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Radiation Technology Against Bioterrorism

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ABSTRACT

The threat of bioterrorism has prompted a reaction from governments and scientists in a rapidly expanding war against unknown attacker. In the United States, the Postal Service has announced new safety measures that include processing mail with electron beam technology to eliminate potentially dangerous microorganisms. The microbiocidal activity of radiation is one of the radiobiological effects that is of considerable interest in medicine and public health. It has already been employed for sterilizing medical equipment and supplies, medicaments, pharmaceuticals, cosmetics and biological tissue.

L. G. Gazsó recommended first to use ionizing radiation for the inactivation of biological weapon agents (VI. Int. Symposium on Protection Against Chemical and Biological Warfare Agents, Stockholm, 1998. and Symposium on Nuclear, Biological and Chemical Treats in the 21st Century, Helsinki, 2000.)

The calculation of inactivation dose depends on three parameters, namely the initial microbiological contamination (number of microbes), the radiosensitivity of microorganism and the assurance of sterility required. The radiosensitivity of microorganism towards high energy radiation varies widely: different types, species and strains exhibit greatly different radiation sensitivity. Certain environmental factors are also able to influence the actual radiation response. The intent of this paper is to provide a broad overview of the importance of radiation neutralizing of bio-warfare/bioterrorism agents, indicate what further work is needed and summarize the recent experiences.

The application of radiation technology for inactivation of bioterrorism agents and the main results of NATO Advanced Research Workshop on "Radiation Inactivation of Bioterrorism Agents" (7-9 March, 2004, Budapest, Hungary, NATO Co-director L. G. Gazsó) are described in this paper.

1. INTRODUCTION

There are not many technologies in modern science, which have attracted so much attention in the academic world than radiation technology. Early application of radiation processing: radiation crosslinking of polymers and radiation sterilization of health care products have developed into substantial industries, while food preservation is widely accepted for some products, such as spices. Radiation treatment of municipal and industrial waste water for inactivation of pollutants, electron beam flue gas treatment have been extensively studied and successfully demonstrated on pilot plants.

The threat of bioterrorism (dispersal of anthrax spores by means of delivered mail) has prompted a reaction from governments and scientists in a rapidly expanding war against an unknown attacker. In the United States, the Postal Service has announced new safety measures that include processing mail with electron beam technology to eliminate potentially dangerous microorganism.

Paper presented at the RTO SCI Symposium on "Systems, Concepts and Integration (SCI) Methods and Technologies for Defence Against Terrorism," held in London, United Kingdom, 25-27 October 2004, and published in RTO-MP-SCI-158.

Main applications of radiation technology:

- radiation sterilization of health care products,
- food preservation,
- radiation processing of industrial and municipal waste water,
- radiation inactivation of bioterrorism agents,
- electron beam processing of flue gases,
- radiation crosslinking,
- radiation curing

The microbiocidal activity of radiation is one of the radiological effects that is of considerable interest in medicine and public health. It has already been employed for sterilizing medical equipment and supplies, medicaments, pharmaceuticals, cosmetics, biological tissues and more recently postal mail and high-risk luggage.

Radiation, as a sterilizing agent, offers a number of advantages that make it an attractive choice in a number of situations (1).

- Radiation causes no significant temperature rise, which permits sterilization of heat-sensitive materials.
- Due to its high penetrating ability, radiation reaches all parts of the object to be sterilized. The items can be pre-packed in hermetically sealed, durable packages, impermeable to microorganisms.
- The chemical reactivity of radiation is relatively low compared with the often highly reactive gases. Hence, the possibility of inducing a chemical reaction that may lead to disadvantageous changes in the products is minimal.
- Since there is no problem similar to convection of heat or diffusion of gases, the effect of radiation is instantaneous and simultaneous in the whole of the target.
- Radiation can be easily adapted for continuous processing as compared with the batch operation used with gas sterilization.
- The radiation is the most reliable of all competing sterilization methods because time (dose) is the only variable that requires monitoring once the process parameters have been established. All the other methods of sterilization depend on simultaneous control of many factors such as temperature, pressure, concentration, humidity and other.

