 h of exposure [45]. In addition, the results indicate that whole blood NTE inhibition reflects NTE inhibition in brain within 24 h of exposure [29].

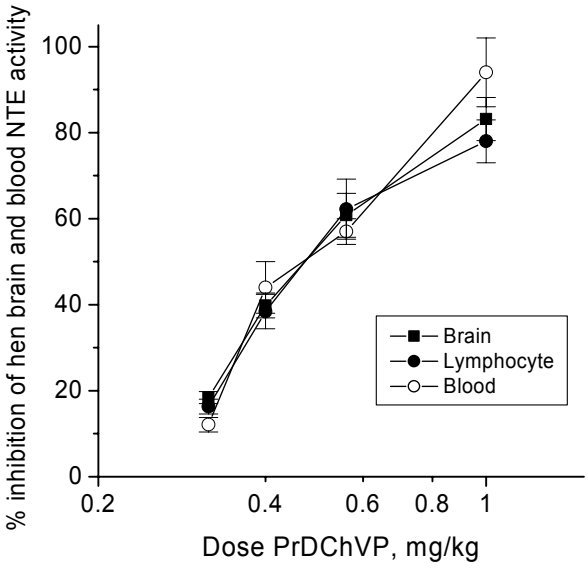

Figure 22.6. The dose-related NTE inhibition in brain, lymphocytes, and whole blood of hens 24 h after injection of the neuropathic OP, O,O-di-1-propyl O-2,2-dichlorvinyl phosphate (PrDChVP). The NTE activity in brain and lymphocytes was determined colorimetrically and in whole blood amperometrically using the tyrosinase carbon paste biosensor. The results are % control value for each tissue expressed as mean ± SEM, n = 3. Control NTE activities, nmol/(min × mg protein), mean ± SEM, n = 3: brain = 30.9 ± 2.8, lymphocyte = 9.0 ± 1.4, whole blood = 0.107 ± 0.013. Adapted from [29] by permission.

To evaluate the time dependence of blood NTE inhibition, we studied NTE inhibition in brain and blood at 4, 24, 48, 72, and 96 h after the injected dose of 1.0 mg/kg [36]. Results are shown in Figure 22.7. NTE activity in both compartments differed significantly from the respective control values (zero time) at all time points ($p < 0.0001$, ANOVA, Dunnett's posttest). During all the measured times, the brain NTE was inhibited (mean ± SE, $n = 5$) 72 ± 4% and the blood NTE 75 ± 3% relative to controls. The results demonstrate that whole blood NTE is a reliable biomarker of exposure to neuropathic OPs during 96 h between the exposure and the measurement [36].

These studies indicate that NTE in lymphocytes or whole blood can be assayed and used as a biomarker of exposure to delayed neuropathic OPs, particularly if blood samples are taken within 24 h of acute exposures. Attempts to measure aging of NTE in lymphocytes or platelets of exposed subjects have not been done. However, if aging of NTE inhibited by neuropathic OPs typically occurs within a half-life of a few minutes [26, 50], aging would be completed by the time that blood or lymphocyte samples could be taken and assayed.

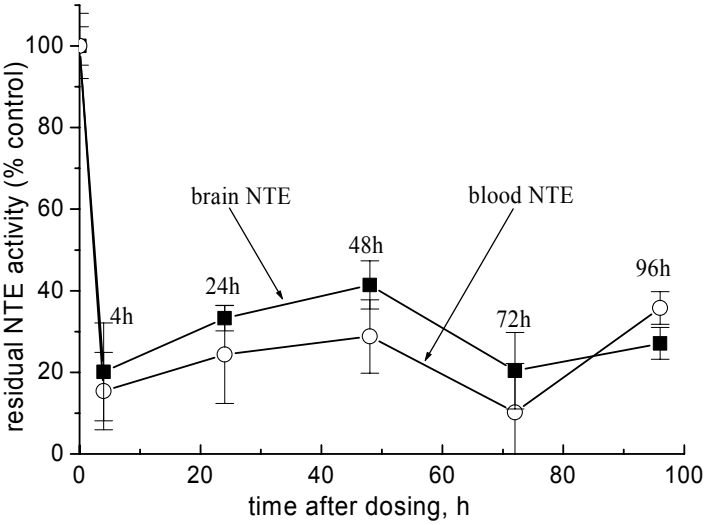

Figure 22.7. Time-dependence of NTE activity in brain and whole blood of hens injected with 1 mg/kg of the neuropathic OP, PrDChVP. NTE assayed colorimetrically in brain and amperometrically in blood; both tissues assayed after freezing and thawing. The results are % control value for each tissue expressed as mean ± SEM, n = 3. Control NTE activities, nmol/(min × mg protein), mean ± SEM, n = 3: brain = 21 ± 2, whole blood = 0.23 ± 0.02. Adapted from [36]. With permission

Nevertheless, as stated above, given the apparent requirement for aging as well as the inhibition of NTE in OPIDN, to help rule out false positives arising from inhibition by nonaging inhibitors (Figure 22.5), future work could be undertaken to identify aged or nonaged NTE, perhaps by using mass spectrometry [9].

In keeping with the concept of relative potency discussed above, inhibition of NTE in blood can be used in conjunction with inhibition of RBC AChE and plasma BChE to assess the likelihood that an exposure to OP compounds would produce cholinergic and/or delayed neuropathic effects. RBC AChE inhibition has been used as a biomarker of exposure to conventional nerve agents or OP insecticides [15, 17, 51]. BChE can be sensitive to both conventional and delayed neuropathic agents, and its inhibition could thus serve as a general biomarker for OPs [52–54]. Lymphocyte and blood NTE have been shown to be the biomarkers of exposure to neuropathic OPs.

To test this suggestion, we determined AChE and NTE activity in brain and blood of hens in 24 h after acute i.m. administration of increasing doses of PrDChVP. The determined *in vitro* relative potency value, k_i(NTE)/k_i(AChE), for this compound was 2.45, suggesting that PrDChVP would have the potential to cause OPIDN at doses lower than LD_{50}. The results of the experiment are presented in Figure 22.8. The inhibition of both NTE and AChE was dose-dependent in brain and blood, and NTE in brain and blood was inhibited at every dose to a greater extent than AChE (*t*-test, $p < 0.05$).

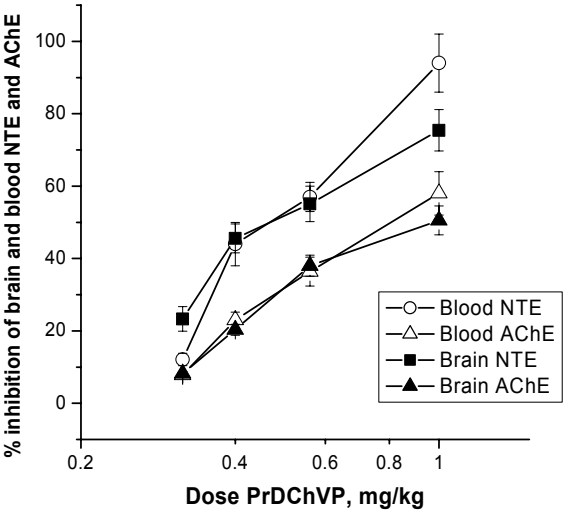

Figure 22.8. The dose-related NTE and AChE inhibition in brain and blood of hens injected with neuropathic OP compound PrDChVP. The results are expressed as means ± SEM, n = 3

The average from the ratios of NTE to AChE inhibition in hen brains at each of the four doses of PrDChVP (linear region [55] was equal to 2.00 and in hen blood to 1.88, in agreement with the *in vitro* k_i(NTE)/k_i(AChE) ratio of 2.45.

The results show that the simultaneous determination of AChE and NTE in blood has the potential to discriminate between exposures to acute or delayed neurotoxic agents.

22.5. Paraoxonase (PON1): Role in OPs Toxicity and Use as a Biomarker of Individual Susceptibility

Human serum paraoxonase (PON1, aryldialkylphosphatase, EC 3.1.8.1) is a calcium-dependent hydrolase that is tightly associated with high-density lipoprotein (HDL) particles [56, 57]. PON1 received its name from paraoxon, its first and one of the most studied substrates. PON1 hydrolyzes the active metabolites (oxons) of several other OP insecticides (e.g., chlorpyriphos oxon, diazoxon, and methylparaoxon) as well as nerve

agents such as soman, sarin, tabun or VX [11], and it plays a central role in their detoxification and toxicity. For example, animals with low paraoxonase levels (birds) are more sensitive to specific OPs than those with high enzyme levels (rats and especially rabbits) [58, 59]. Moreover, recent animal studies have convincingly shown that PON1 plays a major role in the detoxification of OPs processed through the P450/PON1 pathway.

PON1 is a broad-spectrum enzyme that, along with paraoxonase activity, has high arylesterase and lactonase activities [60, 61]. A comparison of PON1-catalyzed hydrolysis of phenyl acetate and paraoxon in rat and human plasma (Table 22.2) shows that the phenyl acetate-hydrolyzing activity of PON1 far exceeds its paraoxon-hydrolyzing activity, and that the paraoxon-hydrolyzing activity in humans is lower than that in rats.

In human populations, serum paraoxonase exhibits a substrate-dependent polymorphism as well as a large variability in plasma levels among individuals. The Q192R polymorphism, Gln(Q)/Arg(R) at position 192, imparts different catalytic activities toward some OP substrates [62]. The polymorphism at position -108 (T/C), in the promoter region of PON1, is the major contributor to differences in the level of PON1 expression and appears to have the major effect on the levels of PON found in plasma of individuals [63]. These two factors determine to a great extent an individual's sensitivity to OPs exposure [64, 65]. The available data on the developmental time course of the appearance of PON1 in plasma showed that the serum PON1 activity is low in newborns and infants, and increases gradually during early development [66].

Table 22.2. PON1-catalyzed hydrolysis of phenyl acetate and paraoxon in rat and human plasma (μmol substrate/(min × mL plasma)

Assay conditions	Rat #1	Rat #2	Rat #3	Mean ± SEM
PON1 – phenyl acetate 0.1 M Tris-HCl buffer pH 8.0, 40 μM physostigmine, 0.1 M $CaCl_2$, 4 mM PhA, λ 270 nm	115.56 ± 8.29 $n = 5$[a]	81.57 ± 10.21 $n = 8$	95.83 ± 2.07 $n = 8$	97.66 ± 9.85 $N = 3$[b]
PON1 - paraoxon 0.1 M Tris-HCl buffer pH 8.0, 40 μM physostigmine, 0.1 M $CaCl_2$, 1.2 mM PO, λ 405 nm	0.0824 ± 0.0021 $n = 4$	0.0741 ± 0.0015 $n = 5$	0.0980 ± 0.0023 $n = 5$	0.0848 ± 0.007 $N = 3$
	Human #1	**Human #2**	**Human #3**	**Mean ± SEM**
PON1 – phenyl acetate 0.1 M Tris-HCl buffer pH 8.0, 40 μM physostigmine, 0.1M $CaCl_2$, 4mM PhA, λ 270 nm	47.12 ± 0.92 $n = 7$[a]	80.65 ± 1.56 $n = 6$	62.85 ± 1.09 $n = 6$	63.54 ± 9.69 $N = 3$[b]
PON1 – paraoxon 0.1 M Tris-HCl buffer pH 8.0, 40 μM physostigmine, 0.1 M $CaCl_2$, 1.2 mM PO, λ 405 nm	0.0173 ± 0.0002 $n = 6$	0.0268 ± 0.0005 $n = 5$	0.0268 ± 0.0014 $n = 4$	0.0236 ± 0.0032 $N = 3$

[a] n = number of replicates; [b] N = number of subjects

These data suggest that a higher sensitivity of young animals to OP toxicants could be explained, at least partially, by a deficiency in PON1 activity [59, 67]. Several major factors (environmental chemicals, drugs, smoking, alcohol, diet, age, and various diseases) have been shown to modulate PON1 activity in either direction [68]. Decreased serum PON1 activity can result in an increased sensitivity to OP toxicants upon exposure [69].

The information outlined above demonstrates that assessment of the esterase status of an organism that includes assay of AChE, NTE, BChE, CaE, and PON1 activities could give a more complete picture of exposure to OPs than assay of any of these ac-tivities alone. As we have seen, the esterase status provides information not only on exposure, but also individual susceptibility, type of toxicity (e.g., acute or delayed), and prognosis of toxicity.

Currently, the activities of blood esterases are determined separately using the corresponding substrates and selective inhibitors and, as a rule, spectrophotometric or less often titrimetric or radiometric techniques. Carrying out separate assays of a set of esterases is a time-consuming and cumbersome process that is ill-suited for high throughput investigations and biomonitoring of large numbers of samples.

The application of modern electrochemical biosensors is a good alternative to currently available methods, mainly due to the high sensitivity achieved in recent years for the key analytes, such as phenol and hydrogen peroxide. Thus, it is now possible to increase the sensitivity of blood esterase assays significantly and to simplify the analysis procedure. An example of such an approach is the development of a tyrosinase-based biosensor for NTE assay described above. We also demonstrated that AChE and BChE can be determined in blood with acetylcholine and butyrylcholine as substrates using the biosensor for choline, and for CaE using the biosensor for ethanol with ethyl butyrate as a substrate [70].

Currently, the ISTC project 3130 continues to be under development. The aim of this project supported by the EC is to develop a smart biosensor system for the simultaneous analysis of a set of blood esterases including AChE, BChE, NTE, CaE, and PON1. This task can be solved by using the biosensors and sensor arrays formed by the universal layer-by-layer (LBL) technology. The latter provides modern levels of sensitivity, specificity, speed, and manufacturability. The sensor array application can provide the measurement of multiparameter analytical responses that can be analyzed using modern mathematical approaches enabling the simultaneous quantitative assay of a mixture of several esterases. The biosensor approach to the esterases assay is described in the current volume in the paper of Kurochkin et al. [49]. The data that were obtained with standard spectrophotometric methods and that provide the esterases activity in rats and human blood (Figures 22.3, 22.4 and Table 22.2) will be used in the Project for the validation of new biosensor measurements.

The advantages of the developed biosensor include the following: fast assessment of the esterase status of an organism using minimal blood sample volumes; fast assessment of individual susceptibility to OPs; effective detection of exposure to OPs and carbamates; and more accurate diagnostics of poisoning with discrimination of acute and delayed neurotoxicity. A broad clinical application of the developed biosensor with the advent of approaches for individualized therapy should also be possible.

The developing approach is not devoid of the limitations inherent in enzymatic methods of biomonitoring. However, the speed, sensitivity, and integrated approach of the method will allow the hazard and appropriate intervention to be interpreted before overt toxic damage has occurred. The development of the proposed biosensor device and methodology of esterase status determination will improve risk assessments relating to human health effects arising from terrorist acts, incidental or occupational exposures, or ecological effects arising from OPs discharges into the environment. It also provides a tool for carrying out epidemiological studies and acquiring a baseline database for these important esterase levels in human populations.

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Chapter 23

Diagnosis of Exposure to Chemical Warfare Agents: An Essential Tool to Counteract Chemical Terrorism

Noort D., Van der Schans M.J., Bikker F.J., and Benschop H.P.
Business Unit Biological and Chemical Protection, TNO Defence, Security and Safety, The Netherlands

Abstract. Methods to analyze chemical warfare agents (CW-agents) and their decomposition products in environmental samples were developed over the last decades. In contrast herewith, procedures for analysis in biological samples have only recently been developed. Retrospective detection of exposure to CW-agents is useful for various applications. It can be envisaged that rapid diagnosis will play a pivotal role in the management of a terrorist attack with CW-agents. In the same context, confirmation of non-exposure of worried citizens is of utmost importance to suppress unrest in the society at large. Also, such methods can be used for forensic analyses in case of suspected terrorist activities ("chemical fingerprints"). These methods will also be valuable in case of a military conflict, e.g., to establish firmly to which chemicals casualties have been exposed to, as a starting point for adequate medical treatment. Finally, the procedures can be used for health surveillance of workers in destruction facilities of CW-agents. This presentation will deal with specific methods currently available for diagnosis of exposure to the most common CW-agents, i.e., nerve agents and mustard agents. Although the presentation will focus on GC/MS and LC/MS analyses of the long-lived protein adducts of these agents, some attention will be paid to metabolites in urine and immunochemical detection of DNA- and protein adducts. The utility of the presented methods will be exemplified on the basis of exposure incidents.

Keywords. Chemical warfare agents, diagnosis, adducts, proteins, mass spectrometry, immunoassay

23.1. Introduction

Qualitative and quantitative information on exposure to CW-agents can be obtained by means of analysis of biomedical samples, e.g., urine and blood. Detection of covalent adducts and metabolites provide forensic evidence in cases of military or terrorist use of these agents. Such methodology can also be used for diagnostic purposes to ensure administration of appropriate medical countermeasures and for monitoring exposure of workers engaged with demilitarization and other defensive activities. This paper deals with the biological fate of the major CW-agents, their metabolites and adducts that are useful as biological markers of exposure, and analytical methods for their detection [1].

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Presently, biomedical sample analysis is unlikely to give information in the early phases of response to a terrorist or military incident and guide the initial response phase of the incident. Therefore this chapter will not only summarize time-consuming and mostly retrospective procedures to analyze adducts of sulfur mustard and nerve agents with proteins by means of LC- and/or GC-MS methods but will also deal with attempts to develop rapid "point of care" methodology mostly based on relatively simple immunoassays. The latter approach may also involve target analyses having a short half life such as DNA-adducts and low molecular weight metabolites in blood and urine. In contrast herewith, CW-adducts with proteins have half lives up to several months, allowing analysis of biomedical samples in specialized laboratories, collected weeks or even months after the incident.

23.2. Sulphur Mustard (HD)

The analysis of urine and blood samples from two individuals who had been exposed to sulphur mustard (HD) accidentally during the destruction of a WW1-shell is illustrative for the approach as laid out in the introduction. Urine and blood samples were collected from both a highly blistered patient (patient 1; 6.5% of the total body surface area) and patient 2 who had only one small blister, starting on day 2 after the exposure and then three weekly samples were taken 4–6 weeks after the exposure. The urine samples were analyzed by means of LC-MS analysis of oxidized derivatives of the highly characteristic metabolite 1,1'-sulfonylbis[2-(methylthio)ethane], (SBMTE), which are formed via a β-lyase reaction of the bis-adduct of HD with glutathione subsequent to partial hydrolysis to the bis-cysteinyl adduct. As expected the highest level of SBMTE was found in patient 1 in the earliest urine sample, with a very rapid decline over the first 4–5 days. The half-life for the excretion of the oxidized derivatives of SBMTE was less than 1 day. Traces of this metabolite were also found in the other patient [2].

Blood samples of the two patients and blister fluid from patient 1 were analyzed for adducts of HD with cysteine-34 in albumin. The albumin was isolated by means of affinity chromatography and was digested with pronase. The resulting tripeptide cysteine(34)-proline-phenylalanine with HD adducted to the cysteine component was analyzed by means of LC-MS/MS. As illustrated in Figure 23.1, the adduct in the blood of patient 1 could be detected up to at least 40 days after the incident resulting in a half-life of 22 days. Interestingly, the adduct level of albumin in the blister fluid taken from patient 1 at 7 days after the exposure was approximately the same as that in blood at the same point in time [3].

A standard operating procedure is available for an immunoslotblot assay using a monoclonal antibody against of the major adduct of HD with DNA, i.e. the N7-adduct with 2'-deoxyguanosine for use in lymphocytes and granulocytes in human blood and in human epidermis [4]. The limit of detection for this immunoslotblot assay corresponds with a 50 nM in vitro exposure of human blood to HD. Moreover, the N7-adduct of guanine was shown to be excreted into urine of guinea pigs after administration of HD at iv doses >0.5 mg/kg [5]. The adduct was excreted mostly within the first 24 h and was analyzed by means of LC-MS/MS. These approaches were not applied for DNA-adduct

analysis in the abovementioned patients, presumably because an immunochemical assay is considered to be less specific than MS-based methods. Obviously, this drawback is not valid for MS-based analysis of the N7-guanine adduct excreted into urine.

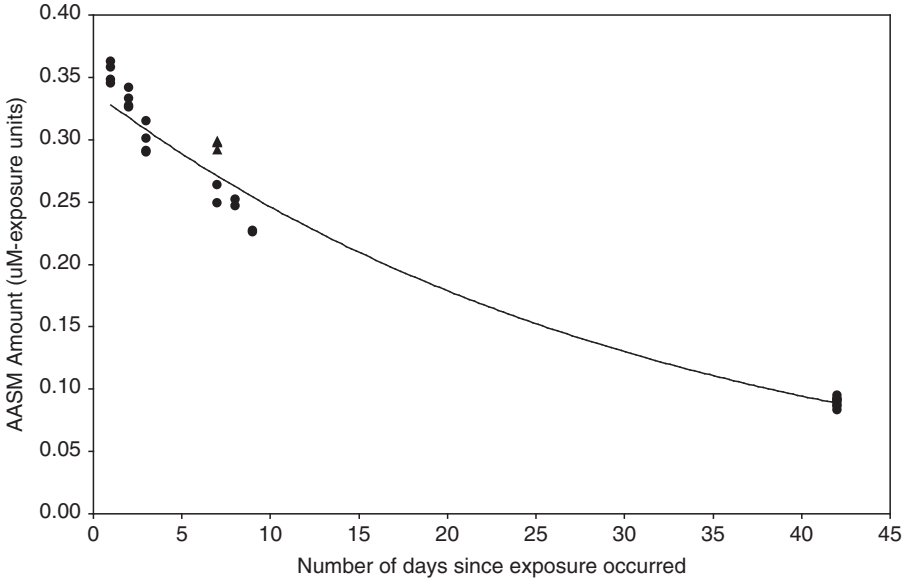

Figure 23.1. Time course of the levels of adduct of HD with cysteine-34 of albumin in blood (●) of a patient (patient 1) accidentally exposed to HD. The adduct level in blister fluid (▲) taken at 7 days after exposure is also given

The immunoslotblot assay has been applied on erythrocytes and granulocytes in blood samples of two Iranian casualties of the Iran–Iraq war, taken 22 and 26 days after the alleged exposure to HD [6]. One victim suffered from skin injuries compatible with HD exposure. The other patient had only vague complaints. The results were compared with those of a GC-MS analysis of the adduct of HD with N-terminal valine in hemoglobin [7]. All analyses proved to be positive. The estimated exposure levels based on the amounts of analyzed adducts were lower for the immunoslotblot assay than for the protein-adduct based analysis. Presumably, considerable repair of the DNA-adducts had taken place in the more than 3-week period in between exposure and blood sampling.

Recently, attempts have been made to use the abovementioned monoclonal antibody 2F8 in a "hand-held point-of-care diagnostic tool", based on lateral flow immunochromatography. In this rapid "sandwich-type" assay the monoclonal antibody is immobilized as the Test Line on to a nitrocellulose membrane. HD-exposed DNA which is extracted from blood (5–10 μL) is added to the Sample Pad. After addition of the sample a protruding absorbent tip is dipped into a vial containing the running buffer that initiate the lateral flow immuno-chromatography. Then, the adducted single-stranded DNA is captured on the Conjugate Pad by the same monoclonal antibody which is covalently coupled to blue-dyed latex beads serving as a visual tag. As the sample runs further on the nitrocellulose membrane, the complex is captured by the immobilized

monoclonal antibody on the Test Line. Results are visually read after 10 min. The proof of principle for this device has been performed [8]. In another study, a similar 2F8-based hand-held HD detection device was generated. This device was able to detect HD on contaminated surfaces as well as HD-vapour.

Since the skin is a major target for HD it would seem obvious to develop immunoassays for protein adducts of the agent in this organ, in addition to those for DNA-adducts (vide supra). Keratins are abundant proteins in stratum corneum and epidermis. Evidence has been obtained that esters of glutamic and aspartic acid in keratins with the hydrolysis product thiodiglycol of HD are the most abundant adducts upon exposure of skin to the agent. Therefore, attempts have been made to raise antibodies against these esters in partial structures of keratins [9]. Although antibodies were obtained that were promising in immmunofluorescence experiments, the overall results so far have been variable. Further alternative attempts are made to obtain promising antibodies [10].

23.3. Nerve Agents

Classical procedures to detect and identify exposure to nerve agents and organophosphate pesticides have serious shortcomings. The intact compound and its metabolites can only be measured shortly after exposure. Alternatively, measurement of cholinesterase inhibition in blood (i) does not identify the organophosphate, (ii) does not provide reliable evidence for exposure at inhibition levels less than 20%, and (iii) is not suitable for retrospective detection of exposure due to *de novo* synthesis of enzyme. The obvious shortcomings of detection based on cholinesterase inhibition are due to the overcomes the last mentioned shortcoming. The new approach is based upon reactivation of phosphylated BuChE with fluoride ions. This converts the organophosphate moiety into the corresponding phosphofluoridate which is subsequently isolated and quantified by means of GC-MS. As in the case of measurement of hydrolysis products, this approach identifies the organophosphate except for its original leaving group. Based upon the minimal concentrations of phosphofluoridate that can be measured in blood it is calculated that inhibition levels of BuChE $\geq$ 0.01% can be analyzed. Evidently, inhibition levels can now be measured that are several orders of magnitude lower than those based on residual cholinesterase activity. The method is limited by spontaneous reactivation and ageing (loss of alkyl from the alkoxy group of the phosphyl moiety) and by the natural life span of the inhibited enzyme.

Application of the new method to serum samples of victims from the Tokyo subway attack and of the Matsumoto incident yielded sarin concentrations in the range of 0.2–4.1 ng/mL serum. Evidently, these victims had been exposed to an organophosphate with the structure iPrO(Me)P(O)X, presumably with X = F (sarin). The amounts of regenerated sarin correlated with the increase of enzyme activity due to reactivation with oxime in the same serum samples. It was concluded that fluoride-reactivated sarin was bound to the active site of the enzyme and not to an unspecific binding site.

Upon analysis of blood samples from rhesus monkeys which had been challenged with a sign-free dose of the five classical nerve agents, regenerated phosphofluoridates could be measured over a period of 2–8 weeks, depending on the structure of the nerve agent. It is concluded that the retrospectivity of the new procedure is promising [11, 12].

As mentioned earlier, one of the limitations for the retrospectivity of the fluoride reactivation approach is due to the ageing process since the aged phosphylated BuChE is not reactivatible with fluoride ions. With this limitation in mind, Noort and coworkers [13] developed an alternative procedure based on isolation of (un)inhibited BuChE from plasma by means of affinity chromatography on a procainamide column, followed by pepsin digestion of the enzyme and LC/electrospray tandem MS analysis of a specific nonapeptide containing the phosphylated active site serine-198 residue. In this approach the aged and non-aged peptide fragment are measured separately. Application of the new procedure to serum samples from the Tokyo incident confirmed the results obtained by means of reactivation with fluoride ions. Currently, *in vitro* inhibition levels of 2–5% can be detected in plasma.

The new procedure could also be applied for analysis of BuChE inhibited by, e.g., diethyl paraoxon and pyridostigmine bromide which demonstrates its wide applicability, especially for biomonitoring of exposure for health surveillance of pesticide workers and of the population at large. Recently, more generic approaches of this particular method were published [14, 15]. In this variant, the respective phosphylated nonapeptides in the pepsin digests were converted with $Ba(OH)_2$ into a dehydroalanine derivative in the presence of a nucleophilic tag (e.g., a thiol or an amine). In this way the phosphylated nonapeptides were transformed into a common tagged nonapeptide for analysis by means of LC/MS, without need for prior knowledge of the structure of the inhibitor. Also, part of the procedure could be successfully automated, which holds promise for future high sample throughput in case of large scale incidents with CW-agents [16].

In an inhibition variant of the earlier mentioned lateral flow immuno-chromatography assay [8] soman and some analogs could be detected using a monoclonal antibody against soman. Also, attempts are being made to develop an immunochemical assay for BuChE inhibited by organophosphate anticholinesterases [17]. Since the target organophosphate is bound to serine-198 in the active site gorge of the enzyme it is hidden for sensing molecules. Therefore, the enzyme is digested with a protease (trypsin, pepsin) releasing the fragment that binds the organophosphate which is now accessible for antibodies. In order to develop a generic immunoassay by analogy with the abovementioned generic mass spectromeric approach, an immunogenic tag, e.g., a 2,4-dichlorobenzylamine moiety, is bound to the dehydroalanine derivative of the target peptide. Several hybridomas were generated which produce polyclonal antibodies that are able to discriminate between the tagged and untagged peptide [17].

23.4. Conclusions

Several procedures have been developed to analyze protein adducts of mustard and nerve agents in biomedical samples by means of mass spectrometric methods in combination with chromatographic separation methods. This approach proved its value in several analyses of samples originating from terrorist attacks and military use of CW-agents.

Further improvements in detection limits and therefore in retrospectivity will hinge on future enhancements in sensitivity and resolution of mass spectrometry instruments in general and of hybrid configurations in particular.

Immunoassays for rapid point-of-care analysis of exposure to CW-agents need further development in order to allow "routine-like" laboratories to contribute to analyses of biomedical samples.

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Chapter 24

Assessing Chronic Exposure to Anticholinesterase Pesticides by Hair Analysis

Tsatsakis A.*, Toutoudaki M., and Tzatzarakis M.
Centre of Toxicology Sciences and Research, Division of Morphology, Medical School, University of Crete, Voutes, Heraklion, 71003, Crete, Greece

Abstract. The present paper explores the possibility of using hair as a suitable biomarker of chronic exposure to anticholinesterase pesticides. Research results already available confirm that hair is a suitable biomarker of past exposure to drugs and pharmaceuticals. Recent experimental evidence suggests that hair is a suitable biomarker for the assessment of chronic exposure to organophosphate and carbamate pesticides. Animal studies have indicated that hair concentrations of the pesticide diazinon are dose dependent. Hair has been used as biomarker of chronic and recent exposure to anticholinesterase pesticides in an epidemiological study conducted in Crete. Experimental data confirmed the presence of organophosphates in hair samples of the rural population examined.

Keywords. Organophosphate pesticides, hair, biomarker, gas chromatography-mass spectrometry, chronic exposure

24.1. Introduction

Toxicological analysis of non-conventional biological samples (e.g. hair, saliva, sweat, and sperm) has significant value and many applications in several areas of medical, forensic and environmental science. These samples may provide important additional information and possess certain advantages (e.g. sampling is time-efficient, practical, cost-effective, non-invasive and a second sample giving similar information to the first can be easily obtained) over the conventional biological samples. Among the non-conventional biological samples, hair can provide the most vital information, with sectional hair analysis being the most widely used application [1].

The main advantage of hair is that it retains trapped information for prolonged periods of time. Analyte stability in hair has been demonstrated in 4,000 years old mummies, in which small amounts of cocaine metabolites were found [2]. This is attributed to the absorption and trapping mechanism that exists in the hair, taking place during keratinisation of the newly formed cells.

* Corresponding Author: Aristidis M. Tsatsakis, Ph.D., D.Sc., ERT, Head of Forensic Sciences Department, Medical School, University of Crete, 71409 Heraklion, Crete, P.O. Box 1393, Greece; E-mail: aris@med.uoc.gr

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Several studies have provided contradicting evidence on the dose–response relationship between chemical ingestion and chemical titres in the hair. Despite the concerns expressed by the scientific community with respect to the role of hair testing, it has already been applied in forensic investigations, historical research, autopsy, adoption cases, exclusion of evidence, serial criminal cases, rape cases, doping control, as well as other scientific and/or legal cases [3]. Another possible application would be in clinical practice. Sectional hair testing may be utilized to check compliance to therapy regime for people under long-term treatment for several diseases [4].

Hair analysis has been successfully used to assess chronic exposure to various chemicals like drugs of abuse, medicines and heavy metals. Recently, analysts give their attention to the possibility of using hair analysis for the assessment of exposure to organic pollutants. The most studied compounds are organochlorine pollutants, like pesticides, dioxins and PCBs [5–13] and currently used pesticides belonging to the families of anticholinesterase agents like organophosphates (e.g. diazinon, malathion) [14–16] and carbamates (e.g. methomyl, carbaryl) [17–18].

Hair analysis is feasible due to the special characteristics of hair, and the most basic ones are described below.

Although hair might appear as a simple and homogeneous structure to a non specialized observer, it is in fact a very complex part of human anatomy. Hair consists of long shafts created of closely packed cells that emerge from the follicles. The mean diameter of a single hair shaft ranges from 15–120 μm, in humans, depending on the type of hair and the body area that each follicle is located. Hair is rich in keratins, a family of proteins with a high content of sulfur. Inside the shaft, keratin forms long fibers linked to each other by sulfur bridges and other types of bonds between keratin and other proteins, creating a very stable structure.

The hair could be described as cross linked polymer containing a large number of chemical functional groups, capable of trapping small molecules. Human hair consists of 65–95% proteins, 15–35% water and 1–9% lipids. Lipids originate from sebum and the secretions of apocrine glands and consist of free fatty acids, mono and di-glycerides, waxes carbohydrates and aliphatic alcohols. Human hair is rich in the aminoacids threonine, aspartic acid, glutamic acid, lysine, cystine and tyrosine [19].

Substances in the blood-circulating system, which enter the hair via the follicle, are trapped and retained in specific parts of the hair. Sweat and sebaceous glands also play a basic role in the process of drug deposition in hair. Water-soluble compounds excreted into sweat and sebum from the skin may also be incorporated in the hair. The removal of drugs depends upon several variables, such as gels or solutions used to wash or treat the hair [20].

24.1.1. Hair Analysis for the Assessment of Exposure to Anticholinesterase Pesticides

Pesticides are substances that are used to prevent, destroy, repel or mitigate any pest ranging from insects, animals and weeds to microorganisms such as fungi, moulds, bacteria and viruses or other organisms that compete with humans for food, destroy property, spread disease, or are considered a nuisance [21–22].

Workers employed in the manufacture and application of pesticides is the most highly exposed group [17]. While acute exposure to pesticides usually leads to intoxication with well defined and studied symptoms and only concerns a small part of the population, the interest of public health officials is focused in understanding and recording the effects of low level long term exposure to pesticides. This is because pesticides are widely released into environment. Consequently, exposure of the general population at some level to several different pesticide residues is almost inevitable [23].

24.1.2. In Vivo Studies in Animals

Recently, researchers have demonstrated that organophosphorus pesticides and environmental pollutants (DDTs and HCHs) can be detected in hair [24–26]. The disposition of diazinon in the hair of experimental animals that were exposed to the pesticide through their drinking water was studied in our laboratory [27–28].

Rats and rabbits were exposed to the pesticide diazinon through their drinking water. Both rats and rabbits were divided to three groups. One served as the control group and the two others received the pesticide in their drinking water at two dose levels. The rats were exposed to two levels of the pesticide, a low one of 2.7 mg/kg/day and a high one of 5.5 mg/kg/day in their drinking water for 45 days. The rabbits were exposed to 7 mg/kg/day (low dose) and 15 mg/kg/day (high dose) through their drinking water for 4 months. At the beginning and at the end of the dosing period, as soon as the hair regained its original length, it was removed from the back of the experimental animals.

A sensitive and selective method for analyzing organophosphate pesticides like diazinon, fenthion and methyl parathion in hair was developed. This method includes preparation of the sample by homogenization of the hair, methanolic extraction followed by liquid-liquid extraction with ethyl acetate and analysis by gas chromatography- mass spectrometry or gas chromatography coupled to nitrogen phosphorus detector. Analysis of the samples of both animals revealed that the concentration of the pesticide detected in hair was dose dependent [27–28].

Table 24.1. Diazinon concentration in the hair of the exposed rats and rabbits

Animal	Group	Group Mean Concentration (ng/mg) of hair ± S.D.	T-test p value
Rat	Control group	0	<0.001
	Low dose (2.7 mg/kg)	0.24 ± 0.01	
	High dose (5.5 mg/kg)	0.53 ± 0.05	
Rabbit	Control group	0	0.023
	Low dose (7 mg/kg)	0.17 ± 0.05	
	High dose (15 mg/kg)	0.23 ± 0.02	

Significance levels estimated between the high and low dose groups n = 5.

24.1.3. In Vitro Studies Using Human Hair

The effects of parameters such as colour of hair, concentration of pesticide and time of hair exposure to the pesticide diazinon were studied *in vitro* using human hair.

Three experiments were conducted in order to study the effect of the aforementioned parameters.

In order to study the effect of time of exposure of the hair sample to the pesticide, a 100 mg pesticide free sample of brown hair was immersed in an aqueous solution of 0.01 mg/mL diazinon for 1 and 4 h, at ambient temperature. At the end of each incubation period, the hair was removed from the solution, washed for 2 min in methanol in order to remove the loosely bound pesticide, and analysed by GC-MS.

Results (Table 24.2) indicated that the measured hair concentration of diazinon was time dependent.

Table 24.2. Measured concentration of diazinon in hair, following incubation of the sample in a 0.01 mg/mL aqueous solution of the pesticide

Concentration of exposure medium (mg/mL)	Time of exposure (h)	Concentration of diazinon in hair (μg/mg)
0.01	1	0.46
0.01	4	2.22

In order to study the effect of the pesticide concentration in the exposure medium, samples consisting of 100 mg of natural brown hair were incubated in aqueous solutions of diazinon at various concentrations (0.01, 0.1 και 1 mg/mL) for 1 h. At the end of the incubation period the samples were removed from the solutions simultaneously, processed and analyzed by GC-MS.

It is easily observed that the concentration of the pesticide in the exposure medium plays a crucial role in determining the levels of the pesticide absorbed by hair. Presumably saturation of the available binding sites in the hair would account for a leveling of the measured pesticide concentration.

Previous studied have indicated that hair colour is a crucial parameter that determines the amount of chemicals that may bind to hair, especially if absorption of the substance in hair occurs through the bloodstream, during the keratinisation step of the hair shaft More specifically it has been found that the concentration of many substances measured in hair is proportional to the melanin content of the hair shaft [29]. In order to study the effect of colour on the detected diazinon concentration, in the case of external contamination of hair with diazinon, human hair samples (100 mg) of different colours were incubated in an aqueous solution of diazinon of 0.1 mg/mL for 1 h (Table 24.3). At the end of the incubation period hair samples were removed from the incubation media, processed and analysed by GC-MS. The results of the experiment are depicted in Table 24.4.

When diazinon is absorbed through external contamination of the intact hair shaft from the environment and not through the blood stream during the keratinazation step of the hair shaft, melanin content does not seem to play such an important role. The results of the conducted experiment show differences in the measured hair diazinon concentration, but these do seem to relate so much to the melanin content of the hair shaft. This could be exposed by the fact that melanin is not directly exposed to the environment. On the contrary, the hair shaft is enclosed by a membrane that protects the cells that carry the melanosomes. The destruction of the membrane is necessary before chemical substances gain access to the melanosomes and the melanin binding sites.

Table 24.3. Measured concentration of diazinon in hair following 1 h incubation in diazinon aqueous solutions of different concentrations

Diazinon concentration of exposure medium (mg/mL)	Time of exposure (h)	Concentration of diazinon in hair (μg/mg)
0.01	1	0.10
0.1	1	1.59
1	1	3.99

Table 24.4. Measured diazinon concentration (μg/mg) in hair samples of different colours incubated in 0.1 mg/mL aqueous solution of diazinon for 1 h

Sample	Colour	Diazinon detected concentration (μg/mg)
1	Brown (dyed)	3.3
2	Light brown	2.2
3	Blonde	1.3
4	Black	0.9

Hence it seems that other factors play a role in the amount of pesticide binding to intact hair through passive exposure from the environment. These parameters would be the state of the membrane, as indicated by the fact that dyed hair absorbed more pesticide than the hair with intact membrane. Other parameters could be the lipid content of the hair. Hair with higher lipid content offer more binding sites to diazinon than hair with lower lipid content.

Finally another factor that could account for the amount of diazinon bound in hair is the diameter of the hair shaft. Hair shaft with smaller diameter offer more binding sites to diazinon per equal weight of sample than those with bigger diameter.

As a summary we should note that when measuring the concentration of a pesticide in hair several factors must be studied before relating the measured pesticide in hair to the exposure of the organism.

24.2. Population Study in Humans

Pesticide exposure of rural and urban population in the island of Crete was assessed using hair analysis.

A total of 463 and 70 head hair samples from rural (South Crete) and urban population (Heraklion city, Crete) were collected and analyzed. Approximately 500 mg of hair was cut from the root, at the back of the head and used for the analysis. Hair samples were analyzed for the currently used organophosphate pesticides (diazinon, methyl parathion, fenthion, malathion, dimethoate and chloropyrifos). Also they were analysed for the hexachlorocyclohexane isomers (HCHs: lindane, a HCH, HCB) and the dichlorodiphenyl trichloroethane isomers (DDTs: op-DDE, pp-DDE, opDDD, pp-DDD, op-DDT and pp-DDT).

The length of the samples from male participants varied from 3 up to 6 cm, corresponding up to 6 months of pesticide exposure, while that of the female participants varied from 8 up to 32 cm, corresponding up to 32 months of pesticide exposure [26, 30].

24.3. Organophosphate Extraction

The first step of the sample preparation procedure was the removal of the external contamination from the hair matrix. Hair was washed twice in water and methanol for 2 min and dried in the oven at 38°C. Subsequently 200 mg of hair was weighed out and pulverized in a ball mill homogeniser. Pulverisation of the hair before methanolic extraction was necessary for optimal recovery of the target analytes.

The powder was transferred in a test-tube with 2 mL of methanol and was incubated at 37°C overnight. The supernatant was transferred to a clean test-tube and methanol was evaporated to dryness under a gentle nitrogen stream. The residue was resuspended in 2 mL of HPLC grade water and liquid–liquid extraction followed with 3 mL of ethyl acetate, twice. The combined organic phases were transferred to a clean test-tube and evaporated to dryness under nitrogen. The residue was resuspended in 50 μL of 100 ng/mL 1,2,3,4-tetrachloronaphthalene (TCN) solution in hexane (external standard) and analysed by gas chromatography – mass spectrometry (GC-MS).

24.4. Results and Discussion

In the present study we assessed the present and past exposure to four currently used pesticides (diazinon, methyl parathion, malathion, fenthion), as well as to two banned ones that are still found as environmental pollutants and their metabolites (HCHs and DDTs) using hair analysis.

The concentration of the detected HCHs was higher in the hair of the rural population, compared to the urban one, but they were detected in both population groups. DDTs were also detected in both groups at very similar concentration levels.

No OPs were detected in the hair of urban population. Diazinon was detected in 2.8% of the hair samples of rural population with concentrations ranging from 2.5 to 5.8 pg/mg, malathion in 1.5% of the samples (5.1–8.4 pg/mg) and chloropyrifos in 2.4% of the samples (5.0–11.3 pg/mg). Methyl parathion, fenthion and dimethoate were not detected in any hair sample (Figure 24.1).

It was observed that the large majority of the examined samples was negative for all of the examined analytes. This is a good indication that these pesticides either degrade fast in the environment and in the organism, or that there was insignificant exposure due to strict observation of the safety rules (respiratory masks, gloves, special clothing) during the manipulation or application of the pesticides. Also the concentrations detected in hair were close or below the quantitation limit achieved presently. For this reason, our next step is to focus to the determination of specific and non specific metabolites (dialkyl phosphates) of organophosphates pesticides in hair [31].

A literature search revealed that not many published studies exist on the selection of pesticides in hair. In report by Liu and Pleil [14] it is stated that three OP pesticides were detected in hair, diazinon at a concentration of 20 ng/g, chlorpyriphos at a concentration range of 33–700 ng/g and Malathion at concentration range of 7–24 ng/g.

Ostrea and co-workers analyzed cord blood, infant hair and meconium samples simultaneously to determine the most sensitive matrix to detect antenatal pesticide exposure [32]. In this comparison analysis of infant hair, cord blood and meconium the authors reached the conclusion that meconium was the best matrix for this purpose.

The pesticides under investigation were propoxur, diazinon, lindane, transfluthrin, malathion, chlorpyriphos, bioallethrin, pretilachlor, DDT, cyfluthrin and cypermethrin. Eight of these pesticides were detected in meconium with a frequency between 0.2% for diazinon to 23.8% for propoxur. Cord blood and infant hair were positive, each for a single pesticide, propoxur and chloropyrifos respectively.

All the above evidence suggests that among other uses, hair analysis despite the uncertainties that exist in the interpretation of the results in relation to the observed concentrations, gives valuable information about exposure to pesticides.

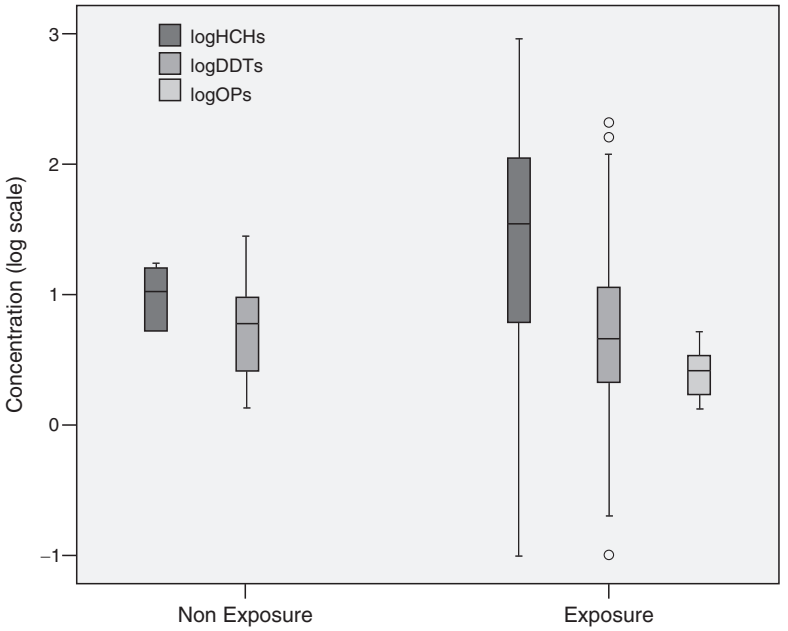

Figure 24.1. Concentration range of the analyzed compounds in the hair of urban (non exposed) and rural (exposed) population of Crete

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Chapter 25

Multidrug-Resistant Acinetobacter Baumannii: A Major Threat Worldwide

Savov E.[1], Mihaylova G.[1], Borisova M.[1],
Stoeva T.[2], Kjoseva E.[1], and Trifonova A.[1]
[1]*Military Medical Academy, Bulgaria*
[2]*Medical University, Bulgaria*

Abstract. Historically, Acinetobacter spp have been associated at the beginning of 70 with opportunistic infections that were rare and modest severity. The last 3 decades have seen an increase in both the incidence and seriousness of Acinetobacters, especially of A. baumannii infections. There is an indication of an increase also in the number of reported A. baumannii bloodstream infections and very seriously wound infection in patients at military medical facilities in Iraq, Kuwait and Afganistan. Together with this fact, A. baumannii infections became very important because of concomitant development of resistance and multiresistance of the strains to antimicrobial drugs with high rates. This paper reflects the problem A. baumannii infections of the literature' background and own data, their clonal spread significance and multidtug/pandrug'/resistance threat worldwide.

Keywords. A. baumannii, infection, multidrug resistance

25.1. Introduction

A. baumannii is a nonfermentative, gram-negative, nonmotile, oxidase-negative bacillus, whose natural reservoir still remains to be determined. Nevertheless, it is found in many health care environments and is a very effective human' threat in the hospitals during the last decades. A. baumannii causes a wide range of serious infections and is a major cause of bacteremia, pneumonia (particularly ventilator-associated pneumonia), meningitis and urinary tract infections [1]. Infections attributed to this organism have been reported around the world and are increasing in incidence. As such, *A. baumannii* is emerging as a cause of numerous global outbreaks, displaying ever-increasing rates of resistance. There are reports of multidrugresistant /MDR *A. baumannii/* from hospitals in Europe, North America, Argentina, Brazil, China, Taiwan, Hong Kong, Japan, and Korea and from areas as remote as Tahiti in the South Pacific [1, 2]. More recently, cases of United Kingdom and U.S military and nonmilitary personnel returning from operations in Iraq and Afghanistan and harboring infections caused by MDR *A. baumannii* are receiving increased attention [3–5]. The problem A. baumannii infections is also an object of investigation in Bulgaria since 1975 and the data received by us are very close to these, cited in the literature [6]. The aim of this study is to estimate the epidemiology

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of A. baumannii infections and the significance of these isolates with respect of their resistance and multidrug resistance' /pandrugresistance/ threat worldwide.

25.2. Materials and Methods

Military Medical Academy is a community hospital with 800 beds. The hospital is a one of the national centers for trauma patients treatment. Antibiotic prescription includes all groups of antibiotics together with carbapenems, quionolones, third and forth generations of cephalosporins. Because of that that the MMA is a multiprofile hospital/ with several surgery units, two ICU/ it can be a pattern for the tendency in the bacterial resistance' development to antimicrobial drugs in Bulgaria, nevertheless the variety, detected in different regions in the world, countries, hospital to hospital in the same country, reported in this respect. The resistance of the isolates to antimicrobial drugs was investigated by MINI API system/ Biomerieux/, automated system VITEC TWO v. 4.1. /Biomerieux/ and disk-diffusion method of Bauer et al. [7] according to the recommendations of CLS I 2005. Additionally for epidemiological investigation, polimerase chain reaction /PCR/ was done according to Grundmann et al. [8].

25.3. Results and Discussion

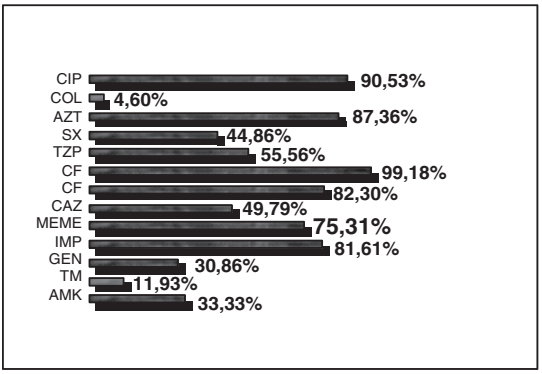
25.3.1. Epidemiology of A. baumannii Infection

Infections attributed to this organism have been reported around the world and are increasing in incidence. It is the cause of 2–10% of all Gram-negative ICU infections in the USA and Europe [1]. The data, received by us show that 15% of the strains, isolated at MMA in 2007 belong to the group of Nonfermenting Gram-negative bacteria, and only A. baumannii takes the sixth place between the first ten most often isolated microorganisms with relative part of 6%. Numerous studies have attempted to determine the impact of A. baumannii on patients in the hospitals. The problem is that A. baumannii causes a wide range of serious infections and is a major cause of pneumonia (particularly ventilator-associated pneumonia), wound infections, bacteremia, urinary tract infections, with an associated mortality of approximately 30% [2]. In this sense our data about 80% of A. baumannii strains, isolated from respiratory system of military and nonmilitary patients at MMA, followed by these isolated from wound secrets 8.5%, urine 6%, and from blood and puncta 5.5% confirm the literature' conclusions. *Acinetobacter* spp. had previously been described as common pathogens in war wounds. Currently, the soldiers from the 2003–2005 military operations in Iraq with wound infection or osteomyelitis caused by MDR *Acinetobacter* were admitted in Brooke Army Medical Center, in San Antonio, Texas. Investigation into the cause of these infections is ongoing, but the source is of infection is not clear [9]. Very similar situation in this respect is the investigation of A. baumannii infections in military and not

military patients at the Walter Reed Army Medical Center /WRAMC/, which is the major US site receiving casualties from the conflict in Iraq/Kuwait and Afghanistan, where 53% from A. baumannii strains are isolated from bloodstream infections [3, 10, 11, 12]. Also, there are many reports, showing that persistent hospital environmental contamination with A. baumannii strains, may play an important role in the nosocomial dissemination of these organisms [13, 14], and probably the colonized soldiers themselves, as previously noted, were the reservoir for MDR *Acinetobacter*, and that this colonization was obtained from the environment. The generated PCR fingerprinting done by us demonstrates that the strains investigated are clonal related and that probably an epidemic strains can spread between the patients in different units in the hospital and that the nebulizers from oxygen providing system in ICU are very often source of infection. /nonpublished data/.

25.3.2. Resistance of A. baumannii to Antimicrobial Drugs

Before the 1970s, most A. baumannii were fully susceptible to antibiotics. With the increased use of broad-spectrum antibiotics and technological improvements in more complicated invasive procedures, conditions were suitable for this ubiquitous pathogen to acquire multiple mechanisms of resistance and infect a vulnerable patient population. Indeed, A. baumannii exhibits a level of intrinsic antibiotic resistance afforded by its decreased membrane permeability and robust efflux systems [1]. There are many reports [2], including our data also, which show that A. baumannii has become resistant to many classes of antibiotics in the last years. Our results for 2007 present very high level resistance and multiresistance to beta-lactams, quinolones, aminoglycosides and carbapenems (Figures 25.1 and 25.2). Similar data for significantly more resistance to ciprofloxacin, ceftazidime, cefepime, amikacin, tobramycin, imipenem, and meropenem for Walter Reed Army Medical Center during 2004 were reported by Hujer et al. [3]. Especially the resistance to carbapenems is very important issue, because these drugs are choice for treatment of severe A. baumannii infections. According to our results [4], OXA 23 and OXA 58 beta- lactamases occured in A. baumannii strains, isolated from different hospitals in Bulgaria, are probably one of the main reason for the resistance to carbapenems found in these strains. These plasmid-encoded enzymes have been discovered also in England, Brazil, Singapore, Spain, Korea, and France [15, 16]. One recent report [1] described the finding that strains of A. baumannii that had infected multiple patients in one hospital contained significantly more integrons in their genomes than the strains that had only infected single patients. The presence of integrons in these strains suggests that the more antibiotic-resistant isolates (containing more integrons) are fit for hospital infection than nonintegron-containing isolates. This suggests that acquisition of multiple antibiotic-resistant determinants in the Acinetobacter genome favors the survival of such MDR strains under selective pressure of many different classes of antibiotics. Given the relative frequency of MDR A. baumannii in clinical infections worldwide, it is important to understand the many mechanisms of antibiotic resistance that this organism possesses.



CIP – ciprofloxacin, COL – colistin, AZT – aztreonam, SXT – TMP-SMX, TZP – tazobactam, CFX – cefotaxim, CF – cefepim, MEM – meropenem, IMP – imipenem, GEN – gentamicin, TM – tobramycin, AMK – amikacin

Figure 25.1. Resistance of A.baumannil to antimicrobials – 2007

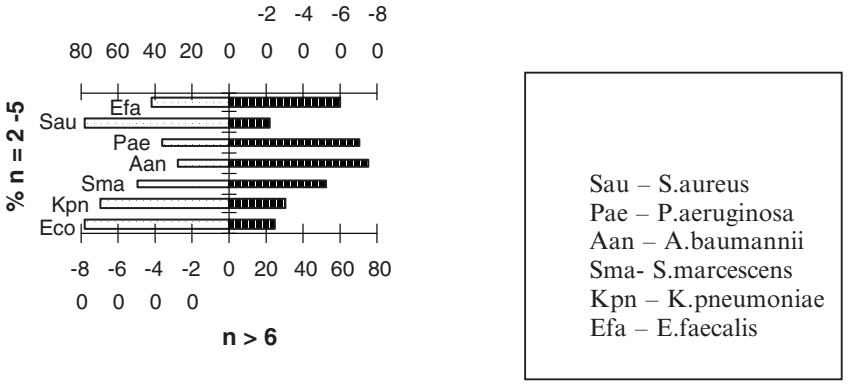


Figure 25.2. Frequency of multiresistance of E. coli, K. pneumoniae, S. marcescens, A. baumannii, E. faecalis

25.3.3. Therapetutic Approaches for Multidrug A. baumannii Infection

Given the tremendous challenge posed by MDR A. baumannii and the predictable emergence and dissemination of mechanisms of resistance to any existing agent, usage of antibiotics should be tentatively but aggressively explored. The observations in vitro and in animal models, although important, are not always applicable in clinical practice [2]. Futhermore, the studies and case series that illustrate the experience with different antibiotics in the treatment of MDR A. baumannii are also difficult to interpret because of potential biases. Few antibiotics have become available to treat MDR A. baumannii. The carbapenems remain the most active of the broad spectrum antimicrobials against

A. baumannii. Among the antibiotics that are considered as agents against MDR A. baumannii tigecycline /a newly licensed glycylcycline/ has received significant attention [2]. Tigecycline has shown excellent in vitro activity against multiple clinical isolates of A. baumannii [17]. Doripenem, a novel carbapenem, also promises to be active against susceptible A. baumannii. In initial in vitro studies, doripenem was not effective against A. baumannii isolates producing bla oxa-23 or bla-Imp4 or MBLs [18]. Combination antibiotic therapy is a strategy often employed in the treatment of MDR A. baumannii. This approach attempts to achieve synergy, particularly against MDR strains. According to our data, the combinations of sulbactam with aminoglicosides /amikacin/ have demonstrated synergy against MDR A. baumannii [19]. Where the dissemination of MBLs accounts for the increasing prevalence of carbapenem-resistant A. baumannii, the combination of colistin and rifampin should be considered. Newer quinolones and some tetracyclines/minocycline and doxycycline/are may be alternative drugs also for multiresistant strans of A. baumannii [2, 5].

Clearly, there is not enough or there is an absence of new antibiotics for treat the MDR A. baumannii which needs supportive methods of aggressive infection control to be implemented together with antibiotic therapy in the hospital setting.

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Chapter 26

Multi-Strip Assay and Multimodal Biosensors for Environmental and Medical Monitoring of Neurotoxicants

Kurochkin I.N. [1,*], Eremenko A.V. [1], Makhaeva G.F. [2], Sigolaeva L.V. [1], Dubacheva G.G. [1], and Richardson R.J [3]

[1] *Department of Chemistry, M.V. Lomonosov Moscow State University, Russia,*
[2] *Institute of Physiologically Active Compounds Russian Academy of Sciences, 142432 Moscow Region, Chernogolovka, Russia*
[3] *Department of Environmental Health Sciences, School of Public Health, The University of Michigan, Ann Arbor, Michigan 48109, USA.*

Abstract. The present report describes two types of biosensor systems. The first is for direct monitoring of neurotoxicants. The second is for monitoring individuals exposed to cholinergic and/or neuropathic organophosphorus compounds (OPs) by analyzing activities of their blood esterases. Both systems are rapid, simple, and sensitive. Assemblies of synthetic polymers, biomolecules, nanoparticles, and electrochemical transducers allow the biosensor systems to be used at the point of care and for field measurements in environmental, medical toxicology, veterinary, and antiterrorist applications. The selective monitoring of anticholinesterase compounds is based on a simple analytical method: the residual cholinesterase (ChE) activity assay after incubation with inhibitors. The specially developed design includes "programmable" strips and an automatic flow-injection amperometric analyzer for ChE activity analysis based on biosensor technology for choline detection. Capabilities include estimating the general toxicity of a sample as well as carrying out selective quantitative and qualitative assays of OPs, and other antiChE agents in mixtures. Combining layer-by-layer electrostatic assembly of enzymes and polyelectrolytes with extremely sensitive amperometric detection of hydrogen peroxide based on carbon electrodes coated by nanoparticles of MnO_2 yields a simple biosensor device capable of sensitive (< 0.1 pM) detection of neurotoxicants in 20 minutes. A new method of simultaneous quantitative determination of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in mixtures is also described in the report; the method is fast and precise. Average error of determination of enzyme activity is 8%. Highly sensitive tyrosinase and choline oxidase biosensors based on nanostructured polyelectrolyte films were developed for these purposes. The methodology of neuropathy target esterase (NTE) activity assay in whole human blood was realized using an amperometric analyzer coupled with a tyrosinase biosensor for phenol detection. These analytical approaches were developed to serve as a monitoring system for individuals exposed to cholinergic and/or neuropathic OPs. Thus, the technologies described here not only have enormous potential for use in responding to terrorist chemical threats, but they also have many civilian applications, which would likely be the larger market sector.

Key words. Biosensors, neurotoxins, blood esterases, monitoring

[*] Laboratory of Ecobiocatalysis, Chair of Chemical Enzymology, Department of Chemistry, M.V. Lomonosov Moscow State University, 119992, Leninskie Gory, Moscow, Russia, E-mail: ikur@genebee. msu.su

C. Dishovsky and A. Pivovarovi (eds.), *Counteraction to Chemical and Biological Terrorism in East European Countries*,

26.1. Introduction

A family of biosensor systems for rapid, simple, and sensitive monitoring of neurotoxicants, as well as biosensor systems for prompt analysis of blood esterases for monitoring of individuals exposed to cholinergic and/or neuropathic organophosphorus compounds (OPs), are described in the present report. Assemblies of synthetic polymers, biomolecules, nanoparticles, and electrochemical transducers allow the biosensor systems to be used at the point of care and for field measurements in environmental, medical toxicology, veterinary, and antiterrorist applications.

26.2. Amperometric Analyzer for Selective Monitoring of Neurotoxicants

The amperometric neurotoxicant analyzer (***EasyChEck*, v3.05X**) is a specially developed automatic flow-injection system (Figure 26.1) for quantitative and qualitative analysis of OPs and carbamates. The analyzer based on cholinesterase enzymes (ChEs) has emerged as a sensitive and selective technique for toxicity monitoring for environmental (water, soil), agricultural (rice, corn), nutritional (milk, juices, meat, fruits) and military applications.

Figure 26.1. Amperometric neurotoxicant analyzer, "EasyChEck"

The assay performed with EasyChEck is based on inhibition of ChEs by toxicants. The analytical response is directly related to choline concentration liberated due to hydrolysis of butyrylcholine (BCh) catalyzed by ChE (Eq. (1)):

$$\text{Butyrylcholine} + H_2O \rightarrow \text{choline} + \text{butyric acid} \quad (1)$$

A highly sensitive amperometric biosensor based on choline oxidase (ChO) is used for the quantitative detection of choline (Eq. (2)):

$$\text{Choline} + 2O_2 + H_2O \rightarrow \text{Betaine} + 2H_2O_2 \quad (2)$$

H_2O_2 is measured amperometrically at 600 mV using a Platinum-Ag/AgCl electrode.

There are two types of the "programmable" strips for quantitative, qualitative, and discriminative analysis using the neurotoxiant analyzer: the General Toxicity Strip and the Discrimination Strip.