Nowadays over 160 gamma industrial irradiators and 1300 electron industrial accelerators are in operation worldwide.

2. RADIATION DAMAGE

The radiation induced inactivation of cells under a given test condition is a resultant effect of a series of complex physical, chemical and biological processes. Traditionally, it has been a practice to consider two quite distinct mechanisms. These have been called direct and indirect actions (2). The alteration in the molecule occurring as a result of absorption of radiation is said to be due to the direct action. The target may be ionized or excited initiating the chain events that leads to a biological change. On the other hand, when energy is absorbed in a certain molecule and transferred to a second molecule in which the chemical change takes place is called the indirect action (Figure 1).

This terminology is used successfully in studies on isolated cellular components, such as enzymes, nucleic acids. Application of this terminology for bacteria and fungi, however, has not been useful because of the chemical structural complexity of the cellular system.

Generally the damage to cells produced by ionizing radiation can be divided into three categories (3).

Lethal damage - which is irreversible, irreparable, and by definition leads to cell death

Sublethal damage - which under normal circumstances can be repaired unless additional sublethal damage is added

Potentially lethal damage - this component of radiation damage can be influenced by environmental conditions (oxygen, temperature, chemicals, etc.)

Radiation action occurs over a broad timescale which extends from the very early physical processes to the very late biological effects, such as mutagenesis and carcinogenesis. The earliest event is the physical stage, which occurs between 10^{-18} – 10^{-12} second. The most important reactions of this stage are the ionization, excitation and dissociation of water, which lead to the formation of radical ions (4).

Ionization $H_2O \rightarrow H_2O^+ + e^-$

Excitation $H_2O \rightarrow H_2O^*$

Dissociation $H_2O^* \rightarrow H^\bullet + {}^\bullet OH$

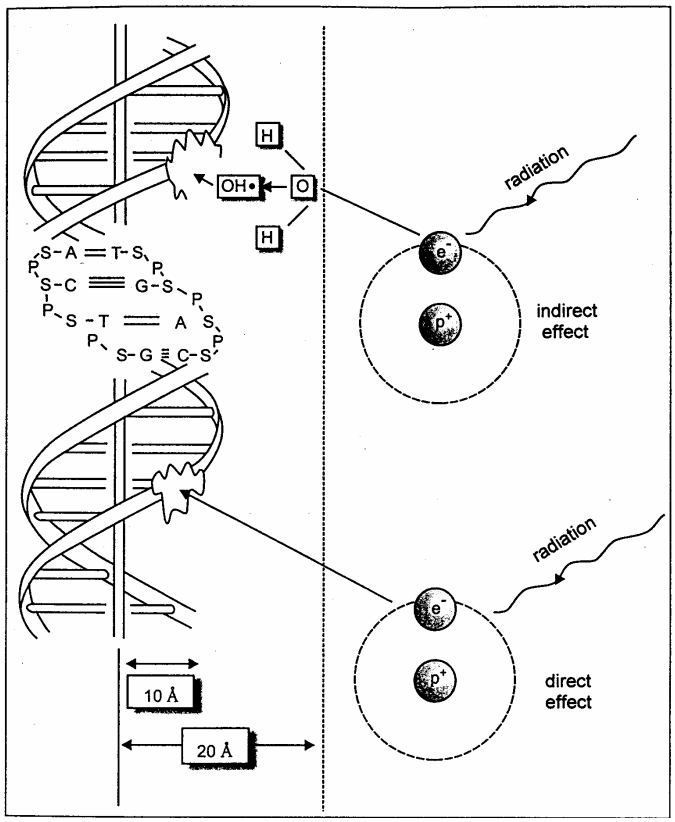

Figure 1. Direct and indirect effects of radiation

The chemical stage occurs between 10^{-2} and 10^{3} second. The most important parts of it are the different reactions between primary products, homogenous distribution of free radicals and the biochemical processes.

The timescale of biological stage can range from hours up to several years (mutagenesis, carcinogenesis).