The discrimination of OPs and other ChE inhibitors is based on application of organophosphate hydrolase (OPH), which hydrolyzes the OPs, rendering them inactive as ChE inhibitors. The difference between ChE activity before and after the action of OPH can be a quantitative estimate of the content in a sample of OPs (e.g., chemical warfare agents or pesticides) and carbamates.

Samples for the analysis can include the following: water; water extracts from soil, food, and forage; and organic solvent extracts from water, soil, food, and forage. For extract preparations from water, soil, and food products, different EPA certified methods (or other adequate methods) could be applied. Typically, these extracts are chloroform or hexane solutions. These solutions should be evaporated and redissolved in 1–2 mL of pure isopropyl alcohol before analysis. The linear range, detection limit, and bimolecular rate constant of inhibition (k_i) for a variety of neurotoxicants (mostly insecticides) are presented in Table 26.1.

Table 26.1. The linear range, detection limit, and bimolecular rate constant of inhibition for neurotoxicants of varying potency against BChE

Neurotoxicant	Linear range	Detection limit	k_i (BChE), $M^{-1}min^{-1}$
DFP	1–50 nM	1 nM	$(3.0\pm0,2)\times10^6$
Paraoxon	20–250 nM	20 nM	$(7.0\pm1,0)\times10^5$
Carbaryl	2.5–25 μM	2.5 μM	$(6.2\pm0,7)\times10^3$
Carbofuran	0.07–1.6 μM	0.07 μM	$(7.2\pm0,5)\times10^4$
Chlorpyrifos[1)]	1–8 nM	1 nM	$(4.2\pm1,3)\times10^7$

[1)] as chlorpyrifos-oxon produced after treatment of sample by bromine oxidation reagent (oxidation by bromine is included in the protocol of sample preparation)

The neurotoxicant workstation is capable of performing all analytical operations automatically with no operator participation. The simple procedure of sample preparation and user-friendly design of the "programmable" strips and analyzer allow for relatively rapid (20–70 min) assay of neurotoxicants.

26.3. Hydrogen Peroxide–Sensitive Amperometric Sensor Based on Thin Films and Nanoparticles of Manganese Dioxide

Direct electrochemical oxidation of hydrogen peroxide on a platinum electrode gives a detection limit of 10^{-6} M; however, it demands high positive potentials. To increase the sensitivity and improvement of analytical, technological, and operational characteristics of the hydrogen peroxide sensor, methods based on thin films and nanoparticles of manganese dioxide have been developed. Hydrogen peroxide formed as a product of enzymatic reactions can be detected electrochemically by a graphite electrode modified with manganese dioxide (Mn (IV)) as a mediator. The oxidation of hydrogen peroxide leads to the reduction of MnO_2 to Mn (II) and Mn (III), which are immediately oxidized electrochemically back to Mn (IV) as MnO_2.

The method of electrochemical modification of graphite electrodes has been applied to formation of a thin film manganese dioxide layer. Nanoparticles of manganese dioxide were synthesized by means of the disproportionation reaction between potassium

permanganate and manganese acetate in a system of reversed micelles containing AOT/hexane/water at different ratios of the reagents and extents of hydration (AOT is bis(2-ethylhexyl)sulfosuccinate sodium salt). Manganese dioxide nanoparticles generally consist of needle-like objects of 1 nm thickness agglomerated in clustered aggregates, as it seen in transmission electron microscopy. A stable micellar suspension of manganese dioxide nanoparticles was deposited layer-by-layer (LBL) on the surface of a graphite electrode, and then the electrodes were washed to remove the residual AOT and dried in the open air at room temperature.

The dependence of the analytical responses to the electrochemical potential was studied for the both types of hydrogen peroxide electrodes with manganese dioxide as a mediator. The maximum analytical responses to H_2O_2 addition were observed in the potential range from 350 to 500 mV. Analytical characteristics for manganese dioxide modified graphite electrodes are represented in Table 26.2.

Table 26.2. Analytical characteristics of manganese dioxide modified electrodes

MnO_2 modified electrode	**Linear range, $[H_2O_2]$**	**Detection limit, $[H_2O_2]$**	**Sensitivity, $A/M \cdot cm^2$**
Thin films	0.01–100 μM	10 nM	1.2
Nanoparticles	0.08–100 μM	78 nM	0.9

The detection limit for H_2O_2 in the nanomolar range makes it possible to use manganese dioxide modified graphite electrodes coupled with choline oxidase in an assay for low concentrations of ChE inhibitors like VX, sarin, and soman with a sensitivity of about 0.1 pM.

26.4. Simultaneous Quantitative Determination of Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) Activities in Mixtures

The activities of AChE and BChE in blood can serve as biomarkers of exposure to ChE inhibitors, such as OPs and/or carbamates [1]. It is important that some of the most potent inhibitors of AChE have been developed for chemical warfare. The most recent devastating demonstration of the availability, rapidity, and lethality of these agents was the release of sarin into the Japanese commuter train system in Tokyo, in which blood ChE activity was used as a tool for confirming exposure to the agents [2].

The LBL technique that has been developed relatively recently has demonstrated its promise as a versatile way to form organized multilayered thin films with predictable physical, mechanical, and chemical properties. The technique is based on the consecutive adsorption of positively and negatively charged polyelectrolytes on a solid substrate [3, 4]. As the LBL technique gives a wide range of possibilities to control surface properties by inserting functional elements such as conductive nanoparticles, enzymes, and mediators into polyelectrolyte assemblies, it has great potential for biosensor design [5, 6].

Highly sensitive tyrosinase and choline oxidase biosensors built on nanostructured polyelectrolyte films were developed for simultaneous quantitative determination of AChE and BChE activities in mixtures [7] based on their different substrate specificity. Substrate pairs consisting of one phenyl ester (phenyl acetate or phenyl valerate) and

one choline ester (acetylcholine or butyrylcholine) were used for AChE and BChE activity determination in the mixture. Analytical responses of sensors were directly related to liberated phenol/choline concentrations. The tyrosinase-based biosensor for phenol detection is built on a graphite rod on which tyrosinase is immobilized by the LBL technique. The molecules of tyrosinase are oxidized by oxygen and then reduced by phenol. Because of the last reaction, phenol is oxidized to quinone, which is an electrochemically active compound that can be reduced to catechol at an electrode to produce an amperometric signal. Similarly, the choline oxidase-based biosensor for choline detection is based on a graphite rod with the surface covered by a peroxide-sensitive layer containing manganese oxide on which choline oxidase is immobilized by the LBL technique. The principle of operation of the choline oxidase electrode is based on amperometric detection of hydrogen peroxide liberated as a result of oxidation of choline by oxygen in the presence of choline oxidase. The detection limits for phenol and choline are 10 nM [8] and 100 nM, respectively. The coefficient of variation ([SD/mean] ×100) for 10 measurements of phenol and choline are 6% [8] and 1%, respectively.

The choline and phenolic sensors described above were used as the basis of an electrochemical multisensor. The multisensor consists of a two-electrode electrochemical cell connected with a two-channel potentiostat. Such a multisensor allows the two main products of enzymatic hydrolysis (phenol and choline) to be analyzed simultaneously (Figure 26.2).

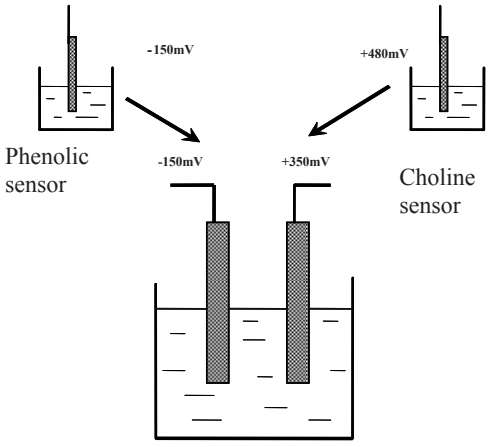

Figure 26.2. Principle of analytical multisensor (from [7])

It was shown that the presence of choline did not influence phenol detection. However, choline detection is impossible at +480 mV due to interfering phenol oxidation. However decreasing the potential to +350 mV makes the influence of phenol nearly negligible while keeping the analytical response to choline virtually the same. The influence of substrates (acetylcholine, butyrylcholine, phenyl valerate, and phenyl acetate) on the amperometric detection of the main analytes (choline and phenol) was also studied. The calibration dependencies were obtained for choline and phenol in the

presence of the aforementioned substrates. It was shown that those components did not interfere with amperometric choline/phenol determination.

Based on the effective kinetic parameters (K_M and V_m) determined for AChE and BChE for all four substrates (acetylcholine, butyrylcholine, phenyl valerate, and phenyl acetate), the optimum concentrations of substrates were chosen as the following: acetylcholine, 1.5 mM; butyrylcholine, 1 mM; phenyl acetate, 0.5 mM; and phenyl valerate, 0.2 mM. Then the factors of sensitivity and linear ranges for AChE and BChE using these four substrates have been determined as described below.

For the initial moment of time, the rate of enzymatic hydrolysis of substrates in the presence of ChEs is calculated according to the equation of Michaelis-Menten (Eq. (3)); each of the terms in the equation has their commonly accepted meaning:

$$v_0 = \frac{k_2^{eff} \times S_0}{K_I^{eff} + S_0} \times E_0 \tag{3}$$

In the case when the reaction proceeds under the action of two enzymes, the equation for the initial rate will be represented as the sum of the initial rates of each enzyme (Eq. (4)):

$$v_0^{\Sigma} = v_1 + v_2 = \frac{k_2^{(1)eff} \cdot S_0}{K^{(1)eff}{}_M + S_0} \cdot E_0^{(1)} + \frac{k_2^{(2)eff} \cdot S_0}{K^{(2)eff}{}_M + S_0} \cdot E_0^{(2)} \tag{4}$$

Multipliers before $E_0^{(1)}$ and $E_0^{(2)}$ were named as the factors of sensitivity k_s of enzymes to substrate S. Then the total rate of hydrolysis of substrate S at the initial moment of time will be the following (Eq. (5)):

$$v_0^{\Sigma} = k_s^{(1)} \cdot E_0^{(1)} + k_s^{(2)} \cdot E_0^{(2)} \tag{5}$$

Thus, after determining the factors of sensitivity of enzymes to chosen substrates and measuring the initial rates of hydrolysis of a pair of substrates by ChEs, we can calculate the content of each ChE in the sample.

For each pair of substrates the factors of sensitivity of ChEs were determined along with the linear ranges of enzyme concentrations in which they can be determined using the appropriate pair of substrates. For this purpose, the dependencies of enzymatic rate of substrate hydrolysis on the concentration of enzyme in a cell were obtained. The factor of sensitivity was calculated as the slope of the calibration curves. The factors of sensitivity and linear ranges are summarized in Table 26.3. The lower limit was accepted as the concentration of enzyme at which the total rate of substrate hydrolysis was twice the rate of its spontaneous hydrolysis.

Analysis of the data presented in Table 26.3 has shown that the widest linear range of the concentration of two enzymes is achieved when using the substrate pair acetylcholine and phenyl valerate. The lower limit of detection for BChE is obtained for the same pair of substrates. This result is explained by a better sensitivity of BChE to phenyl valerate in comparison with phenyl acetate.

Table 26.3. Factors of sensitivity of AChE and BChE for each pair of substrates

Substrate pairs	AChE		BChE	
	k_s, A /M·s	Linear range·10^{10}, M	k_s, A/M·s	Linear range·10^{10}, M
Acetylcholine	1.1±0.1	2.2–32.8	1.76±0.03	7.7–44.4
Phenyl acetate	0.16±0.02		0.19±0.02	
Acetylcholine	0.94±0.01	2.2–28.5	1.6±0.1	2.1–53.2
Phenyl valerate	0		0.63±0.10	
Butyrylcholine	0	2.2–26.3	6.45±0.07	7.7–35.5
Phenyl acetate	0.12±0.01		0.17±0.01	

The response of a bi-electrode sensor consists of two curves (Figure 26.3). The top curve represents the response to the choline oxidase electrode and displays the process of hydrolysis of choline ester. The bottom curve represents the response of the tyrosinase electrode and displays the process of hydrolysis of phenyl ester. On both curves it is possible to allocate the sites of spontaneous hydrolysis of substrates and hydrolysis of substrates at the presence of ChEs. The initial rate of enzymatic hydrolysis was calculated as the difference of the reaction rate after esterase mixture addition and the reaction rate for the spontaneous hydrolysis of the substrate.

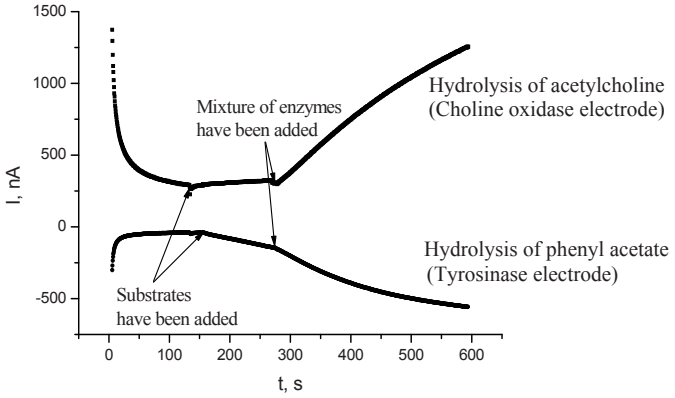

Figure 26.3. Typical response to bi-electrode sensor. Conditions: bi-electrode cell with stirring, concentrations in the cell: AChE – $4.4.10^{-10}$M, BChE – $8.9.10^{-10}$M, acetylcholine – 1.5 mM, phenyl acetate – 0.5 mM, buffer solution 50 mM HEPES with 3 mM KCl (pH = 7.5), room temperature (from [7])

The values of the initial rate of enzymatic hydrolysis, and the calculated factors of sensitivity for each esterase were introduced into the system of the linear equations (Eq. (6)):

$$\begin{cases} R_1 = x_1[\text{AChE}] + y_1[\text{BChE}] \\ R_2 = x_2[\text{AChE}] + y_2[\text{BChE}] \end{cases} \quad (6)$$

Where x_1, x_2 are the factors of sensitivity of AChE to choline and phenyl esters respectively; y_1, y_2 are the factors of sensitivity of BChE to choline and phenyl esters, respectively; and R_1, R_2 are the initial rates of enzymatic hydrolysis of choline and phenyl esters, respectively. Solving this system of linear equations gives the concentration values of AChE and BChE in the mixture.

The deviation of ChEs activity determination was calculated as a difference between specified concentration and calculated concentration for each ChE taken as modulo and expressed as a percentage of specified concentration ($[|C_{spec.} - C_{calc.}|/C_{spec.}] \times 100$). The deviation determined for 15 mixtures does not exceed 17%, which indicates good accuracy for the suggested method. The maximum and average deviations in determining esterase activity using the three pairs of substrates presented in Table 3 were found to be 17% and 8% for AChE, and 13% and 4% for BChE [7]. The minimum deviation in the determination of concentration of ChEs was achieved when using acetylcholine and phenyl valerate as substrates. The maximum and average deviations in determining esterases using this pair of substrates were 9% and 5% for AChE, and 10% and 3% for BChE [7].

Thus, the use of a bi-electrode sensor system allows one to measure AChE and BChE in mixtures with concentrations as low as $3 \cdot 10^{-10}$ M with high accuracy. It enables one to speak about the applicability of the given approach for determining the ChE activity in whole blood. The suggested method does not require any significant time consumption for the sample preparation, and the measurement typically takes a maximum of 15 minutes.

26.4. Neuropathy Target Esterase (NTE) Activity Assay in Whole Human Blood

Neuropathy target esterase (NTE) is thought to be the target protein for neuropathic OPs that produce OP-induced delayed neurotoxicity (OPIDN). Inhibition/aging of brain NTE within hours of exposure predicts the potential for development of OPIDN in susceptible animals [9].

Lymphocyte NTE has also found limited use as a biomarker of human exposure to neuropathic OPs. Although NTE in lymphocytes should be an ideal marker, it takes time, resources, and relatively high sample volumes to separate lymphocytes from whole blood. Therefore, there would be an advantage to being able to detect the lymphocyte (and platelet) NTE directly in whole blood. The standard NTE assay is based on the colorimetric determination of phenol released by hydrolysis of the substrate, phenyl valerate [10, 11]. Because of interfering absorbances, the colorimetric assay as typically conducted cannot be used to assay NTE in whole blood [12].

The problems inherent in a colorimetric NTE assay could be eliminated by using an amperometric technique to detect phenol produced by the NTE hydrolysis of phenyl valerate. Such an approach was developed using tyrosinase-based biosensors. These involve the enzymatic oxidation of phenol via catechol into *o*-quinone, a reaction that consumes molecular oxygen. Electroreduction of quinone to catechol directly on the graphite electrode can be used as a detection reaction for the quantification of phenol. A highly sensitive biosensor was developed for NTE activity using a tyrosinase carbon-paste electrode modified or non-modified by methoxyphenazine methosulfate (MPMS) as a mediator for the amperometric detection of phenol [12, 13].

The differential inhibition method of Johnson [10] with an electrochemical endpoint was used for NTE assay as described in [12, 14] using phenyl valerate as the substrate. Due to the high sensitivity of the tyrosinase carbon-paste electrode (with the detection limit for phenol as low as 20–25 nM), the influence of interfering blood components (ascorbic acid, tyrosine, and others) was diminished by the extensive sample dilution (from 1:275 to 1:2200). The results presented in Figure 26.4 demonstrate a linear dependence of the apparent NTE activity on blood concentration, thus allowing NTE to be detected selectively and with high sensitivity in whole blood, where the usual colorimetric assay is impossible (Figure 4) [15]. Using phenol calibration of the tyrosinase carbon paste electrode, blood NTE specific activity was calculated to be 0.25 nmol phenol produced/(min × mg protein) [12, 15]. The minimum statistically significant decrease in NTE catalytic activity that can be detected with this method is 0.016 nmol phenol/(min × mg protein), when the volume of blood sample taken for analysis is 20 μl.

In order to use the measurement of blood NTE activity as a surrogate of brain NTE, the correlation between the inhibition of the enzyme in brain and that in blood should be known [14]. We studied NTE inhibition in brain and blood at a relatively short time (4 h) after dosing hens with the neuropathic OP, *O,O*-di-1-propyl *O*-2,2-dichlorovinylphosphate PrDChVP (0.32 – 1.0 mg/kg, im) [15]. NTE inhibition was measured in brain by the colorimetric assay and in whole blood using the amperometric assay with the MPMS-modified tyrosinase carbon paste electrode.

Data, presented in Figure 26.5, show that brain and blood NTE were inhibited in a dose-responsive manner 4 h [15] after dosing (linear trend, $p < 0.0001$; Figure 26.5A), and NTE inhibition was highly correlated between brain and blood ($r = 0.997$, $n = 4$; Figure 26.5B).

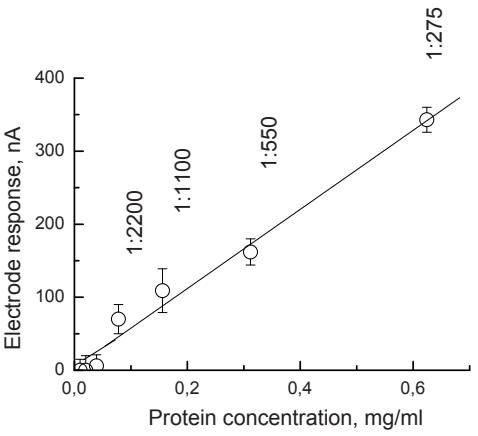

Figure 26.4. Dependence of the apparent NTE activity in human blood (measured as the MPMS-modified tyrosinase carbon-paste electrode response, nA) on final blood sample dilution. Conditions: 0.05 M phosphate buffer solution with 0.1 M NaCl, pH 7.0; applied working potential –150 mV vs. Ag/AgCl; volume of the analyzed blood in each dilution, 20 μl; incubation time, 30 min; blood NTE specific activity, 0.25 nmol phenol produced/(min × mg protein) (From [15]).

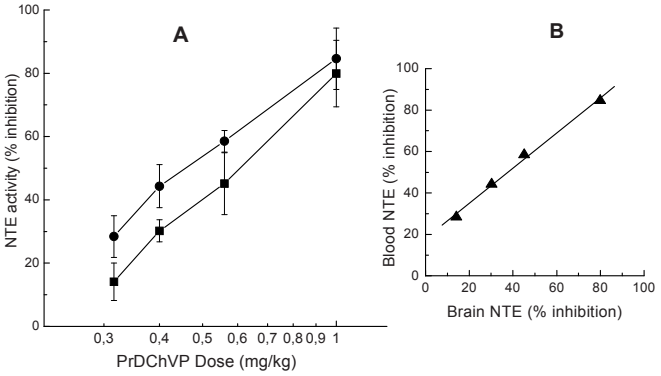

Figure 26.5. (A). Dose-related NTE inhibition in brain and whole blood of hens 4 h after injection of the neuropathic OP, O,O-di-1-propyl O-2,2-dichlorvinyl phosphate (PrDChVP). (B). Correlation between blood and brain NTE inhibition. NTE assayed colorimetrically in brain and amperometrically in blood; both tissues assayed after freezing and thawing. Closed square (—■—) = brain NTE; closed circle (—●—) = blood NTE. Results are % control values for each tissue expressed as mean ± SEM, n = 3. Control NTE activities, nmol/(min × mg protein), mean ± SEM, n = 3: brain = 21 ± 2, whole blood = 0.23 ± 0.02 (From [15]).

Thus, the tyrosinase carbon-paste biosensors are suitable for assaying NTE in whole human and hen blood, which cannot be done using the classical colorimetric technique. Other advantages include the small sample volume required (100 µl), simplicity of sample preparation, and rapid phenol analysis time. A strong correlation between NTE inhibition in whole blood with that in brain suggests that the biosensor NTE assay for whole blood appears to be promising, not only as a biomarker of human exposure to neuropathic OPs, but also as a predictor of OPIDN and as an adjunct to its early diagnosis. Application of the developed biosensors will enable rapid assessment of human exposure to neuropathic OPs. This is important in biomonitoring and epidemiological studies as well as in supporting consequence management and risk minimization of a chemical attack or chemical accident.

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Part 4

DEVELOPMENT OF THE CONTROL ON PROTECTION AGAINST CHEMICAL AND BIOLOGICAL AGENTS

Chapter 27

Development of Measures on Prevention and Liquidation of Bioterrorist Phenomena in Ukraine

Marievsky V. and Doan S.
L.V. Gromashevsky State Institute for Epidemiology and Infectious Diseases of Ukraine Academy of Medical Sciences, Ukraine

Abstract. The paper includes data on the system of prevention and liquidation of bioterrorist phenomena in Ukraine. Peculiar attention is given to proactive measures regarding possibility of their realization in various regions of the world. The paper shows complex and multilateral nature of the problem of citizens' protection from bioterrorism.

Keywords. Bioterrorist phenomenon, prevention, liquidation, indication

In the twenty-first century biological terrorism remains one of the most threatening phenomena of the present days. Relatively low cost, accessibility, easiness of transportation and usage, mass character of annihilation, are preconditions of specific appropriateness of biological weapon for terrorist attempts. Extremely rich micro-world facilitates choosing of causative agents, depending on the purpose of terrorist attack. Striking achievements of molecular biology and genetic engineering give an opportunity to select causative agents with specified biological properties: apocalyptic intentions will demand highly contagious agents with quick spreading, which are capable of inducing acute forms of pathology with high lethality.

In the conditions of military operations, causative agents affecting, for example, males only, with temporary loss of their fighting efficiency, and even with ethical selectivity, can be used.

Geopolitical position of Ukraine, located in the middle of Europe, at the intersection of international transport routes, stirring up of migration processes and so on, demand efficient and properly organized system of counteraction to bioterrorism, both for preventing terrorist phenomena in our country, and pre-acting their occurrence in other countries of the world.

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Factors which promote bringing and spreading of biological pathogenic agents:

- Creation of favorable conditions for flash-like spreading of infectious diseases due to high-speed transport. In 2006 over two billions of passengers used air transport services. Epidemic of disease from one part of the world becomes inevitable threat in the other one during several hours only.
- Geopolitical position of Ukraine, located at the intersection of international transport routes.
- Stirring up of migration processes (flow of foreign citizens, as compared with the year 1991, has grown 33 times).
- Adverse epidemic situation regarding quarantine diseases and highly contagious viral fevers in the countries of Asia, Africa, Latin America being our business partners.
- Possible activation of natural focuses of plague in Russia (in Chechnya, Dagestan, Kabardino-Balkaria, Karachay-Cherkessia).

System of protection from biological weapon should be developed at the governmental level, and it should have multistage, inter-departmental and hierarchically subordinate organization. The most successful method of counteraction to bioterrorist phenomena is prevention thereof. In such conditions, essential role is given to coordinated efforts of special customs authorities, militia, sanitation and quarantine departments, as well as control over research and development activities, museums of storing pathogenic cultures etc. Ongoing highly efficient system of epidemiologic supervision with network of technically equipped microbiological and virological laboratories and unified research-and-information computer system is of considerable importance.

Structure of the system for specific indication of biological pathogenic agents in Ukraine was created in accord with Order No. 127/27 of the Ministry of Health and Academy of Science of Ukraine as of 21.03.2003 “On Improvement of Functioning of Biological Pathogenic Agent Indication System”. It provides for interaction of various ministries and agencies, and guiding function is assigned to the Ministry of Health (Figure 27.1).

System of counteraction to bioterrorism includes six Centers of indication and identification of biologically pathogenic agents, which are in charge of certain administrative regions, and assume specific functions on identification of agents of infectious and parasitic diseases on the whole territory of Ukraine (Table 27.1).

Head offices (laboratories) on indication of biological pathogenic agents in this system are laboratory subdivisions of epidemic specialization under State sanitary and epidemiological service departments of the Autonomous Republic of Crimea, regions, cities of Kyiv and Sevastopol.

Tasks of Head offices (laboratories) on indication of biological pathogenic agents are as follows:

- Indication and identification of all kinds of biological pathogenic agents
- Organizational supervision on the relevant territories
- Personnel training
- Practical assistance rendered to the territorial sanitary and epidemiological stations on the matters of organization and carrying out of laboratory investigations

Executing organs at the local level are municipal and district sanitary and epidemiological stations, and the like stations at water, railway and air transport facilities, which carry out epidemiological survey of the territories concerned; taking of samples from objects supposedly contaminated with biological pathogenic agents, and delivery of the above samples to the territorial Head offices (laboratories) of biological pathogenic agent indication.

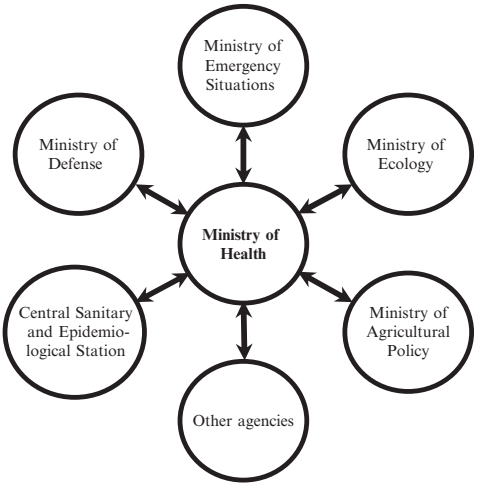

Figure 27.1. Diagram of Biological Pathogenic Agent Indication System in Ukraine

Besides, development of a set of measures in case of occurrence of bioterrorist phenomenon, aimed at liquidation thereof, is of ultimate importance. Should an atypical situation (suspected act of terrorism) arise, it is necessary, first of all, to carry out epidemic investigation with laboratory diagnostics, to assess the scope of spreading the disease and making the adequate management decisions.

Implementation of measures regarding liquidation of terrorist phenomena in Ukraine falls under the competence of both medical forces and facilities (sanitary and epidemiological service, medical and outpatient departments, scientific and medical institutions and so on), but non-medical bodies as well (administrative agencies, extraordinary anti-epidemic commissions, emergency response teams of the Ministries of emergency situations, internal affairs, and defense).

The important element within the system of counteraction to bioterrorism is specific indication of biological agents. Taking into account versatility of biological agents which can be used for terrorist purpose, creation of the relevant “antidotes” within the shortest time possible represents both theoretical and practical task.

All the institutions (laboratories) included into the system of biological pathogenic agent indication work on fulfillment of tasks assigned to them, in accord with regimes

of functioning of "Unified State System for Natural and Man-made Emergencies' Prevention and Response" (Decree No. 1198 of the Cabinet of Ministers of Ukraine dated 03.08.1998).

Regimes of functioning of the agencies operating within the system of biological pathogenic agent indication are as follows:

- Regime of normal activity
- Regime of advanced readiness
- Emergency regime
- Regime of emergency situation

Regime of normal activity means functioning in normal production, industrial, radiation, chemical, biological, seismic, hydrological and hydro-meteorological conditions, in absence of epidemics.

Below listed are the tasks of the regime of normal activity:

- Set of measures on guaranteeing sanitary and epidemiological security
- Supervision and control over technogenic domain, environment, food products, drinking water, and other life-support objects
- Improvement of the staff list and organizational structure of laboratories working on biological pathogenic agent indication
- Scheduled training of workers employed with biological pathogenic agent indication laboratories
- Organization and conducting of studies, participation in conferences, symposiums, congresses, onsite training at biological pathogenic agent indication centers
- Preparation to work in emergency conditions
- Taking measures on improvement of provision of laboratory subdivisions with modern equipment and consumables

Regime of advanced readiness means functioning in conditions of worsened production, industrial, radiation, chemical, biological, seismic, hydrological and hydro-meteorological conditions, should any predictions be made regarding emergency situation occurrence.

Below listed are the tasks of the regime of advanced readiness:

- Analysis of reasons of situation worsening, drawing up the proposals on normalization thereof
- Arrangement of stand-by duty of biological pathogenic agent indication laboratory personnel
- Preparation of out-of-the town dislocation zone and evacuation of SES workers and members of their families
- Establishing of communication with the organs of civil defense of citizens and territories, and with relevant centers, in out-of-town zone
- Bringing to status of advanced readiness of the personnel and material-and-technical facilities used for biological pathogenic agent indication, and clarification of the action plan

Emergency regime is introduced by the Order of the President of Ukraine in case of occurrence and spreading of emergency situation, and it is approved by Verkhovna Rada of Ukraine.

Below listed are the tasks of the emergency regime:

- Introduction of special regime of security, circulation of traffic and personnel of laboratories of biological pathogenic agent indication
- Formation of task forces for protection of citizens and territory, on which the emergency situation occurred, drawing up of the plan of elimination of such situation
- Protection of personnel and property of laboratories, working on biological pathogenic agent indication, from emergency factors
- Provision of functioning of biological pathogenic agent indication laboratories, rendering support to its personnel in emergency conditions
- Communication with coordinating authorities of civil defense of citizens and territories, any biological pathogenic agent indication laboratories on the territory, and biological pathogenic agent indication centers
- Carrying out biological survey on the emergency area with taking of samples of environmental objects with their further conveyance to biological pathogenic agent indication laboratory
- Indication and identification of biological pathogenic agents in samples of environmental objects
- Delivery to biological pathogenic agent indication centers of all biological pathogenic agents and cultures thereof isolated during indication, which could not be classified at previous stages of indication
- Drawing up and submission of reports on organization and conducting of indication and identification of biological pathogenic agents

Regime of emergency situation means functioning at real threat, and during localization and prevention of emergency situation.

Below listed are the tasks of the regime of emergency situation:

- Protection of personnel and property of laboratories from the factors of biological origin in case of threat/occurrence of emergency situations
- Provision of functioning of biological pathogenic agent indication laboratories, rendering support to its personnel
- Securing of communication, in conditions of emergency situation, with coordinating authorities of civil defense of citizens, biological pathogenic agent indication laboratories and relevant biological pathogenic agent indication centers
- Rendering guidance and practical assistance on the matters of organization and taking the necessary measures in conditions of emergency situation
- Participation in task forces on drawing up of the plan for emergency situation localization and elimination
- Indication and identification of biological pathogenic agents in samples of environmental objects, raw materials, food products, drinking water

- Laboratory control over food products and drinking water for specified biological pathogenic agents, and drawing conclusions on possibility of using such products
- Delivery to biological pathogenic agent indication centers of all microorganisms/ cultures isolated during indication, and not classified at previous stages of indication

In our opinion, system meant for counteraction to bioterrorism in Ukraine demands further improvement; in particular, the measures to be taken are as follows:

- Improvement of normative and methodically formalized mechanisms of functioning of Unified multilevel vertically integrated system for diagnostics and monitoring of infectious agents on the territorial, regional, and governmental levels (laboratory network of response to bioterrorist acts)
- Increase in efficiency of interaction with laboratory departments of medical and preventive treatment facilities, as well as research institutes under the Academy of Medical Sciences of Ukraine, National Academy of Sciences of Ukraine, Ministry of Health of Ukraine, Ministry of Emergency Situations of Ukraine in practical and research domains
- Putting into practice of unified analytical system of operative detection of biological hazard, which includes all the basic links of detection of pathogenic biological agents, as well as indication of microorganisms with unknown taxonomic position; agents of "new" and genetically modified infections
- Tightening of the State sanitary and epidemiological supervision over observance of biological safety requirements in the laboratories of those agencies, which are engaged in diagnostics of particularly hazardous infectious diseases
- Provision for financing of measures on strengthening and modernization of material and technical base of laboratories, working on indication of pathogenic biological agents, and training of personnel
- Introduction of molecular-genetic methods of indication and identification of biological pathogenic agents in the laboratories
- Creation of automated computer information system and analytical center for efficient collection and analysis of information provided by microbiological laboratories on detection of unidentified hazardous infection agents
- Implementation of global system of monitoring of the most actual infectious diseases
- Activation of fundamental research on creation of the system on counteraction to bioterrorism in Ukraine

Therefore, elaboration of the efficient system of counteraction to bioterrorism is an important issue of the national security of our country and it poses a global, multidimensional interdepartmental problem.

Table 27.1. Centers of indication and identification of biologically pathogenic agents

State Institute for Epidemiology and Infectious Diseases named after L. V. Gromashevsky under the Academy of Medical Sciences of Ukraine, Kyiv	State Institute for Microbiology and Immunology named after I. I. Mechnikov under the Academy of Medical Sciences of Ukraine, Kharkiv	Lviv Research Institute of Epidemiology and Hygiene under the Ministry of Health of Ukraine, Lviv	Ukrainian Research Anti-plague Institute named after I. I. Mechnikov, Odessa	Central Sanitary and Epidemiological Station under the Ministry of Health of Ukraine, Kyiv	Crimean Anti-plague Station under the Ministry of Health of Ukraine, Simferopol
Tasks of indication and identification of biological pathogenic agents to be fulfilled on administrative territories					
Kyiv, Chernigiv, Cherkassy, Poltava regions	Kharkiv, Donetsk, Lugansk, Sumy regions	Lviv, Volyn, Ternopil, Ivano-Frankivsk, Zakarpatye regions	Odessa, Mykolaiv, Vinnitsa, Kirovograd, Chernivtsy regions	Kyiv city, Zhytomir, Khmelnitsky, Rivne regions	Crimean Autonomous Republic, Town of Sevastopol, Zaporizzhya, Kherson, Dnipropetrovsk regions
Tasks to be fulfilled throughout the territory of Ukraine					
Identification of biological pathogenic agents, not classified at previous stages of indication, which are recombinant/combined ones, and have prion and viroid etiology. Toxins	Identification of biological pathogenic agents, not classified at previous stages of indication, which have bacterial etiology. Mycoses	Identification of biological pathogenic agents, not classified at previous stages of indication, which have Ricketsia and viral etiology	Identification of biological pathogenic agents, not classified at previous stages of indication, which have bacterial and viral etiology of the I pathogenicity group	Identification of biological pathogenic agents, not classified at previous stages of indication, which have bacterial - etiology (zoo-anthroponoses)	Identification of biological pathogenic agents, not classified at previous stages of indication, which have bacterial etiology (cholera and other pathogenic vibrions, legionary, Leptospira)

Chapter 28

Accelerated Analysis for Environment Control on Protection Against Biological Agents

Nesterenko V.
Belarusian National Technical University, Minsk, Republic of Belarus

Abstract. The present work is devoted to creation of the accelerated microbiological analysis for environment monitoring including the purposes of rapid deployment of rescue forces in case of applying most dangerous toxic bacteriological (biological) weapon (plague, smallpox, anthrax, etc., both in individual form and in combinations) which alongside with nuclear and chemical weapon presents large threat for mankind. In the given research the new approach to preparation of hard nutrient media is proposed, namely – impregnation by solution of nutrient materials by a special way of the treated plates from solid inorganic materials. The specimens of different groups of microorganisms, actinomyces, streptococci, yeasty and mouldy fungi grow well on such media. The test results of hard microbiological nutrient media prepared by developed and known ways have shown that the developed way allows to prepare nutrient media with higher exploitative properties provides essential reduction of duration and simplification of preparation process of media to an inoculation. It meets the requirements of express extraction pathogenic microorganisms from environment and their cultivating.

Keywords. Biological pollution, analytical control, hard nutrient media, solid inorganic material

28.1. Introduction

The permanently rising biological pollution of the environment requires organization of legible and operating scientific grounded monitoring quality system of environment objects concerning to such pollution in view of maintenance of a principle of epidemic safety. The solution of these problems with the purpose of environmental protection from biological pollutions, including the case of biological terrorism, is actual today and will be in future [1–3].

One of the most important directions of scientific research in the field of studying and hygienic setting of biological pollution of environment is the development of effect tive monitoring system. In its turn, the basic moment for monitoring implementation of the environment biological factors is developing and perfecting of indication methods of different kinds of biological contaminants including working out of accelerated methods of microorganism extraction from environment. The success of antibacteriological protection in many respects depends on as far as the indication can be fast carried out [4–6].

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The given research is devoted to creation of the accelerated microbiological analysis for environment monitoring including the purposes of rapid deployment of rescue forces in case of applying most dangerous toxic bacteriological (biological) weapon (plague, smallpox, anthrax, etc., both in individual form and in combinations) which alongside with nuclear and chemical weapon presents large threat for mankind.

Mainly, in the practice of modern microbiology at the estimation of environment quality the microbiological analyses are carried out by cultivating microorganisms on different hard nutrient media prepared by dissolution of nutrient materials and special solidifying matter (agar, gelatine, derivatives of cellulose, etc.) in water with the subsequent formation of gel [7].

From known ways of preparing of hard nutrient media the way with usage of agar is the best. However, the process of preparing of this nutrient medium to an inoculation is characterized by large duration (3–5 h) and complexity, including operation of sterilization for which, besides, the special equipment is necessary.

28.2. Experimental, Results and Discussion

In this work the new approach to preparation hard nutrient media is proposed, namely, – impregnation by solution of nutrient materials by a special way of the treated plates from solid inorganic materials. Applying such materials as a basis for hard microbiological nutrient media became possible after the capacity of different microorganisms for active growth and formation of colonies on a surface of such nutrient media was detected in which were used as a sealing basis specially prepared solid inorganic materials but not gel-forming matters [8].

The plates prepared by the given way in sterile conditions are sealed in polyethylene packages where they can be stored a long time without loss of exploitative properties. The duration of preparation of such nutrient media directly to an inoculation equals 5–10 min. The specimens of different microorganism groups, actinomyces, streptococci, yeasty and mouldy fungi grow well on such media.

28.2.1. Example 1

Round plates with a diameter of 90 mm and thickness 8 mm of a solid inorganic material with open pores of a specific size and hardness on a Moos's scale equal 6 were applied. The plates were impregnated with a liquid Czapek's medium and sterilized in autoclave under 175 kPa during 30 min, then dried up at the temperature of 353–363 K up to constant weight. The prepared plates in sterile conditions were put in packages of polyethylene sterilized by ethanol and packages were sealed.

Test of operation properties of a dry nutrient medium prepared by the given method was made as follows. The packages with plates stored for 72 h were treated by ethanol and opened by sterile scissors. Twenty plates were put by sterile tweezers in sterile Petri dishes by one plate into dish. Sterile distilled water was introduced into dishes and was swallowed by plates. The duration of preparation of a medium to inoculation equals 5–10 min.

In comparison a powder of a dry nutrient medium "Czapek's agar" was kept in distilled water for swelling within 1 h, was dissolved under heating on boiling water bath, was sterilized in autoclave under 175 kPa during 30 min, poured out in 20 sterile Petri dishes and was kept under room temperature till solidification. The duration of preparation of a medium to inoculation equals 2–3 h.

In addition for a comparison dissolved, cooled sterile Czapek's medium, stored a flask, was melt down, poured out in 20 sterile Petri dishes and kept till solidification. The duration of preparation of a medium to inoculation is in this case is increased for 1–2 h and equals in general 3–5 h.

The surface of the prepared in this way media was inoculated by suspensions of spores of *Aspergillus niger* and *Penicillium chrysogenum* mould fungi, containing 1 million spores/mL, putting 0.05 mL suspension in three symmetrical points on a surface of a medium in each dish. The dishes were incubated in the thermostat under 301 K during 120 h.

The average diameter of *Aspergillus niger* colonies on a medium prepared according to point 1 equals 15.8 ± 4.7 mm, on agar medium equals 15.6 ± 4.4 mm. The average diameter of *Penicillium chrysogenum* colonies on a medium prepared according to point 1 equals 13.8 ± 3.1 mm, on agar medium equals 14.1 ± 3.3 mm.

28.1.2. Example 2

Dry hard microbiological nutrient media solidified by specially prepared solid porous materials were applied. These nutrient media were kept in usual room conditions during 1 year. All other operations are the same as in point 1. The average diameter of *Aspergillus niger* colonies on a medium prepared according to point 1 equals 15.8 ± 2.9 mm, on agar medium equals 15.6 ± 5.1mm. The average diameter of *Penicillium chrysogenum* colonies on a medium prepared according to point 1 equals 13.9 ± 4.6 mm, on agar medium equals 13.8 ± 3.1 mm.

28.1.3. Example 3

Hard nutrient Czapek's–Dox's medium, in which agar was replaced by a solid porous material with hardness on a Moos's scale equal 5 was prepared. Disks with a diameter 90 mm and thickness 8 mm, which were put in Petri dishes, were made of it. Ingredients of Czapek's–Dox's medium were dissolved in distilled water, then with an received solution disks till saturation were impregnated. Prepared hard nutrient medium was sterilized in autoclave under 175 kPa during 30 min.

The surface of prepared nutrient medium was inoculated by suspensions of spores of *Aspergillus niger* and *Penicillium chrysogenum* mouldy fungi, containing 1 million spores/mL, putting on each disk in three symmetrical points 0.05 mL suspension of one of fungi and after this dishes were incubated in the thermostat under 301 K.

In comparison in the same way poured out in Petri dishes sterile agar Czapek's–Dox's medium was inoculated. The dishes with inoculated agar medium were incubated in the same conditions. Each of four indicated variants was tested on ten Petri dishes.

28.1.4. Example 4

Hard nutrient medium of non-hopped must with strength 7° on polarimeter, in which agar is replaced by a solid porous material was prepared. A medium was inoculated by suspensions of yeast *Candida utilis* and *Saccharomyces cerevisiae* cells with concentration 1 million of cells/mL as is indicated in point 1. In comparison sterile agar musty medium poured out in Petri dishes was inoculated in the same way. All other operations are the same as in point 1. As an example, the comparative data of accumulation of yeasty and mouldy fungi biomass on hard nutrient media with a solid porous material and agar are indicated in the Table 28.1.

Table 28.1. Sizes of microorganism colonies on hard nutrient media with a solid porous material and agar

Species of microorganisms	Duration of incubation (h)	Solidifying basis	Average diameter of colonies (mm)
Aspergillus niger	120	Solid material	16.0 ± 6.1
Aspergillus niger	120	Agar	16.4 ± 6.2
Penicillium chrysogenum	120	Solid material	14.0 ± 2.6
Penicillium chrysogenum	120	Agar	15.0 ± 3.1
Candida utilis	96	Solid material	7.1 ± 0.3
Candida utilis	96	Agar	6.7 ± 0.6
Saccharomyces cerevisiae	96	Solid material	6.8 ± 0.2
Saccharomyces cerevisiae	96	Agar	6.9 ± 0.4

The comparative results in cultivation of microorganisms according to examples 3 and 4 (suspension of *Streptococcus* culture cells, taken from the patient with chronic tonsillitis and suspension of *Actinomyces flavus* spores) on nutrient media, respectively, sterile meat-peptone medium with adding 10% by volume of sterile serum of the bull blood and starch-ammonia medium of composition, namely, starch soluble (amilose) – 10g, $(NH_4)_2SO_4$ – 2 g, 1 g each of K_2HPO_4, $MgSO_4$ and NaCl, water – up to 1.0 L are indicated in the Table 28.2. Solidifying basis is solid porous material and agar.

2.1.5. Example 5

Disks with cultures of microorganisms from points 1 and 2 were autoclaved under 2 at during 30 min, were tempered under 873 K during 1.5 h, cooled in the air, boiled in water 1 h and dried up under 383 K up to constant weight. Then on the base of these disks hard nutrient media were prepared, which were inoculated and incubated as in points 3 and 4. The average diameter of *Aspergillus niger* colonies equals 16.5 ± 3.3 mm, *Penicililum chrysogenum* colonies equals 14.6 ± 2.5 mm, *Candida utilis* colonies equals 7.2 ± 0.2 mm, *Saccharomyces cerevisiae* colonies equals 6.7 ± 0.3 mm.

2.1.6. Example 6

Secondary regeneration of a solidifying basis was conducted. For this purpose the disks with cultures of microorganisms from point 5 were treated as in point 5. The average

diameter of *Aspergillus niger* colonies equals 16.2 ± 3.4 mm, *Penicllium chrysogenum* colonies equals 4.2 ± 2.6 mm, *Candida utilis* colonies equals 7.3 ± 0.3 mm, *Saccharomyces cerevisiae* colonies equals 6.6 ± 0.4 mm.

The results of four species cultivation of microorganisms after two successive stages of solid solidifying basis regeneration, as in examples 5 and 6, are indicated in the Table 28.3.

Table 28.2. Comparative results in cultivation of microorganisms on nutrient media solidified by a solid material and agar

Species of microorganisms	Duration of incubation (h)	Solidifying basis	Average diameter of colonies (mm)
Streptococcus	48	Solid material	1–3
Streptococcus	48	Agar	1–3
Actinomyces flavus	288	Solid material	4.9 ± 0.6
Actinomyces flavus	288	Agar	5.0 ± 0.8

Table 28.3. Results in cultivation of microorganisms after two stages of solid solidifying basis regeneration

Species of microorganisms	Duration of incubation (h)	Solidifying basis	Average diameter of colonies (mm)
Aspergillus niger	120	Solid material[a]	16.5 ± 3.3
Penicillium chrysogenum	120	Solid material[a]	14.6 ± 2.5
Candida utilis	96	Solid material[a]	7.2 ± 0.2
Saccharomyces cerevisiae	96	Solid material[a]	6.7 ± 0.3
Aspergillus niger	120	Solid material[b]	16.2 ± 3.4
Penicillium chrysogenum	120	Solid material[b]	14.2 ± 2.6
Candida utilis	96	Solid material[b]	7.3 ± 0.3
Saccharomyces cerevisiae	96	Solid material[b]	6.6 ± 0.4

[a]After the first stage of regeneration; [b]after the second stage of regeneration.

2.2. Conclusions

Applying solid porous inorganic materials as a basis for hard microbiological nutrient media has advantage before usage of known solidifying matters, as the nutrient media prepared on the new basis, have higher mechanical strength. The solidifying basis is cheaper and accessible and can be used repeatedly.

The test results of hard microbiological nutrient media prepared by developed and known ways have shown that the developed way allows to prepare nutrient media with higher exploitative properties, provides essential reduction of duration and simplification of preparation process of media to an inoculation. It meets the requirements of express extraction pathogenic microorganisms from environment and their cultivating.

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Chapter 29

Counteraction to Chemical and Biological Terrorism in the Republic of Moldova: Problems and Perspectives

Mutaf V.
Chemical and Radiological Division, Civil Protection and Emergency Situations Service, Republic of Moldova

Abstract. The paper deals with main problems and drawbacks which Civil Protection and Emergency Situations Service of the Republic of Moldova come across with during arrangement of counteraction to chemical and biological terrorism, and prospects of development of radiation-chemical and medical-biological protection units in the country with the use of up-to-date international and intrastate experience in this regard. Some examples of international military exercises on the topic of counteraction to terrorism, in which specialized subdivisions of the Republic of Moldova participated, are given.

Keywords. Terrorist attack, explosive materials, chemical warfare agents, biological agents

29.1. Introduction

In the Republic of Moldova, as in other countries of the former Soviet Union, there are industrial productions, research laboratories, military supply depots and other operating or inoperative objects of national importance, which contain toxic chemical, biological, and radioactive substances attractive for terrorists and posing a threat for civilians. Even under proper conditions of their storage and restricted access, these materials can become a subject of terrorist threats or weapon of criminal acts, because of, first of all, human factor based on economic or extremist motives.

List of basic terrorist threats with the use of chemical or biological substances typical for Republic of Moldova

- Accidents with transportation of radioactive waste materials
- Traffic of weapons, explosives, WMD and people through the airports and railroad stations
- Terrorist attack during land transportation of radioactive waste materials
- Terrorist attack in the buildings with many people, cities, industrial complexes, hydro technical facilities/dams, water pipes

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- Attack with explosive materials and radioactive sources ("dirty bomb")
- Release of toxic substances
- Threat, use or attack with chemical warfare agents (CWA)
- Use of biological agents (anthrax, ricin, botulinum toxin, etc.)

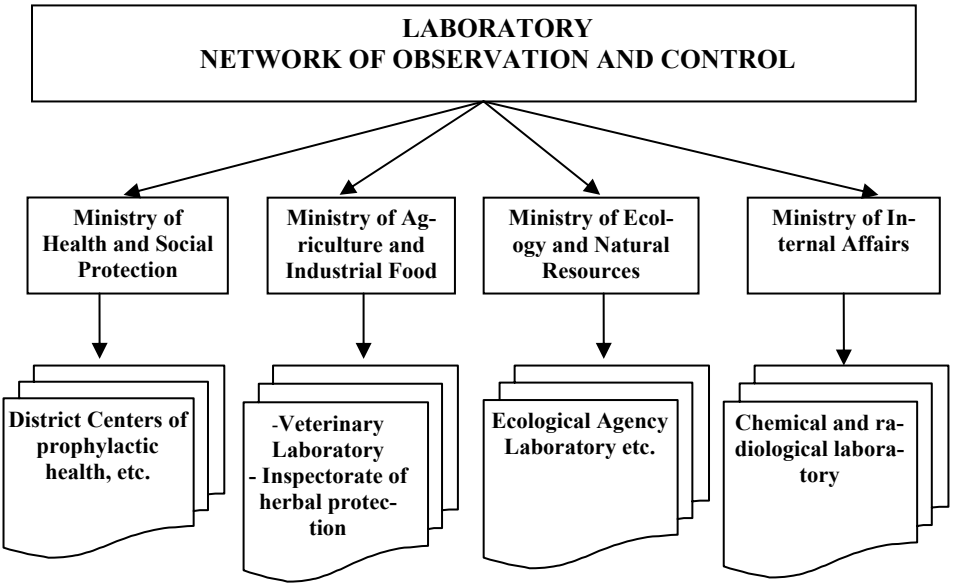
Information about facilities, which use chemical and radiological substances

Nr. d/o	Counties	Radioactive dangerous			Chemically dangerous	
		Total	Sources	Installations	Total	3/4 category
1.	Direction ES mun. Chişinău	62	10	52	41	2/8/31
2.	Direction ES mun. Bălţi	12	1	11	7	6/1
3.	Section ES Anenii Noi	7		7	11	3/8
4.	Section ES Basarabeasca	2	1	1	6	1/5
5.	Direction ES Briceni	2	1	1	4	2/2
6.	Direction ES Cahul	7	3	4	17	4/13
7.	Section ES Cantemir	2		2	8	3/5
8.	Section ES Călăraşi	2		2	5	1/4
9.	Direction ES Căuşeni	3	1	2	14	8/6
10.	Section ES Cimişlia	2		2	12	5/7
11.	Section ES Criuleni	2		2	5	–/5
12.	Section ES Donduşeni	2		2	2	1/1
13.	Section ES Drochia	3	1	2	4	3/1
14.	Section ES Dubăsari				1	–/1
15.	Section ES Edineţ	4	1	3	6	6/–
16.	Section ES Făleşti	2		2	3	1/2
17.	Section ES Floreşti	2		2	5	5/–
18.	Section ES Glodeni	3	1	2	3	2/1
19.	Direction ES Hînceşti	3	1	2	18	8/10
20.	Section ES Ialoveni	3	1	2	12	12/–
21.	Section ES Leova	2	1	1	7	2/5
22.	Section ES Nisporeni	2		2	9	8/1
23.	Section ES Ocniţa	2	1	1	9	5/4
24.	Direction ES Orhei	2		2	13	3/10
25.	Section ES Rezina	2	1	1	3	2/1
26.	Direction ES Rîşcani	2		2	2	1/1
27.	Direction ES Sîngerei	1		1	4	2/2
28.	Direction ES Soroca	3		3	4	2/2
29.	Section ES Străşeni	3		3	19	3/16
30.	Section ES Şoldăneşti	2		2	3	2/1
31.	Direction ES Ştefan Vodă	2		2	14	3/11
32.	Section ES Taraclia	2		2	7	2/5
33.	Section ES Teleneşti	4		4	9	4/5
34.	Direction ES Ungheni	4		4	10	3/7
35.	Direction UTA Găgăuzia	7	1	6	24	11/13
	Summary:	**165**	**26**	**139**	**320**	**128/192**
	Total:	**485**				

Information about quantity of toxic substances in Republic of Moldova

Nr. d/o	Counties	Cl_2 (t)	NH_3 (t)	SO_2 (t)	EM м^3/ acid (t)	Total (t)
1.	Direction ES mun. Chişinău	20–105	9–150	6–7	2_{17}4–120	41–384
2.	Direction ES mun. Bălţi	2–40	4–35	–	1–13	7–88
3.	Section ES Anenii Noi	1–0.6	2–9	4–8	4–9.8	11–27
4.	Section ES Basarabeasca	1–2		3–8	2–3.5	6–13.5
5.	Direction ES Briceni		3–12		1–1.0	4–13
6.	Direction ES Cahul	1–6	2–8	11–15	3–4.5	17–34
7.	Section ES Cantemir			8–12	–	8–13
8.	Section ES Călăraşi	1–1	1–4	1–1	2–3.5	5–8
9.	Direction ES Căuşeni	1–3	3–19	6–20	3–6.0/1	14–48
10.	Section ES Cimişlia	1–2	2–10	7–10	2–2.7	12–25
11.	Section ES Criuleni			2–4	3–4.4/1	5–9
12.	Section ES Donduşeni		1–5		1–1.7	2–7
13.	Section ES Drochia				4–6.8	4–12
14.	Section ES Dubăsari				1–1.7	1–1.7
15.	Section ES Edineţ	1–10	3–20		2–3.5	6–33.5
16.	Section ES Făleşti		2.5		1/1.7	3–5.0
17.	Section ES Floreşti	1–2	2–6		3–4.5	5–10.5
18.	Section ES Glodeni	2–3			1–1.7	3–5.0
19.	Direction ES Hînceşti		2–3	14–25	1–1.7/1	18–30.0
20.	Section ES Ialoveni	1–2	1–3	11–20		12–24.0
21.	Section ES Leova	1–1	1–3	5–15	1–0.2	7–19.0
22.	Section ES Nisporeni	1–1		7–9	1–0.2	9–10.0
23.	Section ES Ocniţa		4–8		5–8.0	9–16.0
24.	Direction ES Orhei	1–3	2–6	4–5	5–7.0	13–21.0
25.	Section ES Rezina	2–40			1–1.7	3–42.0
26.	Direction ES Rîşcani		1–3		1–1.7	2–5.0
27.	Direction ES Sîngerei		2–4	1–2	1–1.7	4–8.0
28.	Direction ES Soroca	1–20	1–3		2–3.5	4–26.5
29.	Section ES Străşeni	1–2	3–8	13–20	2–3.5	19–33.0
30.	Section SE Şoldăneşti		1–3		2–3.5/1	3–6.0
31.	Direction ES Ştefan Vodă	1–1.6	2–6	7–12	4–4.7	14–23.0
32.	Section ES Taraclia			6–6	1–3.5	7–10.0
33.	Section ES Teleneşti	1–1.0	2–6	5–5	1–1.7	9–14.0
34.	Direction ES Ungheni	2–20	1–11	5–6	2–0.4	10–37.0
35.	Direction UTA Găgăuzia	1–50	3–9	16–21	7–8.7	24–35.0

At the same time, special-purpose programs were developed in the republic at the national level, and specialized organs and subdivisions were formed, which are capable to prevent the threat of terrorist acts or operatively eliminate their consequences in case of commitment of such acts. The said authorities include Civil Protection and Emergency Situations Service of the Republic of Moldova (CPESS RM) and National Network for Monitoring and Laboratory Control (NNMLC).



Main tasks of the laboratory network of observation and control:

In case of chemical contamination:

- Establishes the fact of poison-toxic substances contamination
- Determines the types of poison-toxic substances in the air, water, natural reservoirs, soil
- Determines the quantity of poison-toxic substances in the food-stuff, potable water, edible raw material and forage etc.
- Makes the examination of food-stuff, potable water, and forage
- Determines the contamination level with toxic substances of the environment in the heavy traffic transportation areas

In case of biological contamination:

- Establishes the fact of biological contamination
- Determines the types of pathogen biological agents in the environment, people and domestic animals organisms
- Makes the examination of food-stuff, potable water, edible raw material and forage

In case of radioactive pollution:

- Determines the background of gamma-radiation on the site
- Determines the isotopic contents of radioactive pollution
- Determines the level of radioactive pollution of the environment: water, near surface air layer, food, etc.
- Assess the risk of radioactive contamination for people and domestic animals, in the territory and environment
- Makes the examination of food-stuff, potable water, edible raw material and forage
- Radiation control of importing goods at the border

In the course of responding to terrorist threats, CPESS RM can be actually involved at two stages only:

(a) At the stage of demarcation of zones of chemical, radiological or biological contamination of areas
(b) At the stage of elimination of consequences of using chemical, radiological or biological substances

At the stage "a", as a rule, NNMLC units are involved. At the stage "b" the principal operating subjects are subdivisions of radiation-chemical and medical-biological protection.

Civil protection units for counteraction to chemical and biological terrorism:

- Chemical and radiological prospecting service in structure emergency rescue detachment – 1 (Chisinau, 15 persons)
- Chemical and radiological prospecting service in structure emergency rescue detachment – 2 (Balti, five persons)
- Chemical and radiological laboratory (Chisinau, seven persons)
- Chemical, radiological, medical and biological protection section in structure civil protection and emergency situations service (Chisinau, six persons)

Main problems and drawbacks which chemical and biological protection in Republic of Moldova come across with during arrangement of counteraction to chemical and biological terrorism:

- Insufficient of the technical equipment for the chemical and biological protection units
- Uneven placing of the chemical and biological protection units on the territory of country
- Absence of the full strength personnel in the chemical and biological protection units
- Absence of the modern training system for the specialists in the field of chemical and biological protection

Prospects of development of radiation-chemical and medical-biological protection units in the country:

- Creation of additional chemical and biological protection units, at first, for northern and southern regions
- Acquisition of the modern equipment for the chemical and biological protection units
- Full strength personnel in the chemical and biological protection units
- Application of the modern methods for professional specialists training in the field of chemical and biological protection
- Development of international collaboration in the field of counteraction to chemical and biological terrorism

Some examples of international military exercises on the topic of counteraction to terrorism, in which specialized subdivisions of the Republic of Moldova participated, are given:

- "TRANSCARPATHIA 2000"
- "BOGORODSK 2002"
- "DACIA 2003"
- "CMEP TTX 2003, 2004, 2005, 2007"
- "ROUGH AND READY 2006"
- "IDASSA 2007"
- "HUROMEX 2008"

Laws of the Republic of Moldova in the Field of Terrorism Counteraction

Parliament Decree of the Republic of Moldova:

1. №. 44-XIV on 04.06.1998 "Regarding the implementation of the European Agreement on international transportation of dangerous goods"
2. №. 426-XV on 27.07.2001 "Collaboration agreement among CIS countries in struggle against terrorism field"
3. №. 539-XV on 12.10.2001 "Struggle against terrorism"

Government Decree of the Republic of Moldova:

4. №. 45 on 24.01.1994 "Regarding on settlement of transportation of dangerous goods on the territory of the Republic of Moldova and mitigation of the consequences of possible accidents"
5. №. 778 on 14.06.2002 "Regulation regarding operational group for conducting antiterrorist operations"
6. №. 672 on 28.05.2002 "Regarding transportation of dangerous goods on the territory or the RM"
7. №. 961 on 21.08.2006 "Regulation regarding National Network for Monitoring and Laboratory Control"

Chapter 30

Strategies of Preparedness Response to Biological Warfare and Bioterrorism Threats

Paul F.
Medical Support Section, Department of Field Support, United Nations Headquarter, New York 10017, USA

Abstract. Risks associated with deliberate use of biological agents against population and the needs for a strong public health system are well recognized. The potential use of biological agents is mounting every day, especially with growing political dissidence as well as social and economical conflicts in several countries. Countries in Eastern Europe have considerable vulnerability because of economical, social and political difficulties they are facing, along with inadequate response capacity and large number of outfits having continuous low intensity conflicts with established administrative system. There are no doubts that preparedness against biological weapons must be integrated in national disaster preparedness plan, as well as strengthen the core competencies of public health and other national and local authorities to respond to biological crisis, along with strong collaboration with other national agencies, like intelligence, defense and police.

Keywords. Bioterrorism, biological agents, preparedness, public health

30.1. Introduction

The use of microorganisms and toxins as weapons of mass destruction has a long history, with examples dating back to before the fourteenth century. Methods used have ranged from deliberately infecting water supplies with diseased cadavers to passing out smallpox infected blankets to "extirpate" Native American tribes in 1763. Other examples include the use of fomites as the Viet Cong did in early 1960s when they smeared sticks with excrements [1].

The nature of terrorism is changing. It is no longer only hijackers and bombs. Nor it is only chemical attack such as the 1995 Aum Shirinkyo sarin gas in Tokyo subway.