3. RELATIONSHIPS BETWEEN DOSE AND EFFECT

The relationships between dose and effect can be demonstrated by different kinds of survival curves. It is a common practice in the radiation biology to present results in the survival curves, where surviving fraction of organisms plotted semilogaritmically against dose of radiation. (Figure 2.) Originally three types of survival curve were described, namely exponential, sigmoidal and composite (5).

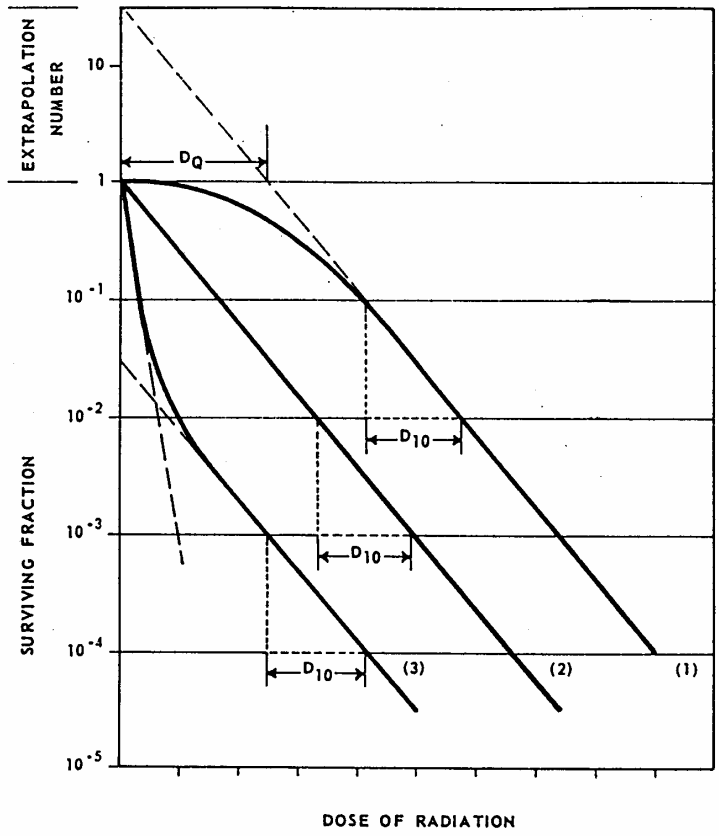

Figure 2. Hypothetical survival curves of irradiated bacteria: sigmoidal (1), exponential (2), and composite (3)

The exponential curve, a straight line when plotted as described above, it indicates that each organism needs only one hit to be inactivated. The exponential curve can be fitted by the next equation,

$$S = e^{-kD}$$

where S is the survival fraction after a single absorbed dose D, and k is the slope of the curve on semilogarithmic plot.

The sigmoidal curve is indicating that each organism needs more than one hit to be inactivated. This type of curve may be described by the so called multitarget single hit expression,

$$S = 1-(1-e^{-knD})^n$$

where the inactivation constant k is the sensitivity of each n target, each of which must be hit to kill.

In the case of composite curve, the population contains a mixture of two or more subpopulation (a sensitive and a resistant one) which separately would follow an exponential dose-effect curve. In the simplest case a mixture consisting of population a and b, the survival curve would be,

$$S = ae^{-kaD} + be^{-kbD}$$

For the practical application of radiation effect, the D_{10} value (decimal reducing dose) was introduced, which is the dose required to reduce the population by a factor of ten. The radiosensitivity of microorganisms is conventionally expressed in term of D_{10} value. The unit of the absorbed dose is the gray (Gy): 1 Gy = 1 J/kg[1]. The old unit, the rad, is 10^{-2} Gy.

4. RADIOSENSITIVITY OF MICROORGANISMS

The sensitivity of microorganisms towards high energy radiation varies widely: different types, species and strains exhibit greatly different sensitivities. Certain environmental factors are also able to influence the radiation response (temperature, oxygen, water, soluble chemical agents).