Bioterrorism is no longer a hypothetical event. A bioterrorist attack has occurred and could occur again at any time, under any circumstances, and at a magnitude far greater than we have thus far witnessed.

Similar with chemical terrorism in some ways, bioterrorism differs primarily in the lag time between the terrorist event and its medical consequences. It therefore poses its own critical challenges, particularly for the publish health community [2].

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In spite of infrequent occurrences of such episodes the potential use of selected agents, with or without genetic alterations, is mounting everyday especially with growing political dissidence as well as religious and resources conflict in several countries [3].

Several factors make biological threats unique as compared with chemical and nuclear weapons. First, facilities and equipment designed for legitimate applications, and also for research and development can be used to produce biological agents. And they are widespread throughout the world. Secondly, the collapse of former Soviet Union [4] and subsequently reduction in funding its massive biologic warfare infrastructure may have resulted in vulnerability for recruitment by states or terrorist organization trying to establish biological warfare programs. The last but not the least, the incredible advances in biotechnology over the past 15 years can potentially be used for production of biological agents, including genetic engineered pathogens, resistant to antimicrobials and classic vaccines.

30.2. Characteristics of Biological Attack and Bioterrorist Attack

In comparison to chemical and nuclear weapons, biological weapons require less complex technology and low cost allowed even poor countries or terrorist organizations to acquire or to product them.

Usually preparedness to respond to a biological attack or bioterrorist attack was directed on a number of potential agents, in particular anthrax, smallpox, plague, botulinum toxin, tularemia, and viral hemorrhagic fevers. Anthrax is a proven risk and of most immediate concern, although smallpox, because it is capable of person-to-person transmission, engendered an equivalent sense of urgency. However, there is a plethora of potential, credible bioterrorist agents. It should be noted that the Soviet Union is known to have weaponized some 30 different biological agents, including drug-and vaccine-resistant strains.

Theoretically, biological agents are ideal agents to be used as weapons of mass destruction. However not all microbes can be weaponized. Several characteristics are required to make a microorganism an ideal agent that can be used as a potential weapon of mass destruction or bioterrorist agent. These pertain to virulence, infectivity, lethality, ease of production, stability in environmental conditions, and post-dissemination retention of features, availability of a susceptible population and lack or inadequacy of resources and tools to prevent and to treat the diseases. while hundreds of pathogenic microorganisms have been investigated for their potential utility as military weapons, relatively few, around 40, have been found capable of meeting specific requirements, and fewer still have been documented to have been weaponized [5].

The US Centers for Disease Control (CDC) has developed a classification system for biological terror agents [6]. The classification is based on the likelihood of the agent being used and the risk posed by each agent. The agents and the diseases they cause are listed in Table 30.1.

Table 30.1. CDC classification of biological weapons agents. Modified from F. Paul

Category	Diseases/Organism	Characteristics
(a) High-priority agents (pathogens that pose a high risk to national, local security)	Anthrax (*Bacillus anthracis*) Botulism (*Clostridium botulinum* toxin) Plague (*Yersinia pestis*) Smallpox (variola major) Tularemia (*Francisella tularensis*) Viral hemorrhagic fevers (filoviruses [e.g., Ebola, Marburg] and arenaviruses [e.g., Lassa, Machupo]	– Can be easily disseminated or transmitted from person to person – Result in high mortality rates and have the potential for major public health impact – Might cause public panic and social disruption and – Require special action for public health preparedness
(b) Second highest priority (moderately risk and relative low mortality)	Brucellosis (*Brucella* species) *Clostridium perfringens* Food safety threats (e.g., *Salmonella* species, *Escherichia coli* O157:H7, *Shigella*) Glanders (*Burkholderia mallei*) Melioidosis (*Burkholderia pseudomallei*) Psittacosis (*Chlamydia psittaci*) Q fever (*Coxiella burnetii*) Ricin toxin from *Ricinus communis* (castor beans) Staphylococcal enterotoxin B Typhus fever (*Rickettsia prowazekii*) Viral encephalitis (alphaviruses [e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis]) Water safety threats (e.g., *Vibrio cholerae*, *Cryptosporidium parvum*)	– Are moderately easy to disseminate – Result in moderate morbidity rates and low mortality rates and – Require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance
(c) Third highest priority (emerging pathogens that could be engineered for mass dissemination in the future)	Emerging infectious diseases such as Nipah virus and hantavirus	– Availability – Ease of production and dissemination and – Potential for high morbidity and mortality rates and major health impact

The classical methods for disseminating the agents are through aerosols or by the use of disease carrying vectors. Explosive bomblets in which there is buster, surrounded by biological agent and enclosed in thin case, explode upon impact and disseminate the biological agent as an aerosol. Spray tanks carried by aircraft and missiles may also be utilized for producing aerosol containing the biological agent.

Disease carrying vectors such as mosquitoes, mites, tics and lice may be delivered by aircraft or missiles in containers which rupture on impact. The biological agents may also be introduced into the food chain or water.

30.3. Preparing for Response

Implications of deliberate use of biological agents are very difficult to be predicted. A well-conducted biological terrorist attack will strain almost all public health authorities and medical care facilities. The primary consequence of a large scale biological attack will be a catastrophically large number of casualties [7].

Response system must be capable of providing a complete package of medical care and additional services in order to mitigate the effects of the attack. However the full range of consequences is very difficult to be evaluated, as the pathogen and the amplitude of attack is almost impossible to be foreseen before the event happens. Too many factors may influence the course of biological crisis. Briefly could be mentioned: location of the attack, density of population, characteristics of biological agent, availability of treatment and prophylaxis, residual environmental damages and hazards etc.

Nevertheless a bioterrorist attack is a criminal act and a complex criminal investigation must be performed.

The full spectrum of consequences require an integrated command and control system, extended at those level of authority able to provide in real time the full support for targeted community. That may include local, regional, federal or national authorities, extension of authority at highest level required for a coherent and efficient control and command. In the mean time a multidisciplinary and multiple professional team is requested to support the common effort to mitigate the effects of criminal act.

Countries in East and South East of Europe have considerable vulnerability because of poverty, inadequate response capabilities and capacities and large number of outfits having continuous low intensity conflicts with new established administrative system. However, high prevalence of communicable diseases and relative frequent epidemics has stimulated national health authorities to strengthen their early recognition and response capabilities.

Successful preparation will depend upon the development of a well-orchestrated plan to be used by first responders. For bioterrorism they will be epidemiologists, infectious diseases experts, medical personnel in emergency room, and intensive care units. The most powerful strategy may be to cast bioterrorism defense as a national security issue first and foremost.

An alternative strategy would be to address bioterrorism response preparedness in a coordinated fashion with broad emerging infectious disease issues. For example, propose this effort as a defense system with concurrent substantial benefits for the public health system, as well as microbial and biomedical science.

A good example is how national authorities and international organizations responded to SARS outbreak.

Response strategies for biological attack and bioterrorism must be developed along with devolvement of a real time surveillance system, with enhanced capabilities for identification of biological agents, for isolation and successful treatment of casualties and efficient prophylaxis measures.

That means that the public health infrastructure must be strengthened in order to ensure a rapid, effective response in the event of another bioterrorist attack.

That includes at least:

- Communication and information
- Laboratory capacity
- Surveillance, detection, and diagnosis
- And strengthening the local response

Educate front line healthcare providers so that the astute laboratory clinician, nurse, or doctor who sees the first patient or wave of patients can recognize an attack early and sound the alarm.

A strong public health infrastructure that can detect cases and deliver the appropriate therapeutics in a timely manner requires resources and organization at the community level. However, workshop presenters representing these organizations noted that there are many significant gaps in our local response capabilities. For example, delivering the stockpile to where it is needed is likely the least of our worries. Rather the challenge will be in distributing its contents. There is a need at both the state and local levels to identify emergency authorities and delegate responsibilities.

A strong local response involves not only local public health agencies, but also hospitals, the law enforcement community, and the community at large. It was observed by WHO that there is a striking disparity in public health capacity not only among countries, but also but also among jurisdictions within countries. That can jeopardize the effectiveness of response.

Strengthening local and state public health agencies will require an infusion of resources, including both trained personnel and financial resources.

It was necessary that every country to evaluate its own system, including its legal system, and implement its own plan of action for organizing and strengthening its response capabilities.

However there are many challenges that must be taken in consideration. From our understanding critical challenges are:

- Preparedness programs to be comprehensive and they may that involve all critical players in a crisis situation. Often planners may confuse the role of first responders with expert/ professionals one; this will conduct to a misapprehend that the last are responsible for action as first responders.
- Budgeting and adequate funding for development of effective surveillance and response systems; traditionally funds have been directed to first responders; this is inappropriate initial direction because without technical expertise first response may be inadequate.
- Involvement of all spectrum of specialist/experts in developing and running preparedness programs; that means that for example medical personnel must be involved in this process, as they are first responders for medical care of casualties. Physicians, nurses, other health care professionals must know how to react in case of mass casualty situations, and must became active participants in the preparedness arena.
- Incident command system is another critical challenge for planners and players too. All parties must know the flow of information, chain of command and control and must obey it, to ensure the coherence and unique command “policy’.

- Preparedness programs must be comprehensive, including not only the final phase-first response to crisis, but more important to develop and sustain surveillance system to detect use/ release of a nuclear, chemical or biological agent. Usually there are dedicated plan for each situation. This may create confusion even within first responders or crisis cell; we consider more effective to develop a comprehensive preparedness and response plan, and with different scenarios for each situation; this approach will bring more coherence and effectiveness within local authorities, first responders, local health authorities, communication, transportation etc.
- Surveillance system is a critical asset for any preparedness plan; the most important task of it is warning of an attack, existence of a threat. It is recommended that surveillance system to be integrated with other similar assets; this will allow a continuous monitoring at different levels of authorities, and not only local, but regional, national or international.
- Critical for an effective response in chemical and biological attack is the need for a specific training for health care personnel. Health care professionals must be able to recognize clinical aspects of chemical or biological attack. This will tremendously help to improve the effectiveness and success of treatment.

30.4. Conclusions

Despite the huge pressure on medical system in crisis situation, preparedness activities for response to a bioterrorist attack are responsibility of local and national government.

To ensure an efficient and real-time response there is a need for authorities to develop feasible response plan, including projected costs and probability of effectiveness and to identify also those mechanisms who will ensure the viability and function of the plan in crisis situation.

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Chapter 31

Antitoxic Action of A. viridans on Exotoxin, Produced by S. aureus

Kremenchutsky G., Yurgel L.,
Stepansky D., Sharun O., and Koshevaya I.
Dnepropetrovsk State Medical Academy, Ukraine

Abstract. One of the most important indices of staphylococcal toxin is leukotoxic activity. During investigation, this characteristic was examined in 94 test samples and 36 control samples of toxin, obtained from two cultures of S. aureus – «Lossman» and «Lor». When analyzing results, we calculated neutrophil damage index (NDI): correlation of the number of damaged neutrophils and total number of found ones. It was established that samples of toxin obtained from test cultures of staphylococci survived during repeated contact with A. viridans, were characterized by reliably decreased ($p < 0.001$) leukotoxic activity. For example, NDI for control samples of undiluted toxin was equal to 0.91 and 0.8; for test samples – 0.26 and 0.62. Variance in leukotoxic activity was observed even upon dilution of test samples of toxin as compared with control ones. So, whereas NDI in 81 times' diluted control samples of toxin was equal to 0.84 and 0.75, this index in test samples appeared to be 0.19 and 0.57, accordingly. Lethal characteristics were examined in 157 test samples and 85 control samples of toxin during intravenous introduction to white mice in quantity of 0.2 mL. Dates of animals' death were duly noted. Evidently, 157 test samples of toxin examined by us turned to be inactive: all animals survived. At the same time, control samples of toxin were distinguished by clearly expressed lethal activity: almost all mice died during 5 days. Only two mice survived of 85 ones. Similar results were obtained during investigation of necrotic characteristics of the same 157 samples of toxin. Toxins obtained from strains of staphylococci not contacted with aerococci caused, as a rule, necrosis of rabbit skin. At the same time, toxins obtained from strains of staphylococci contacted with A. viridans No. 11 caused feebly marked necrotic reaction only. Therefore, A. viridans No. 11 producing H_2O_2, have not only bacteriostatic and bactericide influence on staphylococcus, but also change its characteristics down to loss of virulence thereby. It may be supposed that aerococci completely disturb synthesis or impede formation of any fraction of staphylococcal toxin.

Keywords. Staphylococcus aureus, inhalation, neutralization, A-bacterin, A. viridans

31.1. Introduction

Now toxins, as a specific category, are in the front rank in the publications related to biological weapons [1, 2]. At present time, toxins become more accessible for mass production, than ever before. The term "toxins" means poisonous materials of plants, animals, microorganisms, viruses, fungi, or infectious substances or recombinant molecules, regardless of their origin or method of production, including – (a) any toxic agents

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or biological products which can be created as a result of bio-technologies by any organisms; or (b) any poisonous isomers or biological products, homologues or derivatives thereof [3].

Staphylococcal enterotoxins represent wide-spread source of food poisoning connected with diarrhea, because of intake of improperly processed food. These are proteins varying in their weight from 23 to 29 kDa, which mechanism of action, as may be supposed, consists in stimulation of mass release of a number of cytokines, causing various toxic consequences. This warfare agent is considered as an agent of incapacitation action. Median dose required for incapacitation of human during inhalation of this substance is equal to about 0.4 ng/kg of weight. The relevant lethal dose is approximately 50 times higher [4].

Toxins are released by gram-positive cocci Staphylococcus aureus, which are spread all over the world. Cultivation of a number of stains allows obtaining considerable quantities of enterotoxin of B type. In those cases, when *Staphylococcus aureus* as a contaminant gets to foodstuffs, and resulting toxin is found in digestive system, symptoms like nausea, vomiting and diarrhea are evident during 1–6 h after eating of contaminated food products.

After inhalation of staphylococcal enterotoxin B (SEB) signs of poisoning occur in 3–12 h in the form of sudden rise of temperature, headache, algidity, myalgia and dry cough. In the most serious cases, shortness of breath and precordialgia may occur. In principle, after the first acute stage of disease the major part of patients feel normal, but they will not be able to carry on their ordinary activities during 1–2 weeks [5].

For neutralization of the action of toxin getting into the human organism, it is possible to use neutralizing chemical preparations or antitoxic serums, should the type of toxin be known. These methods are problematic ones, since timely detection of toxin type and use of antidote is not always possible.

Alternative solution, in our opinion, is the use of pro-biotic preparations of live microorganisms, in particular, those producing active forms of oxygen (AFO). Specifically, such preparation is A-bacterin, containing live bacteria – aerococci, generating AFO in the process of oxidation of lactic acid. This preparation is absolutely harmless; it can be used for a long time and is characterized by non-specific neutralizing action on bacterial toxins [6].

We studied the influence of A-bacterin on toxin-forming ability of staphylococci and manifestation of their virulence factors. Two hundred and forty-four strains were investigated, including 161 strains – after sixfold or sevenfold contact with A. viridans No. 167, and after six or seven passages in meat-peptone broth without aerococci. Ability to form toxins and strength of toxin thus formed, as well as virulence factors, were determined by presence and manifestation of leukotoxic, lethal, necrotic and hemolytic activity.

Table 31.1 gives results of testing hemolytic properties of investigated toxin samples regarding sheep and rabbit erythrocytes. During follow-up of results of such investigation, only clearly expressed hemolysis was taken into account. As it is shown by the data from Table 31.1, almost all examined samples of toxin (in test and control groups) demonstrate pronounced hemolytic activity towards rabbit erythrocytes. Variations are not statistically significant ($p > 0.05$).

Insignificant differences ($p > 0.05$) between test and control samples of toxin were revealed regarding sheep erythrocytes (hemolysis in 2 h is an exemption). Namely, of 161 test samples 89 and 130 samples demonstrated well-pronounced hemolytic activity in 2 and 24 h, accordingly (55.2% and 80.7%); among 83 control samples during the same periods – 72 and 65 samples (86.7% and 78.3%). No hemolysis was observed in 31 test samples of toxin (19.2%), and in 11 control samples (13.2%).

One of the most important indices of staphylococcal toxin is leukotoxic activity. During investigation, this characteristic was examined in 94 test samples and 36 control samples of toxin, obtained from two cultures of S. aureus – "Lossman" and "Lor". During analysis of results, neutrophil damage index (NDI) was calculated, i.e. correlation of damaged neutrophils' number with total number of found ones. Results of investigation of neutrophil damage under impact of toxins obtained from control and test strains of the sixth passage of "Lossman" and "Lor" staphylococcus cultures, are given in Table 31.1.

Table 31.1. Neutrophil damage indices (NDI) under action of toxins of staphylococcus culture (M ± m)

Indices	**Undiluted toxin**		**Diluted toxin**	
	Test	**Control**	**Test**	**Control**
	"Lossman" cultures of staphylococci			
NDI	0.62 ± 0.004	0.8 ± 0.002	0.57 ± 0.003	0.75 ± 0.002
Number of observations	25	7	25	7
Level of significance of differences p	<0.001		<0.001	
	"Lor" cultures of staphylococci			
NDI	0.25 ± 0.003	0.91 ± 0.0001	0.91 ± 0.003	0.84 ± 0.0002
Number of observations	69	29	40	28
Level of significance of differences p	<0.001		<0.001	

As based on the analysis of data from Table 31.2 it was established that samples of toxin obtained from test cultures of staphylococci, survived upon multi-fold contact with A. viridans No. 167, are characterized by definitely reduced ($p < 0.001$) leukotoxic activity. So, NDI for control samples of undiluted toxin was equal to 0.91 and 0.8; for test ones – 0.26 and 0.62. Varied leukotoxic activity is observed even upon dilution of test samples of toxin as compared with control ones. For example, if NDI in control samples of toxin diluted 81 times was equal to 0.84 and 0.75, this index in test samples appeared to be equal to 0.19 and 0.57 accordingly. Obtained data are statistically significant ($p < 0.001$).

Lethal characteristics were examined in 157 test samples and 85 control samples of toxin upon intravenous injection to white mice in the amount of 0.2 mL. Dates of animal death were duly noted. Obtained results are given in Table 31.3.

Table 31.2. Hemolytic activity of test and control samples of freshly isolated staphylococcal toxin; n = 4

Staphylococcus strain code	Toxin sample	Q-ty of samples	Hemolysis of erythrocytes					
			Of sheep			Of rabbits		
			N.A.	is evident in (h)		N.A.	Is evident in (h)	
				24 h	incl. that in 2		24 h	Incl. that in 2
«Lossman»	Test	64	10	54	41	1	63	61
«Lor»	Test	97	21	76	48	1	96	80
Total (%; M ± m)	Test	161	31 / 19.2% ± 3.1	130 / 80.7% ± 3.1	89 / 55.2% ± 3.9	2 / 1.2% ± 0.8	159 / 98.7% ± 0.9	141 / 87.5% ± 2.6
«Lossman»	Control	32	8	24	18	2	30	26
«Lor»	Control	51	4	47	47	1	50	46
Total (%; M ± m)	Control	83	11 / 13.2% ± 3.7	72 / 86.7% ± 3.7	65 / 78.3% ± 4.5 *	3 / 3.6% ± 2.0	80 / 96.3% ± 2.1	72 / 86.7% ± 3.7

Designations: test – toxin obtained from cultures of staphylococci survived after sixfold contact with A. viridans No. 167; control – toxin obtained from cultures of staphylococci not contacting A. viridans.

Notes: 1. in the numerator – absolute quantity of samples; in the denominator – the same expressed in percent; 2. *$p < 0.05$ as compared with control one.

Table 31.3. Degree of inhibition of lethal properties of test and control samples of toxin after intravenous trduction to white mice; n = 3

Staphylo-coccus strain code	Toxin sample	Qty of samples	Quantity of samples upon introduction of which the animals						
			Survived	Died in					
				2 h	12 h	24 h	48 h	3 days	4 days	5 days
"Lossman"	Test	50	50 100%	0	0	0	0	0	0	0
"Lor"		107	107 100%	0	0	0	0	0	0	0
Total		157	157 100%	0	0	0	0	0	0	0
"Lossman"	Control	34	0 0%	0	8 23.5%	16 47.1%	3 8.8%	2 5.9%	2 5.9%	3 8.8%
"Lor"		51	2 3.9%	3 5.9%	3 5.9%	20 39.2%	8 15.7%	5 9.8%	10 19.6%	0
Total (%; M ± m)		85	2 2.4 ± 1.6%	3 3.5 ± 2.0	11 12.9 ± 3.6	36 42.4 ± 5.4	11 12.9 ± 3.6	7 8.2 ± 3.0	12 14.1 ± 3.8	3 3.5 ± 2.0

Designations: Test – toxin sample obtained from staphylococci survived upon sixfold contact with A. viridans 167 (test); control – toxin sample obtained from staphylococci not contacted A.viridans (control).
In the numerator – absolute quantity of toxin samples; in the denominator – the same expressed in percent.

Data taken from the table show that 157 examined test samples of toxin turned to be inactive: all animals survived.

At the same time, control samples of toxin were distinguished by clearly expressed lethal activity: during 5 days almost all mice died. Two mice of 85 ones survived only. Significance of difference of lethality rate in test and control groups is high ($p < 0.001$). More than a half of animals from the control group (60.2%) died during 24 h.

Similar results were received during investigation of necrotic properties of the same 157 samples of toxins.

Toxins obtained from staphylococci not contacted with aerococci, as a rule, caused necrosis of rabbit skin. At the same time, toxins obtained from staphylococcus strains which contacted with A. viridans No. 167, caused feebly marked necrotic reaction only.

Consequently, A. viridans No. 167 producing H_2O_2, present not only bacteriostatic and bactericide impact on staphylococcus, but also change its properties down to the loss of virulence thereby. It may be supposed that aerococci completely disturb synthesis, or impede formation of any fraction of staphylococcal toxin.

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Chapter 32

Crops Pests Negative Risk Assessment in the Steppe Zone of Ukraine

Kharytonov M.[1] and Dudka Y.[2]
[1]*Dnepropetrovsk State Agrarian University, Ukraine*
[2]*Institute of Grain Crops, Ukrainian Academy of Agrarian Science, Ukraine*

Abstract. Two types of the grain field pathogen complex formation are selected in dependence of the meteorological conditions of the Steppe Zone of Ukraine. First type is characterized with stress situations (drought, high temperatures) and facultative pathogens predominance. Second type is connected with precipitations abundance and obligate parasites occurrence. Threatens impact on winter wheat seeds germination and affection with root rots were studied in the field experiments. Two ecological factors (air temperature and aphids) are main reasons to the beginning of potato seeds degeneration processes. Ensuring sustainable crops yield productivity is impossible without ecological farming as following: varieties resistance to disease and insects, biological control preparations, rational use of organic and composts and use of microbiological products with multiple effects.

Keywords. Grain crops, potato, tomato, pests, risk assessment

32.1. Introduction

The steppe is most heavily cropped, and occupies most of the southern half of the country (25 million hectares). Of Ukraine's 15.5 million hectares of high fertility soils, the primary steppe soils, the chernozeoms, occupy 77% or 11.9 million hectares [1]. The steppe zone average annual precipitation averages 470 mm, but varies by year from less than 350 mm to more than 550 mm [2]. Yields vary widely from year to year due to this marginal and erratic precipitation.

While wheat is the primary crop, barley, corn, rye and sugar beet are also harvested in the country. Sunflower seeds, flax, sugar beet, tomatoes and potatoes are produced in large quantities.

The Ukraine steppe can be divided in two subzones: northern and southern parts.

Recently collective and private farms have used very little fertilizers and chemicals. Generally speaking the enterprises have been forced into this situation because they do not have enough money to buy inputs. An optimistic interpretation of this situation is that they are coming close to organic farming. It would be good if they adopted the organic farming ideas in full. In order to do it they need to use new knowledge on how to tackle pest problems, the nutrient balance in the soil and the quality of the products. At this time private farmers cannot use ecological farming systems because they are oriented to one-two crops or animal production systems. Taking these into consideration,

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pre-requisites to get sustainable agriculture are with development of mixed farming systems including integrated varieties, pest and nutrition management, conservation tillage, etc. Some prospects to future development this work are connected with means to decrease Colorado Potato Beetles population: BT potato and tomato varieties application, straw mulch and new insecticides treatments with prediction approach, etc. [3–5].

Obviously that tomato and potato plantings should be included in the crop rotation with wheat or maize when favorable conditions to protection from CPB are created (conservation tillage, mulching, Bacillus thuringiensis, etc.).

32.2. Materials and Methods

Phytosanitary monitoring of crops was conducted as system for pests populations scouting including number, activity and reaction on growing agroecological conditions. This system includes development insects, diseases prediction and needs to apply the artificial and nature means to limit pest's negative impact. Crops ecosystems scouting methodology took in account two aspects of crops phytosanitary conditions: crops rotation level and within pest areal. The checklist for pest scouting was used. Main pests will be accounted regarding with accepted ground based methods. Main integration pest protection methodology goal was described. It is not full pests' reduction. We need to mitigate pest population activity to avoid economics for production.

32.3. Results and Discussion

32.3.1. Grain Crops Pest Management Problems

It is possible to note some tendencies of the grain crops phytosanitary condition in the Steppe Zone:

- Turnip moth population growth
- Chinch bug population depression during last 3 years
- European Corn Borer and corn ear worm annually high damage in the maize planting (some hybrids corn cob affection with corn ear worm reached 90.2% in 2003 year)
- Large spreading of the sprouting seeds molding with Fusarium type including corn cob Fusarium rot
- Noticeable growth of ear affection with olive molding caused with raining during period of the ripening and long-term harvesting (2004 year)
- Ecologically-parasite diseases predominance

The winter wheat scouting data for main diseases and insects at the days of their development during 6 years are presented in the Tables 32.1 and 32.2.

Two types of the grain field pathogen complex formation are selected in dependence of the meteorological conditions of the Steppe Zone of Ukraine. First type is characterized with stress situations (drought, high temperatures – 2001–2003, 2005 years) and facultative pathogens predominance. Second type is connected with precipitations abundance (in 2004 year) and obligate parasites occurrence.

Table 32.1. Number of insects in the winter wheat sowing

Insect	Index of scouting	Year of vegetation				
		2000/2001	2001/2002	2002/2003	2003/2004	2004/2005
Turnip moth; (in fall)	Caterpillar/m^2	–	1–2	0.5–1	2–8	5–30
Carabide	Larva/m^2	1.5–4	2–5	2–12	4–20	4–18
Leafhopper (in fall)	Species/m^2	27.6–34.4	10–20	Isolated	10–20	Isolated
Greenbugs (in fall)	Species/plant	0.5	Isolated	Isolated	0–1	1–3
Winter fly	% damaged	12–15	7–12	42–53	3–5	3–4
Greenbugs (booting-heading)	Species/plant	6–8	3–50	Isolated	2–3	0.5–2
Chinch bug	Larva/m^2	8–34	4–23	0.2–0.8	0.1–0.2	0.1–0.5
Corn weevil	Species/m^2	0.5–17	Isolated	0–11	0–3	1–6

The data with pests' occurrence more than ecological level of affection are showed with bold print.

Table 32.2. Diseases development in the winter wheat sowing

Diseases	Year of vegetation				
	2000/2001	2001/2002	2002/2003	2003/2004	2004/2005
Powdery mildew	1.0–10.4	0.1–9.2	0.8	5–15	1–5
Orange leaf rust	19.7–22.8	0–1.0	0.5	60–80	2–5
Septoria Leaf Spot	1.8–4.2	0–5.3	0.3	1–15	2–10
Root Rots	8–20	3–36	1.8	7–15	4–12
Fusarium of Ear	Isolated	Isolated	0	2–10	0–2
Black Germ	16.4–37.6	9.0–32.3	12–27	5–30	5–18
Olive mould	10–20	20–30	–	60–100	15–50

The data with pests occurrence more than ecological level of affection are showed with bold print.

Threatens impact on winter wheat seeds germination and affection with root rots were studied in the field experiments (Tables 32.3 and 32.4).

Vitavax 200FF and Real 200 seem more prospects taking in account threaters impact on winter wheat seeds germination with root rots.

Some decreasing (within 2.0–2.5%) of the root rots intensity of development after seeds treatment.

32.4. Potato and Tomato Pest Management Problems

32.4.1. Abiotic Limitations and Potato Degeneration in Steppe Zone of Ukraine

Pest problems of vegetables are numerous in steppe zone. Regarding to potato and tomato we can mention some diseases, disorders (big bud, late bight, root rots, blossom-end rot, sunscald, internal browning, etc.) and insects (Colorado potato beetle, wire worm, aphids, earth crab, etc.).

Potato is susceptible to air temperature. It should not be higher than 18–20°C so that potato tubers formation could be successful. At the same time it was fixed two

times when total aphids flight reaches the maximum since last week of June and second week of July. It leads to big bud disease development [6]. Thus, two ecological factors (air temperature and aphids) are main reasons to the beginning of potato seeds degeneration processes. That is why farmers should go by the two ways.

First way: to buy the potato tubers brought from northern regions each year, because of degeneration processes in potato are slowed or absent there.

Second way: to start potato planting in early spring or in summer. In this cases tuber formation take place where solar radiation are 11.7–15.8 kcal/cm^2 and 6.8–11.3 kcal/cm^2 correspondingly. For instance, in Moscow region (Russia) tubers formation take place when solar radiation is 7–14.5 kcal/cm^2. Thus, summer potato planting in steppe zone gives possibility to get good potato seeds to the end of September–beginning of October. Spring frosts are an additional negative abiotic factor. It creates the problems to protect the vegetables seedlings against root rots. In fact, that spring frost likelihood is about 60–80% in northern Steppe, 50–60% in the south of Steppe and 80–100% in the east of steppe zone.

Table 32.3. Threaters impact on winter wheat seeds germination with root rots

Seed threater	Seeds germination (%)					
	2001 year	2002 year	2003 year	2004 year	2005 year	In average
Control (without treatment)	70.0	68.2	73.0	76.5	78.7	73.3
Vitavax 200FF, 3 L/t	77.0	75.4	79.3	79.8	83.2	78.8
Dividend, 3% , 2 l/t	76.7	75.6	77.8	78.3	80.2	77.7
Vintsit 050 CS, 2 L/t	74.0	66.0	72.6	74.8	76.3	72.7
Raxil, 6%, 0.4 L/t	76.2	75.8	78.4	79.8	79.0	77.8
Real 200, 0.2 L/t	78.7	76.2	80.1	78.3	80.7	78.8
Sumi 8 flo, 2%, 1.5 L/t	–	–	–	78.3	80.0	–
Dividend star 036 FS, 1 L/t	–	–	–	73.5	74.3	–
Raxil extra, 51.5%, 1.5 L/t	–	–	–	–	81.0	–
LSD_{05}	1.8	6.1	2.4	1.6	3.1	3.2

Table 32.4. Threaters impact on winter wheat affection with root rots

Seed threater	Root rots, intensity of development (%)				
	2001 year	2002 year	2004 year	2005 year	In average
Control (without treatment)	14.7	10.2	5.0	10.5	10.1
Vitavax 200FF, 3 L/t	13.2	7.5	3.7	5.7	7.5
Dividend, 3%, 2 L/t	13.9	7.9	3.9	5.6	7.8
Vintsit 050 CS, 2 L/t	13.8	7.2	3.6	5.4	7.5
Raxil, 6%, 0.4 L/t	13.4	8.5	3.8	6.0	7.9
Real 200, 0.2 L/t	13.4	8.5	3.7	6.3	8.0
Sumi 8 flo, 2%, 1.5 L/t	–	–	3.9	5.8	–
Dividend star 036 FS, 1 L/t	–	–	2.9	5.3	–
Raxil extra, 51.5%, 1.5 L/t	–	–	–	5.3	–
LSD_{05}	1.2	1.1	0.9	1.1	1.1

Late blight occurrence on tomatoes in 1997 showed local pest and pesticide management problems. That year weather conditions were very favorable for Phytophtora infestans development, and Phytophthora infestans developed as an epiphytotic. Therefore, several days were enough that foliage injury on tomato bushes changed from 1–5% to 25–50%.

Comparative assessment of tomato yield shows that losses in 1996 reached 80% (Table 32.5). Recent studies of Phytophthora infestans in Russia and Belorussia [7] showed the late blight occurrence has begun to appear in less favourable temperature and air humidity conditions. It is connected with the crossing of mating types A1 and A2 to the forms which are highly aggressive. Root rots are the second factor that limits tomato yield.

There are several ways to mitigate the negative abiotic and biotic factors influence including straw and plastic mulch application, good nutrition management, and tubes planting in early spring or in summer, the resistant to Colorado potato beetle varieties growing in potato case. First year's two potato varieties with gene BT were evaluated within vegetable varieties stations' network in Ukraine. "Atlantic" variety's yield has reached 23.4 t/ha in the central part of steppe zone (Nikopol Varieties Testing Station). Late blight occurrence was 5%. It is known "Atlantic" variety was distinguished by high susceptibility to the late blight in the north of Ukraine.

Nutrition management prospects are connected with several technologies of agicultural wastes utilization including vermicomposting, bacterial fertilizers, biofungicides [8]. Next, an example of joint vermicompost, biofungicide and bacterial fertilizer application is presented for tomatoes.

Table 32.5. The tomatoes yield (t/ha) in Dniepropetrovsk region in 1995–1998

District	1995 year	1996 year	1997 year	1998 year	1997/1998 years (%)
Apostolovsky	4.3	13.0	2.5	9.8	19.2
Vasilkovsky	5.25	14.7	2.16	7.3	14.7
Verkhnedneprovsky	10.4	11.25	2.62	6.8	23.3
Dnepropetrovsky	12.8	24.8	1.54	17.0	6.2
Krivorozhsky	24.1	19.1	14.1	11.3	73.8
Krinichansky	3.91	8.29	0.45	2.24	5.4
Magdalinovsky	7.12	9.2	0.13	12.24	1.4
Mezhevskoy	6.93	11.7	0.91	3.37	7.8
Nikopolsky	10.5	28.5	7.5	15.06	26.3
Novomoskovsky	11.36	14.6	0.97	5.72	6.6
Pavlogradsky	9.16	13.9	13.3	4.34	95.7
Petrikovsky	10.1	16.79	1.12	7.12	6.7
Petropavlovsky	6.4	11.5	0.55	8.48	4.8
Pokrovsky	10.4	15.8	1.73	11.13	10.9
Pyatikhatsky	3.9	11.8	1.4	5.65	11.9
Sinelnikovsky	10.28	13.14	0.83	3.82	6.3
Solonyansky	8.0	13.8	1.1	4.33	8.0
Sofievsky	2.6	10.2	1.3	8.19	12.7
Tomakovsky	13.68	24.0	2.17	7.49	9.0
Tsarichansky	12.4	13.6	1.87	3.0	13.8
Shirokovsky	2.69	12.5	2.14	9.07	17.1
Yurievsky	3.31	14.1	0.10	1.31	1.0

32.5. Tomato Experiment

32.5.1. Production of Healthy Tomato Transplants

Peat, manure and vermicompost were applied in hothouse conditions. There is a positive effect for the health and survival of tomato transplants, thus giving a greater of reliable yield. The plot size was 10 m^2 in experiments 1–2, with four replications.

The tomato fruit yield was evaluated on 20 bushes of each plot. Experiments 1–2 were conducted in 1998–1999 on the vegetable-varieties experimental station situated also within educational farm "Samarsky" in Dniepropetrovsk district. The *Trichoderma*-based mycofungicide was incorporated into each compost treatment to protect seedlings from root rots. Indigenous black soil was the control. The experiment was continued under field conditions.

Figure 32.1 illustrates that the greatest effect was obtained on joint vermicompost (biohumus) + Trychoderma treatment. This superiority was kept after the seedlings were planting in fields plots.

32.6. Conclusion

Ensuring sustainable crops yield productivity is impossible without ecological farming as following: varieties resistance to disease and insects, biological control preparations, rational use of organic and composts and use of microbiological products with multiple effects.

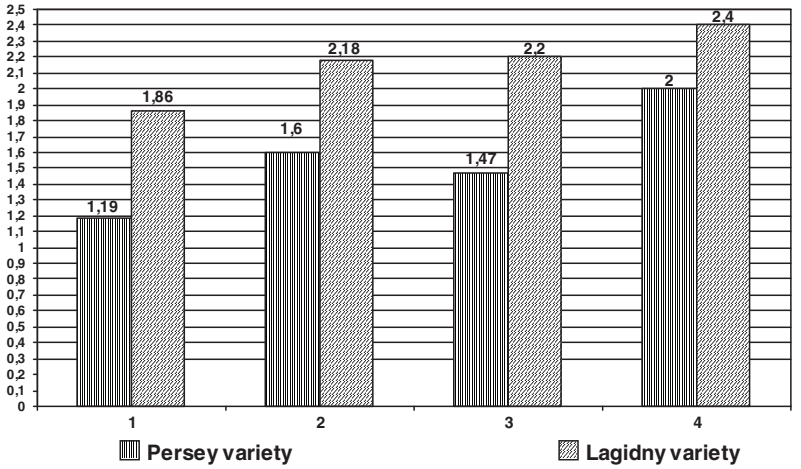

Figure 32.1. Biocompost impact on the tomato seedlings bioproductivity (weight of five dry seedlings, g); experimental treatments:
(1) control = black soil; (2) manure + trychoderma; (3) peat + trychoderma; (4) biohumus + trychoderma

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Chapter 33

Potash Branch in Ukraine as a Possible Object of Terrorism

Grin G. and Semenov E.
National Technical University "Kharkiv Polytechnical Institute", Ukraine

Abstract. There is a characteristic of the Ukrainian potassium industry objects that may become the objects of chemical or biological terrorism. Among the considered counter measures the authors single out: technical re-equipment of the idle objects of potassium industry in Ukraine, processing of the cumulative brines into marketable chemical products, conservation of the potassium mines with the creation of health resorts and clinics.

Keywords. Potassium branch, mines, the object of terrorism, counteraction

33.1. Problem

In Ukraine as well as in any agrarian country, agriculture requires the application of potassium fertilizer into soil [1, 2]. Sugar beet, maize and wheat are especially notable among crops. In the food ration of these crops up to 40% of the general number of nutrients are comprised of potassium. Home consumption of potassium in Ukraine comprises approximately 2 million tons p. a. (in terms of K_2O) [3]. Potassium is very important for humans, as well as for plants, especially for the cardiovascular system.

But at the present point in time the potassium industry is out-of-action. Until recently the general bulk of potassium fertilizer in Ukraine was produced by two giant enterprises – "Polymineral" state mining-chemical enterprise in Stebnyk (Lvov oblast) and "Oriana" public corporation in Kalush (Ivano-Frankovsk oblast).

The beginning of the potassium industry of Ukraine decline refers to 1983, when the ecological catastrophe happened in Stebnyk. The embankment of potassium waste storage was broken. As a result near $5 \cdot 10^6$ m^3 of brines poured out into Dnestr river. Salt mines in Stebnyk are gradually filled up by corrosive brines that dissolve their walls. The collapse of mine walls as the result of their destruction may lead to an earthquake. The soil above the mines sinks and collapses gradually.

Potassium objects in Kalush are also in critical state. The embankment of brines storage is gradually washed away, waste products are kept in the storage, which was washed away by rains. Meanwhile there is a river 50 m from the storage. Because of this there exists a high risk of protection embankment destruction and brines ingress into water bodies.

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Meanwhile idle potassium mines and enterprises may become the objects of terrorist acts of different international terrorist organizations because of their critical, destroyed state. In case of directional operation idle salt mines, protection embankments, open casts, that are in a miserable taking, may be used and destroyed. As a result of these actions there will be an inevitable ingress of brines into ground waters and other water bodies of Moldova and Western Europe. These brines comprise near 400 g/L of KCl, NaCl, $MgCl_2$, K_2SO_4, Na_2SO_4, $MgSO_4$. Such concentration of salts is destructive for the whole flora and fauna, besides there may be some problems with potable water. Meanwhile one should not rule out the application of chemically dangerous substances and infestants of different infectious diseases.

33.2. Problem-Solving Methods

We considered the methods of solving the problems of potassium production in Ukraine that were suggested by different authors as the ways of counteraction to chemical and biological terrorism. The most important ways of solving the potassium crisis in Ukraine were singled out in the process of considering the proposed methods. They are: possible revival of potassium industry by means of reconstruction, restructing and involving different investors [3], complex processing of the cumulative brines with the obtainment of highly marketable products [4–6], conservation of brines with the adoption of nature-conservative measures [3, 6].

Calculation data are cited in the work [3], according to them the program of mine closure will take place in the period from 1997 till 2015 year. It will require about 1489.0 million hryvnas. According to the profit of the potassium plants the calculated program of the potassium industry revival will begin to pay for itself in 12–17 years. There is an introduced concept according to which, the construction of new not very high-powered potassium plants instead of giant enterprises, is the most reasonable. But it is necessary to take into account the peculiarities of brine composition of every mine. Besides several “Potash of Ukraine” government programs were launched on the instructions of the Ministry of industrial policy in Ukraine. It should be mentioned that the cited solutions require high capital expenditure and long terms, while the flooding of salt mines and dissolution of roadway walls continues and the situation becomes worse day by day [3].

Several methods of the cumulative brines complex processing are suggested. They make it possible to obtain not chloride potassium fertilizer and potassium–magnesium fertilizer, chloride–magnesium concentrate, common salt and rare-earth elements concentrates. In this respect Ukraine has a beneficial geographical location and can achieve a definite status in the market segment. The authors [7–8] suggest processing K_2CO_3 from KCl using brines as the source of potassium ions and extracting KCl from them.

However the application of brines processing variants mentioned above, requires the construction of new manufactures in the region of salinas or transportation of brines to the existing chemical enterprises that also involves expenditure. It should be mentioned that the complex brines processing is necessary in order to raise profitability.

As nature-conservative measures the most interesting is the organization which is situated near the tourist therapy complex in Stebnyk not far from the resort in Truskavets. Such cases are well-known in the world. One of the salt mines in Poland has been functioning as a profitable tourist complex for many decades. Turning the existing brine potassium open cast into lakes and organizing a health-resort zone is also suggested. It is necessary to mention that the existing problem of potassium manufactures in Ukraine is tightly interconnected with the problem of their environmental safety. The solving of the existing problems is possible only at the governmental level with the involving of different investors and investments, including foreign ones. The existing problem is not the problem of only our country. It is also the problem of neighboring Western Europe states.

33.3. Conclusions

Thereby, we considered the methods of solving the problems of potassium sector in Ukraine that were proposed by different authors. Each of the mentioned methods has its advantages and disadvantages. Only doing the total economic calculation of all the possible ways and carrying out the entire comparison characteristic will make it possible to say which of them is the most rational.

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Chapter 34

Water Antiseptic Preparations for Decontamination of Pathogenic Microorganisms

Pivovarov A., Kravchenko A., and Tischenko A.
Ukrainian State University of Chemical Engineering, Dnepropetrovsk, Ukraine

Abstract. The paper includes data of experimental investigations of drinking water decontamination in the plasm-chemical reactor; optimal conditions of the process are determined; distinctive features of activation processes occurring as a result of contact action of low-temperature non-equilibrium plasma are shown. The most efficient fields of application of the above technology in conditions of emergency situations and terrorist threats are discussed.

Keywords. Water media, threat, bacterial cell, plasma, investigation, inactivation

34.1. Introduction

Plasma-chemical activation of water and water solutions is based on obtaining liquid media, which possess new, not known before, or insufficiently explored physical and chemical properties [1, 2] in special technical plasma-chemical systems. These activated water solutions can be used in medicine for treating wide range of diseases and traumas; as a bactericide preparation, preservative agent, disinfectant and sterilizer; in veterinary medicine for treating infectious diseases of cattle, animals and birds, with complete or partial exclusion of antibiotics or other artificial preparations having an impact on people's health under consumption of products of animal origin; as antiseptic prophylactic hygiene-and-sanitary drugs or disinfecting preparations featuring cost-effectiveness and simplicity as to their chemical composition under a threat or commitment of the acts of terrorism of biological origin.

The processes of the water and water solutions treatment with non-equilibrium low-temperature plasma (GDP) allows to increase the efficiency of water activation, to provide the enhanced stability of the activated water parameters over time, and to increase the degree of water activation by creating more stable cluster structure in the water, being activated.

Water treatment with (GDP) is changing parameters and reaction ability of water due to the combination of electron-ion influence, photo-effect and UV irradiation of water. At the beginning stages of water treatment with contact, non-equilibrium plasma, number of transformation such as formation of ions, excited water molecules and formation of secondary electrons and free radicals take place. These reactions result in the

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chain mechanism of water decomposition, and formation of peroxide and super-peroxide compounds, responsible for the degree of water activation. In addition to the above-described processes, the effect of contact, non-equilibrium plasma causes formation of the hydro-peroxide radicals in water. Especially intensely, said radicals are being formed in the water, containing dissolved oxygen for the purposes of the process intensification.

Chemical reactions and active radicals, formed as a result of the GDP treatment, define reaction ability of the activated water. In such a system where two phases (gaseous and liquid) are present, and electric current is being formed between an electrode, immersed in the water and an electrode, placed into a gaseous environment, the boundary of phases where, due to its complex nature, intensive physical and chemical transformations take place, is of special importance. The thickness of a water layer and the distance between electrodes, allow to provide for uniform treatment of adjoining layers, and thus to increase efficiency of water activation.

Considering the above mentioned processes, it is obvious that treatment of water with GDP results in fundamental changes in the structure of activated water: each of ions of H_3O^+ is surrounded by five negatively charged molecules of water, and forms meta-stable, non-charged cluster compound $H_3O^+_{aq}(H_2O^{0,2e})_5$. The accrued data on the length of existence of meta-stable cluster compounds give evidence that, for example oligomer $5H_2O{\cdot}e_{(aq)}$, being negatively charged before it's breakup and may participate in the formation of a large number of meta-stable compounds.

Thus, breaking up, fragments of meta-stable cluster formations replicate themselves, and in doing so, facilitate process of electron exchange. Precisely because of this continuous process of formation and breaking up, cluster structure of water possesses stability, and new, previously non-existing physical and chemical characteristics.

Works [3, 4] include data concerning efficiency of GDP usage for disinfecting water media seminated with pathogenic and conditionally pathogenic microorganisms. Experimental investigations were conducted as to processes of disinfecting drinking and run-off water for bacteria below: salmonella, clostridia, and bacteria of colibacillus group, lactose-positive colibacillus, enterococci, and viruses of poliomyelitis and hepatitis B. It was shown that as a result of plasma treatment of liquid media under investigation, featuring various degree of microorganism semination, death of almost all indicator groups of bacteria in drinking and run-off water occurs. The only exceptions are spore bacteria - clostridia. Their amount in the process of treatment with GDP decreased insignificantly.

Bacterial cell has 0.5–5.0 mcm size, and its shape may vary between cylindrical, spherical or curled ones. Average mass of bacterial cell is $4 \cdot 10^{-12}$ g [5]. The cell is covered with a shell being durable and stable to the external factors, 10–40 nm thick. This shell is composed of proteins, muco-polysaccharides, muco-proteins, lipoids, sugar. Important feature of many bacteria is their membranes swelling, with formation of mucous layer (capsule). The above stated layer reaches a thickness comparable with cell size (0.2 mcm), and looks like high-viscosity gel, including glucose, sucrose, fructose, gluconic acids, gelactane, and other substances. Capsules represent the perfect shield from destructive action of antibodies in a great number of pathogenic bacteria. Sometimes, mucus encloses several hundred cells, forming so-called zoogelia. Besides,

on the surface of various cells are some appendices, namely, flagella, fimbria, etc. They consist of protein and create a kind of "reinforcement" of mucous layers, generating specific hydrophilic system. Under the cell shell, there is a cytoplasmic membrane, 5–10 nm thick, featuring presence of lipoprotein and ribonucleic complexes. Membrane acts as an osmotic barrier, concentrating nutrients inside the cell and promoting secretion of metabolic product outside. Cytoplasmic membrane includes a cytoplasm-complex of colloid substances and cell organelles, viscosity of which 10^3–10^4 exceeds water viscosity. Cytoplasm of each cell contains up to 10^4 ribosomes sizing 20–40 nm, consisting of protein, phospholipids and RNA. There is no strongly pronounced nucleus in most cases. On superfine sections, nuclear vacuole is observed only, with DNA filament bunch inside. Some kinds of bacteria are capable to form spores at a certain stage of their development, with the functions similar to cysts of protozoa. Spore formation is usually connected with unfavorable conditions of the environment, such as, reduced moisture content, absence of nutritional support, etc. Only one spore is always formed in the cell. Process of spore formation begins with compaction of cytoplasm in the nuclear zone and its isolation together with DNA-partition generated from cytoplasmic membrane. Further, on a cut section of cytoplasm two-layer membrane is formed, together with spore capsule, which fulfills protective functions, due to it's low permeability to water and dissolved substances, ensuring high stability of spores to external actions. Spores of many bacteria retain their vital ability even at high temperatures (for example, spore of Bacillus subtilis survives after 3 h of boiling).

Mechanism of microorganism inactivation is not completely clear so far. Disinfection efficiency depends on the ability of antimicrobial agent to interact with microorganism and on the degree of damage caused by the latter. Character of interaction and degree of damage are influenced by many factors, including structure and concentration of antimicrobial agent, duration of the contact, availability, formula, amount and type of microorganisms. Antimicrobial agent should be available in concentration, sufficient for destructing the required amount of microorganisms. Some researchers suppose that cytoplasmic membrane blocking by inactivator is enough for bacteria inactivation. Possibility of inactivator interaction with the membrane component is not excluded too. In the opinion of the other researchers, disinfecting reagent should penetrate into a cell. Dysbolism is considered as the secondary phenomenon. It was supposed that DNA is the main object of importance as regards the effect of all antimicrobial agents on microorganisms, since these agents cause irreversible changes in its molecules. Consequently, inactivator should reach nucleotide. In this connection, it is evident that the rate of bacteria extinction is determined by the rate of disinfecting agent penetration into the cell and speed of the cell dying as a result of metabolism failure. Bactericide effect is provided for the most part by the formation of oxidation-active substances as free radicals, or their products [4]. As early as in 1955, A. Kelner et al. [6] have found that free hydroxyl and hydro-peroxyl radicals formed outside bacterial cells were influencing microscopic living objects.

In the experiments performed, the presence of active oxygen in the activated water was verified by the following: cathode, made of platinum and submerged into water, was subjected to GDP, and in a certain time period, catalytic reaction of hydrogen with release of oxygen-containing compounds was observed, also accompanied by flashes in

a water medium. Agility of the charged particles as well as a number of carries of the activated water has been experimentally established.

The water, processed with GDP, possesses a number of special parameters: accumulation of hydrogen peroxide and super-peroxide compounds, and retention of compounds with increased oxidising abilities as well as stable cluster structure.

34.2. Experiment

Below there are the agents chosen as the objects of investigation:

Water media: distilled water (H_2O, molecular weight – 18.016 g/mol; density at 20ºC $\rho = 995$ kg/m^3; dynamic viscosity $\mu = 1.028$ mPa · s; pH = 6.15, the simplest stable compound of hydrogen and oxygen; liquid without taste, odor and color; solutions of inorganic compounds in distilled water with the aim of determination of their influence on subsequent antibacterial activity; artificially prepared water solutions of hydrogen peroxide; **drinking water**.

Lyzoformin 3000 – made in Germany (Lyzoform Company). Disinfection, cleaning and sterilization of instruments (0.25%, 0.5%, 1.0%, 1.5%, 2.0%, 8.0% solutions)

Sterilium – made in Germany (BODE Chemie Hamburg Company). Antiseptic for hands and skin. Type – solution. Composition (per 100 g): α-propanol 45 g, 1-propanol 30 g, mecetronium ethyl sulfate 0.2 g, myristyl alcohol, glycerin, food dye, aromatizer.

Microorganisms – Staphylococcus aureus, Pseudomonas aeruginosa, Staphylococcus epidermidis, E. coli, Pseudomonas aeruginosa, Candida albicans, Streptococcus pyogenes, Proteus vulgaris, Klebsiella ozaenae. Cultures were cultivated using agarized beef-extract broth in test tubes at the temperature of 37ºC.

Experiments were conducted with the use of compact laboratory unit including systems of power supply, vacuumization and thermostating of the reactor of discrete action. This is achieved by positioning two or more pairs of the unlike electrodes into a water layer of between 30 and 100 mm on the opposite sides of the "water-air" border at a distance of between 4 to 15 mm from such level accordingly, said electrodes being made of a material which does not have catalytic effect on hydrogen peroxide and super-peroxide compositions, exposing the water to GDP, with voltage at each pair of electrodes being 500–1,500 V, a current being 25–150 mA, the temperature being below the natural boiling point of water and pressure being between $1.5 \cdot 10^4$–$5 \cdot 10^4$ Pa.

Daily cultures of microorganisms were grown beforehand in the nutrient medium (agarized beef-extract broth). Culture meal in the physiological solution was prepared directly before the experiment. Diluted culture was seeded as a "lawn" on the nutrient medium in Petri dishes. Microorganism sensitivity to the media treated with cold plasma was determined by means of disk superposition [7]. The essence of the method is as follows: the culture sensitivity to corresponding media, including antibiotics, is determined by the diameters of growth inhibition zones. Microorganisms are divided into classes according to their sensitivity. They may be sensitive, medium-sensitive, moderately stable and stable. A culture with growth inhibition zone up to 10 mm is considered as low-sensitive, whereas high-sensitive culture features growth inhibition

zone over 10 mm. Cold plasma treated media were introduced into metal cylinders (maximum number of cylinders in one Petri dish was 4). Prepared samples were exposed to thermostating at the temperature of 37ºC during 24 h, and zones of microorganisms' growth inhibition were measured thereafter.

Firstly, investigation was performed of antagonistic action of chemical hydrogen peroxide on test cultures in concentrations approaching to those in activated water (Table 34.1).

Table 34.1. Studying of influence of hydrogen peroxide solutions in various concentrations on microorganism growth

Microorganisms	Hydrogen peroxide concentration in the solution (%)			
	0.001	0.01	0.05	0.1
	Value of microorganism growth inhibition zones (mm)			
Staphylococcus aureus	0	0	13	19
Staphylococcus saprophiticus	0	0	0	11
Staphylococcus epidermidis	0	0	12	18
E. coli	0	0	10	12
Pseudomonas aeruginosa	0	0	0	11
Candida albicans	0	0	11	16
Streptococcus pyogenes	0	0	0	0
Proteus vulgaris	0	0	8	13
Klebsiella ozaenae	0	0	10	14.5

Data of Table 34.1 shows complete absence of antagonistic action of water solution of synthetic hydrogen peroxide of 10–100 mg/L concentration, poor antagonistic action of water solution of 500 mg/L concentration and more stable one for concentration of 1,000 mg/L.

Data obtained before proves a degree of dependence of antimicrobial efficiency of hydrogen peroxide, under usage in reduced concentrations, on time necessary for disinfection (index D). Index D (Table 34.2) decreases with the increasing concentration of hydrogen peroxide. Higher values of index D mean reduced antimicrobial activity. The data given are well correlated with that of Table 34.2.

Complex of active compounds including hydrogen peroxide, which are formed under treating water and water solutions with GDP, possesses high oxidation-reduction ability and causes destruction of surface structures and internal membranes in microorganisms. Disturbance of integrity of cytoplasmic membrane leads to reduction of activity of a number of ferments related to membrane and systems of DNA reparation.

Table 34.2. Values of index D, min

Microorganisms	Hydrogen peroxide concentration (%)		
	3.0	1.0	0.1
E. coli	1	3	11
Pseudomonas aeruginosa	<1	1	13
Bacillus subtili	5	11	35
Candida albicans	17	54	216
Aspergillus fumigatus	10	38	360

OH radical is a strong oxidizer (in alkali medium E^o ($OH_{(aq)} \rightarrow OH + e^-$) = 1.9. In acid medium E^o = 2.73, in neutral one E^o = 2.32. Therefore, under transition from neutral medium to alkali one its efficiency, as an oxidizer, is reduced [8]. H-atoms, hydrated electrons $e_{(aq)}$ and hydrogen peroxide H_2O_2 and its superoxide compounds, depending on pH of the medium, can be oxidizers, and oxidation abilities in acid solutions are displayed by $e_{(aq)}$ and H_2O_2, and H atom acts as such in alkali solutions. In the presence of dissolved oxygen, H and e_{aq} react therewith, forming free radicals HO_2 and O_2^- [9]. From values E^o ($H_2O_2 \rightarrow HO_2 + H^+ + e_{(aq)}$) = 1.7; E^o ($H_2O + OH \rightarrow HO_2 + 2H^+ + 2e_{(aq)}$) = 1.35 и E^o ($HO_2 \rightarrow O_2 + H^+ + 2e_{(aq)}$) = −0.3 V it follows that HO_2 radical is a weaker oxidizer than OH radical, and, as a reducing agent, it is considerably less effective than H atom. For ion-radical O_2^- , reduction reactions are more typical, E^o ($HO_2 + OH^- \rightarrow O_2^- + e_{(aq)}$) = 0.4 and E^o ($O_2^- \rightarrow O_2 + e_{(aq)}$) = −0.33 V. Under recombination, HO_2 and O_2^- are transformed into hydrogen peroxide (H_2O_2).

The data above allows to predict the processes of microorganism inactivation in the activated water medium.

Table 34.3 states data of antagonistic activity of water activated with GDP, and Table 34.4 includes similar properties of artificially prepared water solutions of Lyzoformin and Sterilium. Data of Tables 34.3 and 34.4 proves that activated water, the same way as antiseptics, has antagonistic effect on the microorganisms under study. Besides, we should note similar suppressing ability of solution treated with GDP towards Cornebacterium diphteriae gravis, being of particular importance for epidemiological practice. Here the value of growth inhibition zones lies in the range of 15–20 mm, indicating the sensitivity of the given microorganisms to the solutions under study (Table 34.5).

Table 34.3. Antagonistic activity of activated water (distillate), treated with contact non-equilibrium low-temperature plasma (parameters of water activation with GDP: τ = 20 min; C_{H2O2} = 500 mg/L; pH = 6.4; I = 130 мА; U = 650 V)

Microorganisms	Value of microorganism growth inhibition zones (mm)
Staphylococcus aureus	20
Staphylococcus saprophibicus	10
Staphylococcus epidermidis	13.5
E.coli	12.5
Pseudomonas aeruginoza	12
Candida albicans	11
Streptococcus pyogenes	8
Proteus vulgaris	14
Klebsiella ozaenae	9

Similar pattern is observed for the other investigated microorganisms as well.

Table 34.4. Survival rate of microorganisms in water solutions of Lyzoformin and Sterilium

Microorganisms	Value of microorganism growth inhibition zones (mm)			
	Lyzo-formin 1.0%	Lyzo-formin 1.5%	Lyzo-formin 2.0%	Sterilium
Staphylococcus aureus	20	23	24	13
Staphylococcus saprophibicus	13	14	16	8
Staphylococcus epidermidis	20	24	25	11
E. coli	15	14	17	8
Pseudomonas aeruginosa	10	14	14	8
Candida albicans	20	19	20	13
Streptococcus pyogenes	15	16	16	20
Proteus vulgaris	20	25	26	34

Table 34.5. Dynamics of survival rate of microorganisms in water solution activated with GDP after its boiling and subsequent cooling culture – Pseudomonas aeroginoza

Activated water	Control time		
	0	60 min	24 h
	Number of microorganism colonies		
– Time of GDP activation 20 min	670	0	–
– Time of GDP activation 60 min	1,709	4	0

Antagonist activity of water solutions treated with cold plasma was tested during 1, 5, 10, 15, 20, 40, 50, 60 and 180 days after its preparation. No special conditions were created for storage. The solutions were kept in glass flask in the open space. During observation, insignificant decrease in activity took place only. Data of inhibition of Staphylococcus aureus growth can serve as an example (Table 34.6). In certain cases, the above stated properties were retained even after 6 months of those solutions storage. All this is the evidence of long-term integrity of antibacterial properties of water and water solutions treated with non-equilibrium contact plasma, and possibility to use them in the difficult epidemiological conditions.

Table 34.6. Variation of antagonist activity of distilled water, treated with GDP depending on storage period ($pH_{init.} = 6.85$)

Storage period, days	Value of growth inhibition zones for Staphylococcus aureus (mm)	Peroxide compounds' concentration in water (mg/L)
1	22.0	340.0
15	22.0	336.0
20	20.0	306.0
30	20.0	304.0
90	17.0	301.7
150	15.0	226.0

As it is known [3, 10, 11], coli form bacteria (CFB) represent the basic tested index of epidemic safety of drinking water. With 10^3–10^6 initial concentration of these bacteria in 1 ml, full bactericide effect was obtained at 4-, 5- and 6-min exposures of contact plasma action on water. Actually the same results were received for lactose-positive intestinal bacilli (LIB) and gram-negative bacteria. On total microbial count (TMC) in the framework of experiment the growth of single colonies was registered. Salmonellae, pathogenic coli bacilli present in drinking water in quantities of units, tens and hundreds of microbial cells in 1 mL, died in 2 min. Quantity of clostridia was reduced by one to two orders, but no full bactericide effect was noted, as with any generally accepted methods of water disinfection (chlorination, ozonization, UV-irradiation and so on), except special methods for particular conditions. Figure 34.1 shows dynamics of bacteria death in drinking water in time under action of contact plasma thereon.

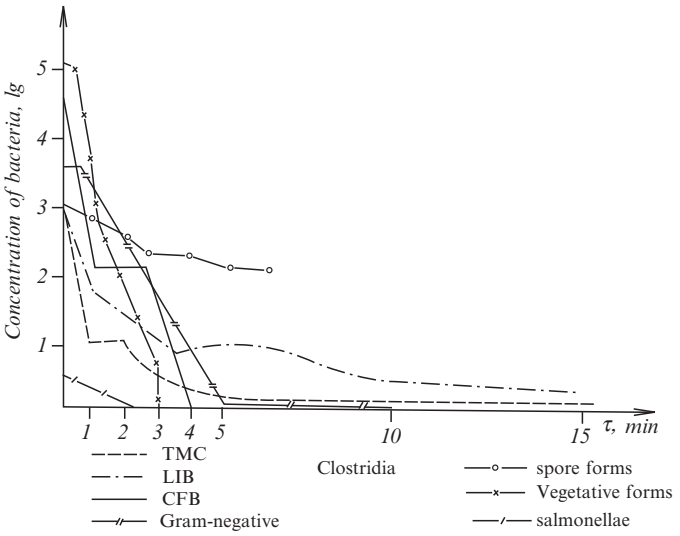

Figure 34.1. Dynamics of microorganism death in drinking water

The represented data show that almost all indicator groups of bacteria in drinking water (TMC, LIB, CFB, gram-negative bacteria) die in 3–5 min of water treatment, except for spore forms – clostridia: vegetative forms are reduced by two orders, whereas spore forms are not actually damaged, and they survive in water.