Viruses

In general it is accepted that viruses are more resistant than bacterial spores. Single-stranded simple viruses are more sensitive than double-stranded complex structures (6). Radiosensitivity of 30 viruses was studied by Sullivan at al. (7). D_{10} values of viruses suspended in Eagle's minimum essential medium containing 2% fetal bovine serum ranged from 3,9 kGy to 5,3 kGy. The radiosensitivity was significantly affected by suspending media. The fully dry viruses are more resistant, as hydratation proceeds the radiosensitivity increases.

Bacteria

The bacteria show more complexity than viruses. From series of radiation microbiology studies, it can be concluded (1):

Among the vegetative bacteria, Gram-negative organisms (D_{10} ranging between 29 Gy - 240 Gy) are more radiosensitive the Gram-positive species (D_{10} ranging between 180 Gy - 890 Gy).

Bacterial spores are considerably more resistant than vegetative species. The anaerobic spore formers like Clostridium (D_{10} values ~ 2.2 – 3.4 kGy) are more radioresistant than aerobic Bacillus spores (D_{10} ranging between 1.2 and 5.0 kGy).

Besides of the differences between the species, there are a number of factors concerned with the environmental conditions can greatly influence the actual radiosensitivity. For instance the D_{10} values of Salmonella typhimurium were significantly different, when the suspending medium was phosphate buffer (D_{10} = 210 Gy) or fish meat, where D_{10} value reached the 1.74 kGy (8). The different supporting surfaces can also alter the radiosensitivity of bacteria.

The bacterium Micrococcus radiodurans isolated from irradiated meat is the most radiation-resistant organisms known. D_{10} values can reach 10 kGy. The radiation resistance of this strain has been attributed

to its exceptional repair capabilities rather than to an altered susceptibility to radiation of its genetic material per se (9). Thus, the ability to repair DNA double-strand breaks has been reported. The specific nature of this repair is still not clear, though it is certain that M. radiodurans possesses DNA excision repair and DNA recombination activities. The taxonomy study has been suggested that Micrococcus radiodurans and its relatives (M. radiophylus, M. radioproteolyticus) are distinct from conventional Micrococcus species (10). Structural observations on these organisms emphasize the unique features of their cell wall and membranes. A new name was introduced as Deinococcus radiodurans.

Fungi

Most studies of the inactivation of fungi by irradiation have been made on asexual spores. Germinating spores, mycelia and other morphological structures of fungi might have different radiation responses (11). The radiation sensitivity of fungi is influenced not only by genetic factors but also by the number of cells in a spore (effect of multicellularity), the number of nuclei per cell (effect of multinuclearity). The haploid yeast cells are more sensitive than diploid ones (effect of ploidity). Ten species of fungi representing the genera Alternaria, Aspergillus, Cladosporium, Curvularia, Fusarium and Penicillium were examined by Saleh et al., (12). D_{10} values of fungal conidia in water for Aspergillus niger is 420 Gy, for Cladosporium cladosporoides 300 Gy and for Curvularia geniculata 290 Gy. D_{10} values for dematiaceous fungi (in agar medium!) ranged from 6 to 17 kGy and for moniliaceous fungi were less than 3 kGy. Yeast appear to be about as sensitive as non-spore forming bacteria.

At present an immense quantity of data is available in the literature. Unfortunately, most of these data were obtained under different experimental conditions. Regarding the considerable influencing effect of environmental condition to the actual radiosensitivity, to achieve a correct comparison is very difficult.

5. DOSE MODIFYING FACTORS

The radiosensitivity of microorganisms can be influenced by some factors other than genotype of species. The responses of cells to a given dose can be altered in different ways. This is possible because response depends on physical factors (quality of radiation, temperature, etc.), on chemical factors (oxygen, water content, chemical agents, etc.) and the biological or physiological factors (growth phase, amount of DNA). (Table I.)