Pathogenic bacteria (salmonella) died during 2–3 min of plasma discharge action. Consequently, treatment of drinking water with contact plasma for 4 min and more promotes ensuring standard quality of water.

Dynamics of inactivation of various concentrations of coliphages in drinking water at various time of contact plasma action is given in Figure 34.2. As it follows from the given data, action of contact plasma during 1 min leads to reduction of coliphage concentration for more than one order at high levels of water contamination (10^3 PFU/mL).

With further increase of action to 3–5 min, concentration of coliphages is reduced to a lesser extent, and it makes, on the whole, approximately two orders. Therefore, maximum inactivation of coliphages (with initial concentration equal to 10^3 PFU/mL) is observed at 5-min action of contact plasma, and upon 10-min action no coliphages are found in water.

It was determined by experiments that maximum rate of coliphage death (about 1.5 orders) was noted during the first minute of contact plasma action. Further increase of the time of treatment allows reducing the coliphage concentration, on average, by 0.4–0.5 logarithm during each following minute. The degree of coliphage inactivation at high level of drinking water contamination (tens thousand PFU/ml) after plasma treatment during 1 min was equal to 95.71%, which fact was conditioned by presence in suspended material of both high-stable populations, and less stable populations of microorganisms. Further increase in the time of contact plasma action resulted in growth of the inactivation degree. At maximum time of treatment (10 min) quantity of coliphages decreased in mains water to 99.92%. At initial contamination of drinking water at the level of tens and hundreds PFU/mL after 1-min treatment quantity of coliphages was reduced, on average, by 61.67% and 42.46%, accordingly, and only increase in duration of contact plasma action on water to 3 min resulted in more substantial inactivation of coliphages (99.66% and 93.6%). Analysis of obtained data showed that regardless of initial contamination of drinking water, nearly equal decrease in the level of coliphage content in drinking water was observed at 3-min action of contact plasma. Later on, the time required for complete inactivation of coliphages depended on initial concentration of microorganisms in water.

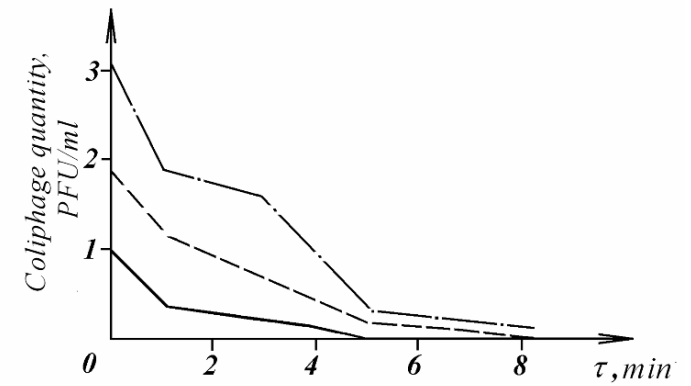

Figure 34.2. Dynamics of coliphage inactivation in drinking water at their various concentrations

Investigations carried out with the use of falling film plasma reactor of continuous operation proved efficiency of the offered method of aqueous media decontamination.

It should be noted that depending on the method of achieving the given thickness of the layer of bacterized liquid, dynamics of bacteria death is different. For example, with changing thickness of solution layer due to variation in the reactor diameter, period of time required for achieving the definite percent of bacteria death is more than that with the same thickness of the layer obtained by using distributing devices of specified capacity.

As a result, at maximum thickness of liquid film, percent of surviving bacteria is also somewhat higher. Observed phenomena are quite explainable, taking into account that density of flow of UV-radiation and active particles, as well as specific current densities on the surface of inter-phase boundary, are varying in the inverse proportion, with changing radius of the reactor (Table 34.7).

Table 34.7. Degree of inactivation of E. coli depending on thickness of treated layer of bacterized liquid (duration of treatment – 0.17 c)

Thickness of liquid film (mm)	Concentration of single individuals/L		Degree of inactivation (%)
	Initial	Eventual	
0.2	$3.1 \cdot 10^5$	0	100
0.2	$3.1 \cdot 10^6$	0	100
0.5	$2.3 \cdot 10^5$	0	100
0.5	$3.8 \cdot 10^6$	Single individuals	>99.9
0.7	$2.3 \cdot 10^5$	Single individuals	>99.9
0.7	$3.1 \cdot 10^6$	$10–10^2$	>99.9

Table 34.8 shows results of experiments on inactivation of various microorganisms in aqueous solutions subjected to plasma treatment, depending on the number of recycles of treated solution. Conditions of experiments are similar to those given in Table 34.7.

At higher concentrations of microorganisms (about tens and hundreds thousand in 1 L of the solution) duration of exposure, as it is evident from data of Table 34.8, should be increased. Coliphages turned to be more stable to action of low-temperature plasma electrolysis among any other investigated microorganisms. In this case, bactericide effect is observed only with three-/four-time passage of treated liquid through reaction zone.

Table 34.8. Survival rate of microorganisms depending on the number of recycles of solution subjected to plasma chemical treatment

Recycle quantity	Poliomyelitis virus, concentration of individuals/L		Coliphages, concentration of individuals/L		Paratyphoid bacteria, concentration of individuals/L	
	Initial	Eventual	Initial	Eventual	Initial	Eventual
1	$4.1 \cdot 10^2$	Single individuals	$8.0 \cdot 10^2$	$1.2 \cdot 10^2$	$2.0 \cdot 10^2$	0
	–	–	$2.0 \cdot 10^6$	$3.0 \cdot 10^3$	$3.0 \cdot 10^3$	Single individuals
2	$4.1 \cdot 10^2$	0	$8.0 \cdot 10^2$	Single individuals	$4.0 \cdot 10^4$	0
	–	–	$2.0 \cdot 10^6$	$4.0 \cdot 10^1$	$4.0 \cdot 10^5$	Single individuals
3	$3.7 \cdot 10^5$	Single individuals	$8.0 \cdot 10^2$	0	$1.5 \cdot 10^6$	0
	–	–	$2.0 \cdot 10^6$	Single individuals	–	–
4	$3.7 \cdot 10^5$	0	$2.0 \cdot 10^6$	0	–	–

Promising outlook of using non-equilibrium contact plasma for disinfection of waters, containing pathogenic microorganisms, viruses and coliphages, is shown by recent works in this area [12–14].

34.3. Conclusion

Activated water and its solutions can be used both for external use (treatment of septic wounds, in burn centers, in stomatology, for washing of putrescent areas, etc.), and for controlling infection diseases in combination with known medicinal preparations, in particular, when the matter concerns extreme environment including biological and chemical acts of terrorism. Besides, activated water can serve as a source of creation of new efficient preparations for treating diseases poorly explored, for preventing and suppressing episodes of focal diseases of unknown origin, and means of disinfecting medical instruments in the absence of known antiseptics. Possessing high antagonistic properties, water solution formed in water solution under plasma action, can be an efficient general-purpose disinfectant used in health care, medical-and-biological industry and other fields of sanitation.

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Chapter 35

Theoretical Investigation as Instrument of Prediction of Effect of Chemical Terrorism

Gryn G. and Kozub P.
National Technological Polytechnic University "KhPI", Ukraine

Abstract. The technique of forecasting of environment pollution based on the thermodynamic analysis of reaction routs in case of damage industrial units and storing places of various industrial, domestic materials and especially toxic chemical products it is proposed and developed. For this the special algorithm of determination of minimum number reactions was theoretically grounded and the rules for description and classification of reagents were applied. Finally, analysis of thermodynamic and physico-chemical properties of basic inorganic substances was carried out and it was shown that clear relationship with their and constitutive properties (structure, dimension, mass) is taken place. The examples of use of proposed technique for forecast of results of environmental pollution by vanadium substances, investigation of processes of hydrocyanic acid production and oxidation of graphite in technology of synthetic industrial diamonds is given.

Keywords. Pollution effect, prediction, thermodynamic analysis

35.1. Introduction

In spite of some lessening of international tensions after times of cold war an expectation of quiet life now changes on fear of terroristic acts which go from Muslim countries and countries with despotic regimes. And due to increase of professional and educational level of executors its actions come more and more sophisticated and large-scale.

Under this conditions the enterprises which produce, process, transport or store of chemical substances comes especially attractive targets for terroristic activity.

It should be noted that for large-scale enterprises it gives more consideration to the questions of safety to assume in advance that they are the potential target of war and terroristic activity. Similar approach is applied and for design of places of storage of chemical substances (especially toxic).

For small-scale unit and new or young technologies such approach applies not always because experimental research of possible consequences of its damage require a lot of works. But meanwhile just such medium and small size units and technologies can be great threat for environment and people.

As last examples it can be given periodically emergent spills of ammonia on chemical plants, pollution of ambient territory by chlorine gas near water treatment plant and

C. Dishovsky and A. Pivovarovi (eds.), *Counteraction to Chemical and Biological Terrorism in East European Countries*,

basins, recent spill of white phosphorus under its carriage by rail, poisoning of Danube by cyanides from gold-mining plant of Rumania, intoxication of alluvial and ground water near sludge-catchment basins of metallurgical works and power plants.

In most cases the main cause of such anthropogenic disasters are independent or individual circumstances without goal-seeking behavior but very often they are caused by human factor and therefore they can be provoked any time and any person.

It should be noted that for all abovementioned cases the consequences of impact on environment and people are more or less known and previous experience of its liquidation already is developed. For more complicated cases as for example fire on storage site of chemicals, pesticides or toxic substances, destruction of containers of toxic liquid wastes, fire on dump or storage site of military equipment and ammunition consequences of incidents come unpredictable and so measures for its liquidation cannot be found in short time.

As a result forecasting of consequences of damage of such units performs using of previous experience and measures for their liquidation develop commonly also only after at least one incident such type.

Theoretical calculations of probable chemical interaction between reagents under specified conditions with a glance of probability of its appearance both during just incident and during its liquidation should be obvious solution of this problem. But for now such technique and even basic principles for it is not developed.

35.2. Basic Principles of Technique of Theoretical Prediction of Consequences of Chemical Terrorism and Other Anthropogenic Disasters

The main cause of absence of technique of theoretical prediction of consequences of anthropogenic disasters (irrespective of causes of its appearance) is the high complexity of assigned task, which includes great of smaller and relative independent task and problems. And all of them require of use not only experimental and theoretical investigations in field of pure chemistry, but also application of sufficiently complex mathematical methods.

Such theoretical calculations must include algorithms of calculation of probable interactions between different substances in different aggregate states taking into account changes of conditions of reaction environment during chemical process, methods of choice of set of reagents – participants of chemical process both like molecular particle and like radicals, methods of determination of collection of necessary and sufficient chemical equations between such reagents and above all the presence the data base of thermodynamic properties for all reagents.

35.2.1. Basic System of Chemical Reactions

Appearance of emergency situation that is dangerous for environment and people in any way is caused both by side chemical processes in reaction mixture and by its interaction with components of environment. So as result the amount of probable reagents increases radically and it gives to drastic increase of amount of probable interaction reactions between this reagents.

Application of traditional for chemical technology technique of calculation of such amount of interaction leads to practically unsolved problems in field of computational mathematics – the problem of choice of system of linear-independent equation, minimum necessary amount of reagent for such equation, minimum necessary amount of such equation etc.

Application of calculation technique based on mathematic methods only does not allow to solve the assigned problem because growth of amount of reagent leads to exponential growth of amount of interaction equation. So as more efficient it was chosen calculation algorithm based on the basic laws of chemical thermodynamics and kinetics.

So as one of the basic rules it was proposed limitation of the order reaction that is basic principle of chemical kinetics. According to this rule only interaction with no more than two structural units (molecules, radicals, ions, atoms) can be assumed as participants of equation set for further calculation.

Such approach eliminates all complex interactions from calculation process leaving though possibility to calculate all necessary indices for them as combination of indices of simple reactions and drastically decrease the number of chemical equations for calculation.

As second proposed rule was the rule of stepwise changes of oxidation rate of elements during interaction. In this case it is supposed that oxidation or reduction of any substance runs in several steps and substance with high or low oxidation rate can not be received without receiving of substance with intermediate oxidation rate. It allows to interpret all oxidation and reduction interactions as sum of fixed number of simple reactions.

But anyway, application of this two rules only simplifies the problem of formation of set of chemical equations leaving an ambiguity of choice of reactions for processes without changes of oxidation rate (i.e. exchange or fusion interactions). So for leaving of such ambiguity it was proposed to select for equation set the interaction with less complication structure of substances (increase of mass, number of bonds, type of elements etc.). This rule is similar previous rule and can be formulate as rule of stepwise increase complexity.

Our practice of calculation shown that application all this rules allows to perform unambiguous and objective choice of chemical equation which allows completely describe equilibrium composition of reaction mixture irrespective of number of its components. Therewith in contrast to set of reaction systems which are formed with traditional technique the number of equations in proposed reaction set growth linearly to number of reagents and this fact leads to drastic simplification of calculation algorithm.

Yet one advantage of such approach is possibility of use of set of simple equations for thermodynamic characteristics any complicate process with any components of reaction mixture. And this fact allows to consider this set of reaction as “basic” set which uses for calculation of equilibrium state of reaction mixture. The set of reaction which forms as combination of reactions from basic set can be considered as “extended” set and can be used for analysis of complicated processes which is interesting in point of view of safety of production, environment or people.

35.2.2 Basic Set of Reagents

Proposed approach allows to solve the problem of choice of equation set but meanwhile poses the new problem – the problem of objective choice of components of reaction mixture.

Importance of this problem can be shown on well-known example of using of complex substances in hydrometallurgy. So, thermodynamic calculation without complex substances in solution shows practically full insolubility precious metals in acid and alkaline solutions but addition of complexing agents (cyanides, thiocyanates, thiosulfates etc.) leads to shift of equilibrium in the direction of more solubility of metals as complex salts.

Thus, in some cases limitations on number of reagent in reaction mixture can lead to completely wrong conclusions both about equilibrium state composition and about probability of carrying out of specified chemical processes.

On the other hand, growth of number of reagents leads to increase of number of probable interaction between their. And in some cases (i.e. for the processes with probability of formation of substances of polymer structure) the number of probable reagents and interactions between their can increase infinitely.

To solve this problem it was proposed several rules which give possibility of objective determination of components which are necessary and sufficient for thermodynamic calculations of reaction system.

First of all, as the necessary condition it was been assumed that in reaction mixture during reaction any substances can be arise (interact) which do not have limits concerning stoichiometry and geometry of bonds. Application of this rule shows that really equilibrium composition of reaction mixture is depended only from elemental composition but not of the set of reagents in initial reaction mixture. It allows to develop the unified algorithm of choice of reagents which depends only from elemental composition of initial reaction mixture.

Secondly, carrying out of thermodynamic calculations for various chemical processes gives the possibility to impose the rule of prohibition of common existence of substances with strong reduction and oxidation property. And it was established that redox properties of reaction system in general can be characterized by integrated parameter which has sense similar to ORP for solutions and which also is function only elemental composition but not set of reagents.

Application of this rule allows exclude (at least on the stage of estimate calculation) sufficiently large number of reactions with small thermodynamic probability under specified conditions.

For example, under small concentration of oxygen-containing substances in layer of carbon it does not need to examine probability of formation of high oxides of many elements and vice versa for thermodynamic calculations with participation of oxygen one can not take into consideration reactions with participation of metallic alkaline and alkaline-earth elements because thermodynamic probability of its existence in such form is very small.

As result, the number of basic reactions can be significantly reduced without introduction of essential errors in results of calculation of equilibrium state parameters.

35.2.3. Uniform Algorithm of Calculation of Thermodynamic Properties

Developing of objective rules for choice of reagents allows to simplify algorithm of thermodynamic calculation but points to next problem – absence of thermodynamic data for vast majority of probable chemical reagents.

So, for example, up to date the biggest tables of thermodynamic data have only data for about 15,000 inorganic substances while theoretically only two-elemental combination can be more than 30,000. Furthermore, even for this relatively small number of substances data is not complete. Data for some substances do not have standard thermodynamic values (heat formation, entropy, heat capacity) for other substances it is absent temperature dependence of heat capacity.

Treatment of available experimental data has allowed to reveal general relationships in dependencies of thermodynamic values from temperature for all its phase state. As result fairly simple mathematical equation which approximates experimental data even with more accuracy than traditionally used Debye and Einstein equations or never mind polynomial dependencies has been proposed.

Feature of proposed equation is consistency of its form with thermodynamic theory and very good adequacy to experimental data (about 0.2% error for 95% of experimental data) on the wide range temperature interval – from 0 to 5,000 K. Additionally it was established that the coefficients of equation are related with structural properties of substances that gives the possibility to begin solution the most important task – forecasting of thermodynamic properties of substances with lack of experimental data.

Due to regression analysis of experimental data it was established dependencies of coefficients from such properties as type of constitutive elements, structure of bonds between them, its mass and other. It was shown that in first approximation coefficients can be calculated as additive values. Furthermore, it was established periodic character of properties of substances subject to placement of its constituent elements in the periodic table of elements.

Thus, application of these rules allows to carrying out thermodynamic calculations on practically all important for chemical technology range of temperature and pressure without changes in calculation algorithm and using of conditional statements and even without availability of experimental thermodynamic data.

35.2.4. Algorithm of Calculation of Equilibrium State

Development of the abovementioned rules significantly has simplified mathematical aspects of task of prediction of behavior of complicated reaction systems but has not allowed to liquidate calculation problems completely.

Thus, one of calculation problems was the necessity of operation as for small values (concentrations) as also with its logarithms (for calculation of thermodynamic parameters) that leads to optimization task with boundary conditions and sophisticate algorithm of calculation of minimum of Gibbs energy.

As solution it was decided to carry out optimization in the coordinate system based on logarithm concentration but not concentration. It has allowed to significantly simplified calculation algorithm, increase of reliability and accuracy of calculation.

Next calculation problem was calculation of equilibrium concentration for solid-phase reactions because in this case concentrations of reagents do not include in the equation for calculation of changes of Gibbs energy (it supposed that concentrations is equal 1).

For solving of this problem and basing on theory of Prigogine–Defey [1] for non-equilibrium thermodynamics it was proposed to use not affinity of chemical reaction proper but its production on amount of "stoichiometric mixture" which can be interpret as minimum of amount of reagents necessary for specified chemical reaction. Then such production can be considered as specific free energy of specific reaction in the set of reaction of all reaction system.

Thus, such approach allow to develop of algorithm of calculation of equilibrium composition for any number of components in reaction mixture and for any combination of phase of this components.

35.3. Examples of Application of Proposed Technique

Development of technique and adjustment of its separate elements was carried out with regard to concrete technological and environmental processes. It gives the possibility to verify the results of its application with actual data, and modify the algorithm of calculation as the need arises.

As processes for which proposed technique was successfully applied can be mentioned the process of manufacture of hydrocyanic acid, treatment of spent vanadium and nickel–molybdenum catalyst, recovery of nickel and manganese from waste waters of production of synthetic industrial diamond, process of chemical plating of abrasives with nickel, process of gas-phase oxidation of graphite and oxidation of graphite in alkaline melts, process of recycling of spent nickel–cadmium and nickel-metal-hydride cells.

35.3.1. Forecasting of Environmental Pollution by Vanadium-Containing Substances Caused by Heat and Power Stations

Prediction of consequence of transfer of vanadium substances which are formed during fluid combustion on the heat station near Kharkiv (Ukraine) was one of the first tasks for which technique of theoretical forecasting of consequences of anthropogenic impact on the environment based on thermodynamic calculation was applied [2].

As the main sources of toxic vanadium substances it was considered ashes formed from combustion of black oil and throws out directly to environmental air and slag accumulated on the territory near industrial unit and mostly pollute ground waters.

Traditional approach to calculation of degree of environmental pollution suppose calculation of precipitation degree of solid particle subject to typical meteorological situation in nearest surroundings of investigated object but without consideration of interaction of toxic substances with substances of environment. And comparison of experimental data and calculation shows that such approach dos not allow to describe real environmental situation.

So for more accurate calculation the technique based on accounting of chemical interaction of vanadium substances with environment was applied. In this case for calculation one takes into consideration composition of atmospheric aerosols under various meteorological conditions (cumulus and frontal cloudiness, cyclonic and anticyclonic activity, their probability, typical direction and strength of wind etc.), composition of ground and alluvial waters, composition of soil and subsoil cover.

All this data gives the possibility to determine basic areas of pollution of vanadium substances not only nearest surrounding of heat plant but establish consequences of permanent impact of such object on all territory of Ukraine and neighboring countries (see Figure 35.1). Furthermore, the calculations allow to get some conclusions about composition of toxic substances in most dangerous areas.

This approach can be applied and for other processes, for example, as arising from time to time emissions of chlorine gas on the water treatment station and in basins, fires and spillages on the storages of pesticides and herbicides, near sludge storage basins and storage place of spent rocket fuel, ammonia pipeline, storages of liquid and solid chemical reagents and other.

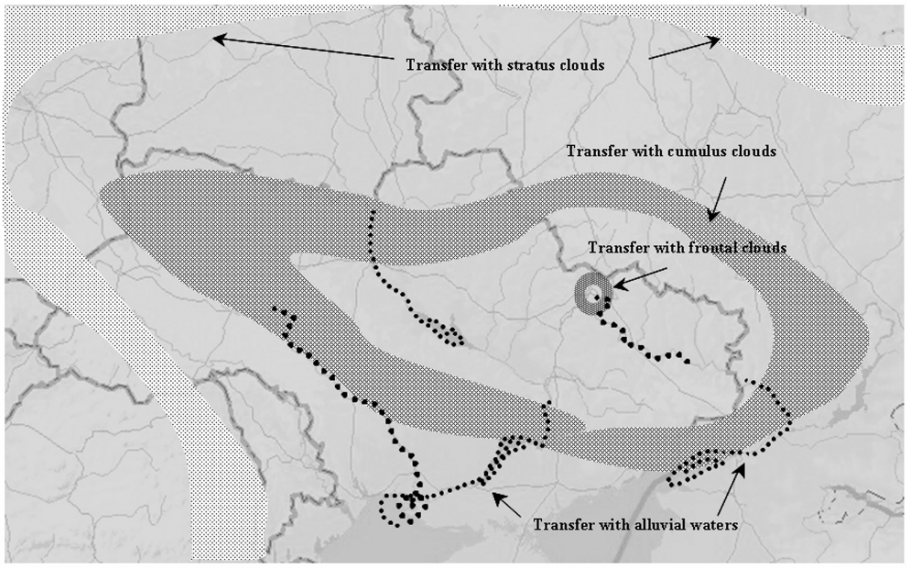

Figure 35.1. The most dangerous area of environmental pollution by Kharkiv heat plant

35.3.2. Hydrocyanic Acid Production

Another task for which proposed technique of forecasting of behavior of complicated chemical systems was successfully applied was technology of industrial production of hydrocyanic acid [3].

In spite of long time of use of this technology its nature was investigated not properly because only initial reaction mixture have five basic component and not less then five additives, the time of formation of hydrocyanic acid is about 10^{-7} s, temperature of

reaction varies from 700 to 1,200°C, and process carried out both catalyst and homogeneously.

Laboratory scale experiments for this process are very expansive and not provide necessary adequacy to industrial condition in any case so for dissolving of this problem it was applied the technique of theoretical investigation of chemical processes.

As result it was established that thermodynamically formation of hydrocyanic acid is most probable trough the formic acid and that the main cause of small conversion degree is the interaction of ammonia with hydrocyanic acid after its formation, it was proposed explanation of influence of hydrogen and other gases on the chemical process (Figure 35.2).

Disclosure and explanation of basic and by-product reaction routes allows elaborating mathematical model of all chemical process and carrying out its deep optimization increasing product yield from 57% to 74%.

35.3.3. Technology of Gas-Phase Enrichment of Diamond–Graphite Concentrate

Very high efficiency of proposed technique was demonstrated for elaboration of new technology of chemical enrichment of synthesis product in production of synthetic industrial diamonds. The time of experimental works was reduced up to 6 months [4].

For this chemical technology it was investigated only 35 basic chemical reactions between catalytic substances (oxides, carbides, metal state) and reaction mixture (carbon, oxygen, carbon oxides) which have formed "basic" reaction system. But for deep exploration this system and revealing of possible routes of chemical process was used extended system with more than 300 complicated reactions.

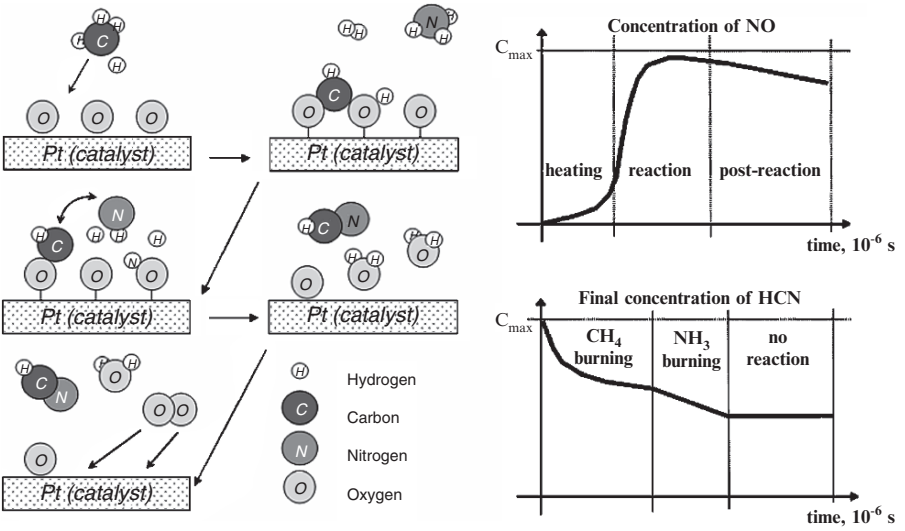

Figure 35.2. Chemistry and kinetics of hydrocyanic acid formation

These theoretical investigations allow to establish possibility of changes of routs of oxidation process subject to composition of reaction mixture and its temperature. It was found causes of catalytic effect of vanadium substances on oxidation process of graphite and inhibitory effect on oxidation process of diamond. It was established difference between catalytic impact of vanadium oxides and molybdenum oxides.

Mathematical model of process gives possibility to calculate optimum parameters for technological process before laboratory and even industrial experimental investigations and therefore it saved great deal time. Main result of research is that time of chemical process was reduced from 7–8 h to 40–60 min.

35.4. Summary and Perspectives

So, our investigations point to probability and perspective of use of thermodynamic analysis for prediction of behavior of chemical systems any complexity including possible effect of accident in industry for any set of initial substances. For now this technique mostly was used for design of chemical technologies and dumping sites of domestic and industrial wastes. But it can be used also for forecasting of behavior of several chemical objects under the influence of aggressive environment on its which can arise as a result of many causes including war or terrorism.

This technique is very useful for scientific research and development of new technologies even in such state, but it will more and more interesting after elaboration more dependencies between thermodynamic, structural and physico-chemical properties of substances. This will give the possibility to forecast not only equilibrium composition of reaction mixture but also its properties including toxic impact on people and environment well in advance of tragedies on chemical objects.

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Chapter 36

Museum-Depository of Pathogenic Microorganisms in the System of Ensuring Bio-Safety

Marievsky V., Doan S., and Polischuk E.
L.V. Gromashevsky State Institute for Epidemiology and Infectious Diseases of Ukraine Academy of Medical Sciences

Abstract. Ukrainian Museum-Depository is a basic scientific and organizational center on studying of dominating agents of opportunistic infections, including intra-hospital infections, and collection of actual strains of microorganisms pathogenic for humans, isolated in the regions of Ukraine during outbreaks and in the course of preclinical research. All-round investigation of strains allows revealing in due time significant changes in biological properties of circulating agents of infectious diseases.

Keywords. Storage, infectious disease, biological identity, bacteria, strains, taxons

Awareness by the world community of potential threats for the mankind which arise from spreading of agents of infectious diseases both in natural and artificially created epidemic processes, in particular, as well as growing threat of bioterrorist acts, gave rise to intensification of fundamental and applied research studies in the domain of improving the methods of indication and identification of biological pathogenic agents, toxins thereof, development of measures of specific/non-specific protection, prevention, medical treatment of patients, systems of monitoring for early detection of epidemic complications.

Permits for production and introduction of bio-preparations in various branches of industry are given by the relevant entities or specialized institutions. However, no institution or entity may guarantee long-term maintenance of viability and biological activity, as well as genetic stability of microorganism strains, which are introduced in production. Biological materials can create potential risks, affect the workers and environment. Therefore, the problem of improvement and development of modern systems for protection of people and environment during fulfillment of works by the agents of infections is the issue of high priority. At the present stage, great importance is given to eco-toxicological studying of the cultures of microorganisms, in particular, genetically modified ones.

Taking into consideration mentioned above, depositing of microorganism strains in specialized governmental agencies is very important from the point of view of ecological and biological safety regarding control over microorganisms' movement, and maintenance of their genetic stability. Besides, the said deposit can be used for solving a number of issues, in particular, in case of emergency, for production monitoring and product control.

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Storing of industrially prospective, reference strains of microorganisms, diagnostically important clinical isolates, particularly, agents of opportunistic infections (so called conventionally pathogenic microorganisms with acquired determinants of pathogenicity factors) and other biological materials gives an opportunity to solve many strategic issues and problems, in particular, ensuring of maintenance and replenishment of the national asset of Ukraine – gene pool of patented, industrial, industrially prospective strains of microorganisms and other biological materials and guaranteeing of observance of biosafety standards during storage of deposits, which promotes proper control over toxicohygienic, eco-toxicological and epidemic situation in Ukraine. Control over spreading of these strains which is provided by conditions of storing and delivering from the museum-depository of strains of microorganisms pathogenic for humans (MPM), is an essential integral part of measures meant for counteracting bio-terrorism.

MPM at the State Institute for Epidemiology and Infectious Diseases named after L. V. Gromashevsky under the Academy of Medical Sciences of Ukraine was established in 1992, and the depositary was formed on its base in 1995. Statutory documents of the Museum and Depositary which define the legal basis of functioning thereof were approved on the directions below:

- Organization of depositing, investigation, storage, and maintenance in active condition, of all strains of microorganisms which have industrial, diagnostic, and controlling significance, as well as any patented strains of microorganisms being the agents of infectious diseases in humans, strains-antagonists, or strains used as control test cultures
- Organization of investigation, identification, certification and production of samples of actual strains suitable for long-term storage, which can have diagnostic value in future or be used for development of new immuno-biological preparations
- Investigation of physical, biochemical, and morphological properties of collection strains with a view to ensuring their biological identity throughout the whole period of their storage in collection
- Execution of the required documents for each collection strain, printing of collection catalogue with designation of storage of separate strains in the museum branches, creation of computer database, for subsequent entering in the international information network
- Securing of accurate recording of reception and movement of strains in accord with valid normative documents of the Ministry of Health of Ukraine
- Provision of research institutions and enterprises of Ukraine with typical, production, actual strains and test cultures

Objects of storage at MPM are strains of fungi, bacteria, viruses pathogenic for humans and conventionally pathogenic, which are included into relevant documents of the World Health Organization and European Economic Community.

As at 31.12.2007, the basic fund collection covers about 4,500 strains, including 225 toxins of bacteria, 35 fungi species (185 strains), 15 types of influenza viruses, enteroviruses, agents of tick-borne encephalitis (48 strains). Acquisitions for the year make nearly 300–400 strains of bacteria, fungi, viruses etc. of microorganisms. In accord with the requirements of anti-epidemic regime and specialization of research institutions,

located in various regions of Ukraine, as based on the principle of complementarity of collection funds, MPM is functioning within the structure of several branches meant for storage of unique or particularly dangerous strains. The branches below are included into the Museum structure.

State Institute of Microbiology and Immunology under the Academy of Medical Science of Ukraine, which stores about 900 strains, 44 taxons of bacteria and 4 fungi species, collection of Bordetella, corynebacteria, Shigella and Salmonella, including production strains for producing serums and diagnostic preparations.

On the basis of the State Institute of Dermatology and Venerology under the Academy of Medical Science of Ukraine, collection of agents of dermatologic and venerologic human diseases was formed, which consists of 84 strains of 42 taxons.

Ukrainian Research Antiplague Institute under the Ministry of Health of Ukraine is specialized in studying and storage of particularly dangerous pathogens. Collection covers over 700 strains of 27 taxonomic groups, including vaccine strains of brucellosis, anthrax etc. In 2007 this collection was supplemented by more than 50 strains of agents of tularemia and Yersinia.

Collection of rickettsia and arboviruses is kept at the branch of Lvov Research Institute of Epidemiology and Hygiene under the Ministry of Health of Ukraine. This Institute is independently included into the list of national scientific assets of Ukraine.

The branch of Governmental Agency "Ukrainian Antiplague Station" under the Ministry of Health of Ukraine has formed collection of choleric vibrions, and this entity is engaged in research activity meant for improvement of diagnosing diseases caused by pathogenic vibrions, and development of atypical cultures for producing diagnostic preparations.

MPM branches also acts as subsidiaries of the National Depository of microorganisms pathogenic for humans. Such principle of MPM work organization, on the one part, allows to maintain gene pool of rare biological objects which guarantee retaining of bio-versatility of Ukraine, and to promote development of biotechnology, microbiology, medical diagnostics in conditions of minimum budgetary funding, and on the other part, ensures proper storage of biological pathogenic agents with restricted access thereto.

The Museum-Depository is developed through inter-collection exchange with museums of microorganisms of research, educational, and controlling institutions of various countries; by means of transferring the author's collections of workers of research institutes, sanitary-epidemiological and medical establishments, agencies of the Ministry of Health of Ukraine, as well as enterprises of pharmaceutical and immunobiological industry, which use strains of microorganisms pathogenic for humans, or strains-antagonists (industrial and control strains) in their activity.

Museum-Depository is a basic scientific and organizational center on studying of dominating agents of opportunistic infections, including intra-hospital infections, and collection of actual strains of microorganisms pathogenic for humans, isolated in the regions of Ukraine during outbreaks and in the course of preclinical research. All-round investigation of strains allows revealing significant changes in biological properties of circulating agents of infectious diseases in due time.

Registration and long-term storage of microorganism strains in the depository of microorganisms pathogenic for humans are effected in full compliance with directive documents of Ukraine.

MPM, together with branches thereof, carries out investigations aimed at verification of condition of collection funds as to compliance of samples of various microorganism species with primary certificate characteristics after long-term storage.

Therefore, creation of museum-depositary of microorganisms pathogenic for humans is of great importance for controlling storage and movement of biological pathogenic agents in Ukraine, and such activity promotes enhancing bio-safety as a whole.

Chapter 37

Usage of Portable Thermo-Spray Device for Treatment of Wounds Contaminated by Microbes

Khudetskiy I., Krivtsun I., Furmanov Y.,
and Gvozdetskiy V.
E.O. Paton Electric Welding Institute National Academy of Science of Ukraine, A.A. Shalimova Institute of Surgery and Transplantology Academy of Medical Science of Ukraine

Abstract. Use of pathogenic biological agents during terrorist operations as the basic or accompanying amazing agent makes the big danger to the population and seriously complicates rendering assistance by the victim. We receive the patent for a way of treatment of wounds which allows to stop simultaneously bleedings in a wound and to disinfect it. Researches with use of the portable device developed by E.O. Paton Electric Welding Institute NAS of Ukraine, A.A. Shalimova Institute of surgery and transplantology AMS of Ukraine. Therefore, this experimental investigation reflects results of pre-clinical tests of thermo-spray method of treatment of infected wounds, as well as equipment created for its realization. Upon carrying out of the necessary clinical tests and creation of production sample of equipment this method can be recommended for use in military medical practice, in the emergency conditions, as well as during rendering of surgical service to citizens in normal conditions during treatment of infected wounds.

Keywords. Wound, treatment, infection, bacterial inoculation, thermo-spray device

At present time, there is a great variety of casualty agents which may be used for terrorist acts [1, 2]. The main task of terrorists, as a rule, consists in causing maximum damage to life and health of people, and impeding rendering help to injured persons. Eventually, the above actions are aimed at formation of stable atmosphere of panic and fear of terrorists in citizens [3].

Consequently, usage of pathogenic biological substances as basic or auxiliary casualty agents during terrorist operations poses a great hazard for peaceful citizens and seriously impedes rendering help to injured persons.

Rather extensive arsenal of preparations and methods for sanitation and disinfection of contaminated wounds is used in modern surgery [4–6]. Unfortunately, even the most advanced antibiotics and antiseptics have a restricted effect towards resistant pathogenic microorganisms. Physical methods of action on bacterial flora of the wound are also not always successful, and the relevant devices are actually not available on a commercial scale.

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This problem is particularly important in the face of the enemy, during terrorist acts, including those with the use of highly pathogenic microbial cultures.

First-aid medical treatment of people suffered as a result of terrorist act, in the presence of wounds contaminated with highly pathogenic microorganisms, appears to be very difficult and dangerous for medical workers. The doctor rendering initial medical assistance should make not simple decision which procedures should be performed first: arrest of bleeding or decontamination of the wound. Besides, it should be taken into account that traditional means usable for undamaged skin cannot be used, as a rule, for decontamination of wounds [7].

The Electric Welding Institute named after Academician E. O. Paton under NAS of Ukraine and the National Institute of Surgery and Transplantology named after A.A. Shalimov of the Academy of Medical Sciences of Ukraine carry out long-term developments and tests of equipment in the domain of hyperthermic surgery – electric welding of living biological tissues, plasma argon surgery and new method of thermo-spray welding and sterilization of tissues [8].

We've received the patent for the method of wounds' treatment, which allows arresting of bleeding in the wound and antiseptic treatment thereof simultaneously [9].

Medical tests were carried out in the department of experimental surgery in the form of sterile surgical operations and manipulations on three species of laboratory animals – white rats, rabbits and pigs of 25–30 kg weight. All operations were conducted under narcosis, and manipulations were accompanied by obligatory use of local anesthetics. Removal of animals from experiment was made by narcotic drugs overdosing.

At the beginning of investigations, impact of the developed method on cultures of various microorganisms – E. coli, Staphylococcus aureus, blue pus bacillus (Ps. aeroginosa) – was tested in the experiments with Petri dishes. Doses and time parameters of action for transferring their results and experiments on animals were chosen.

The least complex tests were carried out on small animals, more complex ones – on medium-sized animals. Final investigations, in the form of operations of various complexities, were pursued on large animals.

Three adequate models of septic wound, depending on duration of the infection stay therein, were created. The first model is the wound infected directly upon its occurrence. The second one represents the wound in which the infection factor stayed for 1–2 days. And, finally, the third model is the wound with formed purulent content, in which the infection process was developing for 7–10 days.

It was found that growth stopped after exposure of E. coli culture to 100–110°C during 3–5 min on 1 cm^2 of surface, and 3-min exposure of Staphylococcus aureus to 80–90°C on the area of 1 cm^2. Growth of Ps. aeroginosa in the culture stopped under the same conditions of treatment that were applied for Staphylococcus aureus.

The next stage was transfer of infection into surgically created wounds, which were made with the use of scalpel on the back of white rats (up to 1.5–2.0 cm long). It was found that parameters of treatment in these conditions somewhat changed towards increase. For example, for destruction of Staphylococcus aureus culture the temperature of 120°C and 5 min of treatment on 1 cm^2 were required.

Sterilization of the wound, infected with E. coli and blue pus bacillus, was achieved at temperature of 110–120°C during 3 min.

The wounds were identified for presence of pathogenic microorganisms therein directly upon contamination and immediately after treatment; control inoculation was made in 1 day after manipulation.

In the experiments on rabbits, infection was made by means of contamination of microbial bodies firstly with E. coli and blue pus bacillus, and after that with all of three microorganisms simultaneously (Figure 37.1).

Figure 37.1. Model of a contaminated wound at the rabbit

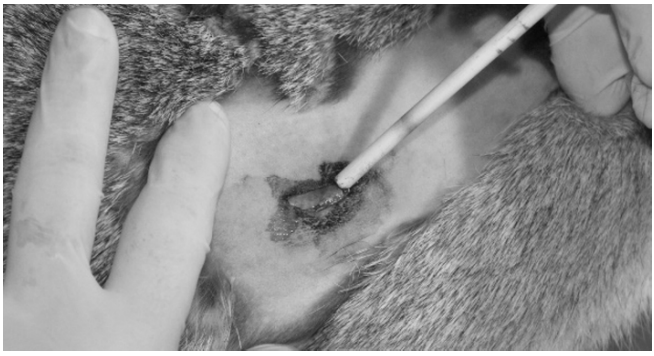

Figure 37.2. Thermo-spray processing chronically a contaminated wound at the rabbit

Surfaces of wounds were treated at the temperature of heat carrier equal to 120°C during 3 or 5 min on 1 cm^2 of the wound surface.

The above series of experiments resulted in complete elimination of microorganisms in wounds at temperature of 120°C during 5-min hyperthermic treatment of 1 cm^2 of its surface (Figure 37.2).

At the same time, these experiments were repeated on pigs which were wounded under narcosis in the hind limb by 4 mm-caliber bullet from “Alfa” revolver, model 420.

Wounds were infected in a similar way, treated as per developed method right on the operating table, and identified before and after treatment.

Results of bacterial inoculation were similar to those obtained for incised wounds.

All wounds were investigated histologically; steps of their healing in the periods of up to half-year were traced. Wounds after sanitation healed without any specific deviations as compared with control wounds, which were not infected (Figure 37.3).

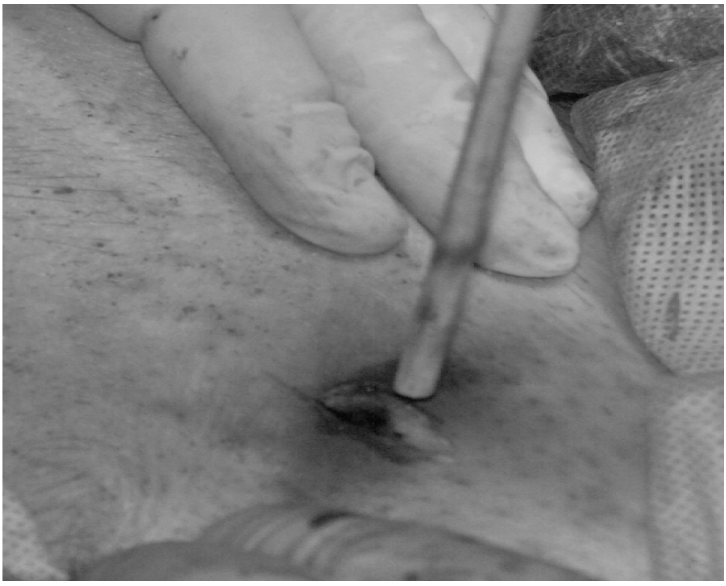

Figure 37.3. Thermo-spray processing of an infected bullet wound at a pig

In the experiments on large animals, infection and disinfection of resection surface of liver was performed (Figure 37.4).

After medial abdominal incision, liver was drawn to the wound, and partial resection of its tail part was made. Arrest of bleeding was performed in accord with developed method and it was completely effective.

Surface of pancreas was infected by contamination with E. coli and blue pus bacillus with Staphylococcus aureus, and after 5–10 min exposure it was treated under hyperthermic method at 120°C during 5 min on 1 cm^2. Wounds of abdominal cavity were stitched after taking of identification smears in accord with the method mentioned above.

No post-infection complications on the part of abdominal cavity were observed in any of the said cases (eight pigs were operated).

Final bacteriological identification was made after removal of animal from the experiment, during autopsy.

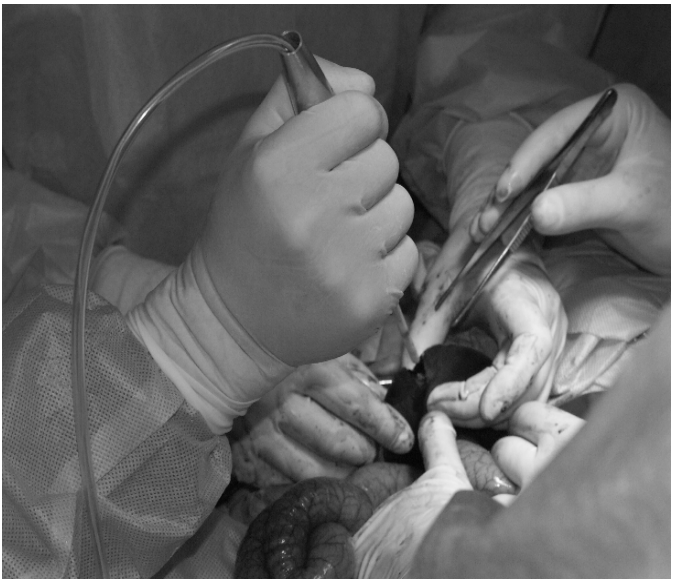

Figure 37.4. Thermo-spray processing of an infected bullet wound of a liver of a pig a simultaneous stopping of a bleeding

Smears from resection surfaces were sterile ones; tissue of pancreas demonstrated post-operation changes only, healing was not accompanied by deformation or creation of suppurative focuses, which could occur in case of retention of pathogenic microorganisms in the wound.

Final model of formed septic wound was achieved by placement of gauze tampon, infected with liquid substrate with pathogenic microorganisms, into sub-dermal area.

Immediately after implantation of such tampon, the wound was closed up tightly and opened in 7–8 days after occurrence of well-marked fluctuation and signs of inflammation of tissues surrounding the tampon.

Purulence was removed by washing of the wound with hydrogen peroxide, purulent necrotic tissues were removed by acute way, and surface of the wound was treated at temperature of 120°C during 3 and 5 min on 1 cm^2 of surface. Repeated identification of wounds has shown complete sterility, and consequently efficiency of treatment.

Starting with the year 2008, conditions of experiments were made more stringent – Candida, enterococcus and Klebsiella were added to three cultures used before. Therefore, practically all set of antibiotic-resistant pathogenic microorganisms, inoculated from patients in the Institute clinic, was represented. Tests were carried out according to methods similar to above ones.

It emerged that combination of Candida and Klebsiella at their introduction into wounds was disinfected during 1.5 min at temperature of 120°C. When enterococci are added to these contaminants, exposure is to be increased to 3 min; it ensures full efficiency of treatment. The aggregate of six microorganisms in one wound is eliminated

by combining the temperature and time of exposure – 120°C during 5 min on 1 cm^2. The same combination of parameters remains effective with the use of the above procedure of septic wounds' treatment with 7–10 days' duration of infection process development.

In parallel with investigation of disinfecting effect, haemostatic possibilities of proposed thermo-spray method of wound treatment were investigated.

Operations of creation of models for subsequent haemostasis with the use of hyperthermic treatment were conducted, apart from the above model of pancreas operation, at wedge resection of rabbit skin, resection of spleen, on wounds of animals' back skin. Complete haemostasis without recurrence of bleeding was achieved during treatment of tissues, without usage of surgical forceps on the bleeder, during 30–50 s (parenchymal bleeding). Actually, in a matter of seconds it stopped during treatment of tissue above the jaws of forceps. Visually, damage of tissues was considerably less pronounced, than at standard dia-thermo-coagulation haemostasis.

Therefore, this experimental investigation reflects results of pre-clinical tests of thermo-spray method of treatment of infected wounds, as well as equipment created for its realization. Upon carrying out of the necessary clinical tests and creation of production sample of equipment this method can be recommended for use in military medical practice, in the emergency conditions, as well as during rendering of surgical service to citizens in normal conditions during treatment of infected wounds.

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Chapter 38

Estimation of the Role of Antropo-Zoonosis Invasion Agents in the Counteraction to Bioterrorism

Boyko A.[1], Brygadyrenko V.[2], Shendryk L.[1], and Loza I.[2]
[1]*Department of Veterinary, Dnipropetrovsk State Agrarian University, Ukraine*
[2]*Department of Zoology & Ecology, Dnipropetrovsk National University, Ukraine*

Abstract. As a result of investigations, the role of Coleoptera order of insects as reservoir hosts of helminthes of Strongylata and Rhabditata suborders was determined. It was proved that average quantity of helminthes revealed in accord with intensity and extensity of insect infection rate, has considerable advantage as compared with point estimation of larvae quantity in the environment. The offered method of larvae counting is very promising one, and it allows to use insects as an object of biological control over helminthes hazardous for humans and animals.

Keywords. Bioterrorism, helminthosis, insects, invasion, Strongylata, Rhabditata

38.1. Introduction

Traditionally it is assumed that action of biological weapons is based on using of disease-producing germs and toxins thereof. In a more comprehensive sense, infected transmitting agents, sources of infectious disease agents (arthropods and rodents) and injurious insects of agricultural plants are also referred to biological weapons [4]. Transmission method of biological weapon usage, which is connected with deliberate spreading of artificially infected blood-sucking insects and ticks, is known. This method is based on the fact that many arthropods easily take in certain causative agents of serious infectious diseases and retain them for a long time.

However, serious diseases can be also caused by helminthes, since their attack results in very complex mutual relations between two living organisms – parasite and host, and these relations tend to long-term or chronic course of disease. Parasites carry out their activity through trophic links (nutrition), which provide for high efficiency of their reproduction in evolutional and ecological aspects. Today the human life environment is drastically changed, as well as natural habitat of parasite spreading. Besides, cattle breeding technologies vary as well, and therefore, new transmitting organisms arise, which promote development and spreading of helminthes. In accord with WHO data, over 16 million fatal cases per year (about one third of all cases registered) are

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caused by infectious and parasitic (helminthic) diseases, which in accord with isolated classes of parasites are called nematodoses, trematodoses, cestodoses [5, 6]. In spite of "adherence" to warm and damp climate, helminthes are spread everywhere. Undoubtedly, helminthoses are found more often in the countries with undeveloped economy and improper financing of medical and social programs. In Europe, helminthes parasitize in every third citizen [7]. According to parasitological monitoring data, actually every resident of Ukraine is infected with helminthes at least once during his/her life. Annual incidence rate of helminthoses in Ukraine is 1,333 cases per 100,000 citizens. Information provided by regional sanitary and epidemiological stations and Ukrainian Center of sanitary and epidemiological supervision points out continuous growth of helminthoses' prevalence in Ukraine.

Helminthoses are characterized by wide range of clinical presentations: from asymptomatic to extremely severe ones, posing a threat to human life. Now about 300 species of helminthes, which can provoke diseases in humans, are known; in Ukraine there are nearly 25–30 parasites considered particularly hazardous. In accord with WHO data, over 4.5 billion people in the world are affected by parasitic diseases, and helminthoses account for 99% of all parasites [3]. There are too many reasons for inefficiency of counteraction to helminthoses in Ukraine.

1. Inadequate estimation of invasion impact on the level of human health and course of many somatic diseases, on the part of health departments
2. High level of contamination of the environment with helminth eggs as a result of discharge of un-treated effluents and wastewater of cattle-breeding farms
3. Uncontrolled migration of citizens
4. Growth in number of stray animals
5. Helminthoses prevention measures are limited to treatment of newly detected cases of disease only
6. Non-specific symptomatology of a number of helminthoses
7. Low awareness of standard methods of helminthological investigation for helminth eggs

Considerable part of people's and animals' parasitoses is of general nature (anthropozoonoses), their agents can develop both in human organism, and in animal one. Among potentially hazardous invasions, the most common forms are toxoplasmosis, opisthorchosis, echinococcosis, trichinosis, ascaridiasis, and strongyloidiasis [1].

However, reporting on helminth incidence rate is not put in order so far. First of all, it relates to helminthes parasitizing in domestic animals. Among them, rhabditatose-strongyloidiasis invasions prevail. Unfortunately, helminthoses do not fall into research domain of the majority of scientists, because of their low prevalence as compared with bacterial, viral, fungal, and protozoal infections in humans [4]. But nevertheless helminthoses pose a potential threat to public health of certain countries.

38.2. Methods

The area of investigations is Dnipropetrovsk region, located in steppe zone of Ukraine. The relief represents combination of ravines with water-parting aligned sections. Such

territories are divided by valleys of rivers-tributaries of Dnepr. Plain sections of water partings are used for ploughing-up. Cattle owned by private persons are freely grazing on natural pastures, located on hollow slopes and river valleys with meadow and steppe vegetation.

For studying the role of insects as reservoir hosts capable of transferring helminthes to various distances from the place of infection, the relevant investigations were carried out; such investigations included selection of insects on pastures, their dissection and determination of species of helminthes found therein. Estimation of epizootic situation as regards cattle helminthoses was made taking into account condition of pastures and rate of animals' grazing on them.

38.3. Results

38.3.1. Hazard of Helminthoses Invasions for Animals and Humans

In natural conditions of living, parasitism in animals remains in ecological balance: degree of invasion of certain representatives of helminthes makes 0.2–2.3%. As a result of impact of anthropogenic factor, sometimes all ruminant animals on pastures are affected by helminthes. The level of invasion in cattle is higher in spring-summer period than in autumn-winter one (Figure 38.1). At present time, there are a great number of pastures, which are unfavorable from the point of view of helminthoses, in Ukraine. Should cattle-breeding complexes be created for hundreds of animals, they are considered as "limited population", and rigid control over observance of veterinary and zoo-hygienic norms is provided. Quite often, falling of some species of helminthes into the said "limited population" leads to the necessity of slaughtering and industrial processing of all animals, thus causing material damage.

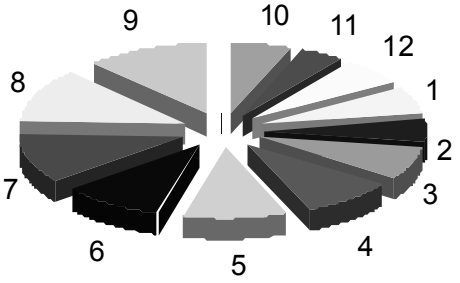

Figure 38.1. Annual dynamics of the level of helminthoses invasion in cattle from October 2002 till September 2003

In the process of evolution, a number of helminthes parasitized in invertebrates, then in vertebrates, and finally in humans. Evolution hadn't invented special human parasites; the same species of helminthes as observed in animals are typical for humans

as well. Human helminthoses are characterized by signs of chronic intoxication and allergization of the organism, impairment of functioning of immune system, respiratory apparatus, digestive tract, myalgia, lymphadenopathy, iron-deficiency anemia. As a result, health indices and quality of patients' life are reduced. Besides, helminthes are hazardous by their mechanical impact on human organism, and ability to induce serious complications such as obstruction of ducts of pancreato-biliary system, abscesses of liver and pancreas, perforation of intestines, and intestinal obstruction. Clinical diagnostics of helminthoses is rather difficult, since they more often display non-specific symptoms and syndromes: general weakness, headache, nervousness, sleep disturbance, local or systemic skin itch, shedding of hair, nail fragility, disturbance of appetite, transitory pain in stomach [8, 9]. Methods of laboratory examination adopted in the most of policlinics not always allow identifying parasites. Symptomatogy of the most parasitic diseases has low specificity. Clinical presentations are stipulated by long-term presence of the agent in the patient's organism (with no specific treatment provided), which is determined by duration of parasite life or frequent re-invasions, intensity of invasion and character of immune response of the patient. The following symptoms prevail: fatigability, deterioration of appetite, irritability, sleep disturbance, in children – mental and physical retardation. As a rule, general practitioners do not associate the above signs of organism asthenization with presence of parasites that in turn results in late and even incorrect diagnostics. Depression is often observed in case of prolonged course of intestines parasitosis, and "immersion in disease" is common [2, 3].

38.3.2. Ways of Spreading of Helminth Populations (Strongylata, Rhabditata), Role of Insects as Reservoir Hosts Thereof

Strongyloidiases and rhabditatoses are helminthoses arising as a result of parasitization of coelelminths of *Strongylata* and *Rhabditata* suborders. Depending on localization of causative agents in the host body, helminthes are divided into two groups. The first group includes nematodes, which parasitize in sexually mature stage in digestive apparatus, and the second group includes helminthes living in respiratory apparatus of animals and humans [9].

Mode of infection is as follows. Larvae of helminthes go out from eggs in the organism of diseased animal and with faeces get into the external environment, where they remain viable during 4–6 months, provided damp substrate is present. Together with feed, larvae are swallowed by invertebrates (earthworms), and they spend the winter and are maintained during droughty period in the above worms. No scientific papers about the role of insects in life cycle of the given group are available. At the same time, insects are one of the most numerous classes of animal world (in the aspect of species), and they can play the role of mechanical carriers of helminth larvae to various distances, increasing the risk of people infection with helminthoses.

The authors investigated the role of certain insect species in spreading of helminthes from territories of pastures to nearby settlements. Helminthes of Strongylata and Rhabditata suborders related to nematodes were taken as an object of investigation. Representatives of these helminth groups parasitize in digestive system and respiratory apparatus. In most cases, their main hosts are horses, pigs, birds, but often humans are also infected.

38.3.3. Results of Using the Method of Helminth Accounting with the Help of Insects – Reservoir Hosts

In the course of previous investigations it was found that epizootic situation is unfavorable as for helminthoses, and in particular – strongyloidiases and rhabditatoses [10]. Using the method of cultivation of coproscopic material of the cattle, larvae of seven species of helminthes of *Strongyloides* class (species *Strongyloides*, *Dictyocaulus*, *Bunostomum*, *Haemonchus*, *Oesophagostomum*, *Chabertia*, *Nematodirus*) were found. From three to six species of parasites fell at one animal. Most often (in 70–100% of cases) such species of helminthes as: *Bunostomum sp.*, *Haemonchus sp.*, *Strongyloides sp.* were detected. With a view to determining the insect role in the nematode development and spreading cycles, we took species which existence was connected with stay and development in cattle excrement. Hundred samples of each insect species from pastures of different areas of Dnipropetrovsk region were examined.

Investigations proved availability of larvae of these helminthes in the body of insects of Coleoptera order (*Oniticellus fulvus*, *Onthophagus ovatus*, *Onthophagus taurus*).This being the case, 20% of *Oniticellus fulvus* insects were infected with larvae of various nematode species. In 15% of them, larvae *Rhabditata* (*Strongyloides*), and in 5% – *Strongylata* (*Dictyocaulus*) were found. Larvae of *Strongyloides* (44%) and *Dictyocaulus* (24%) were revealed in 55% of examined *Onthophagus ovatus* insects.

No other St*rongylata* representatives or eggs of these helminthes were found. Maximum quantity of nematode larvae in each insect was equal to nine ones, and larvae of *Strongylata* greatly exceeded *Rhabditata* larvae in number (Figure 38.2).

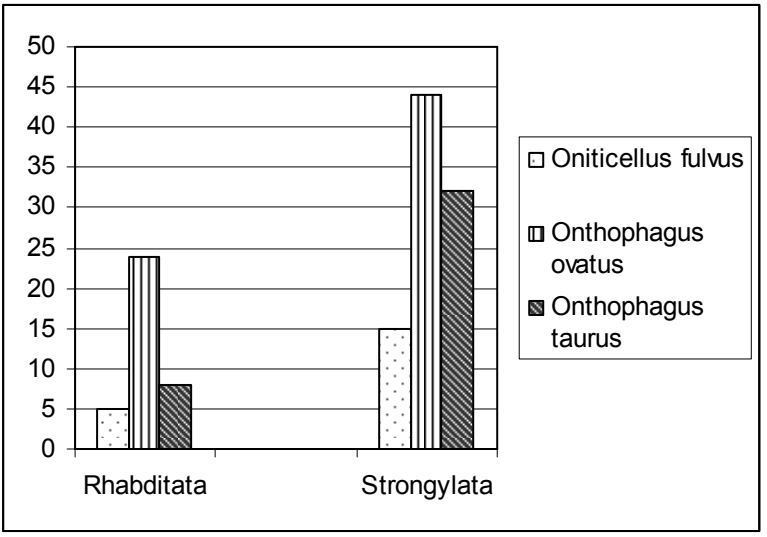

Figure 38.2. Indices of extensity of Coleoptera beetles infection with larvae of *Strongylata* and *Rhabditata* suborders (%) in conditions of Steppe zone of Ukraine (2007)

In the environment, larvae of nematodes of *Strongylata* suborder are developing during 8–16 days, depending on species. This phenomenon proves the fact that in fresh (1–3-day) cattle excrement no such helminthes were found, since they have not formed yet. Larvae *Dictyocaulus sp.*, which achieve invasiveness for 5–7 days, were revealed.

Results of the investigation show that the cattle, in conditions of steppe zone, is infected with mixed helminth invasion (seven nematode species of *Strongylata* and *Rhabditata* suborders). Representatives of insects of Coleoptera order, living in cattle excrement, are "reservoirs" of these nematode larvae and at the same time bioeliminators of their eggs. Coleoptera insects (*Oniticellus fulvus*, *Onthophagus ovatus*, *O. taurus*) are reservoir hosts in life cycle of nematodes – causative agents of helminthoses, promoting preservation of helminthes' invasion and their spreading on the territory of Ukraine.

Method of helminth accounting by examination of insect intestines is efficient and harmless one; it can be widely used for estimation of epizootic situation in certain regions of the country.

38.4. Conclusions

As a result of investigations, role of insects in preservation of the invasion source in the environment was confirmed, and their role in biology of helminthes representing Strongylata and Rhabditata suborders was determined.

Investigation of helminthes' species composition by revealing them in the insects proved to be an efficient method not requiring considerable material costs, having wide spectrum of action, and allowing precise determination of averaged indices of parasites' number. This method is harmless for humans and animals, and it is suitable for use in various natural and climatic regions.

Due to detailed study of biological peculiarities, and relations between helminthes and insects, the scientists can use insects as an indicator group, in particular, during estimation of prevalence of strongyloidiases and rhabditatoses.

Average quantity of helminthes found in accord with intensity and extensity of coprobiontic insects' infection rate, has considerable advantage as compared with point estimation of quantity of Strongylata and Rhabditata suborders' helminth larvae in soil.

Conducted investigations confirmed that further study of biology and mutual relations of helminthes and insects is very promising one, and it allows to use insects as objects of biological control over helminthes hazardous for humans and animals.

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