Table I. Dose modifying factors

Physical	Chemical	Biological
Quality of radiation	Oxygen	Amount of DNA
Dose rate	Water content	Growth phase
Dose fractionation	Sensitizers	Cell cycle
Temperature	Protectors	Age
	$^{\bullet}$OH-scavengers	Sex
	Antioxidants	
	Thiol-reactive agents	

The dose modification can be expressed by the dose modification ratio (M) - this is the ratio of dose under reference conditions to test conditions to produce the same level of effect.

$$M = D_R/D_T$$

5.1 Physical Dose Modifying Factors

The radiation damage is highly depending on the quality of radiation. The radiation quality can be caracterized by the Linear Energy Transfer. LET is defined as the energy lost by particle per unit length of medium. To describe the difference between high LET (fast neutrons, accelerated heavy ions, etc.) and low LET (γ-ray, X-ray, etc.) radiation, the Relative Biological Effectiveness was introduced. RBE is a ratio of absorbed dose of a reference irradiation (D_R) to the absorbed dose of test radiation (D_T) to produce the same level of biological effect,

$$RBE = D_R/D_T$$

The value of RBE depends on the radiation dose, the dose rate and the biological system. The relationship between LET, RBE and OER (Oxygen Enhancement Ratio) can be seen on Figure 3.

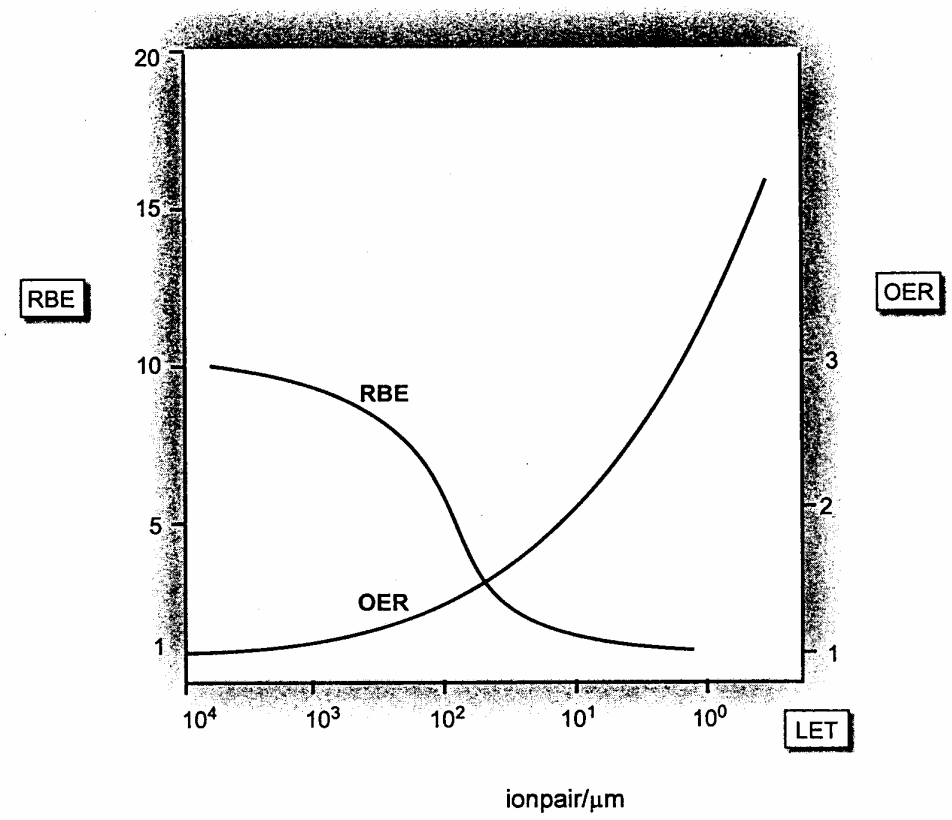

Figure 3. Relationship between LET, RBE and OER

The radiobiological importance of high LET particles:

- relative biological effectiveness is increased
- oxygen enhancement ratio is reduced

- repair of radiation damage is less
- the age response function is suppressed.

Dose rate differences between γ-rays are too small to be of any significance with respect to microbial inactivation, but clearly the large difference between ^{60}Co gamma-ray and accelerated electrons. High dose rate may decrease the efficiency due to the radiolytic depletion of oxygen (13).

Very soon after X-ray was begun to be used clinically it was recognized that the radiation response was usually reduced if the total dose was delivered in fraction rather than single shoot. In the case of *dose fractionation* a second shoulder appears in the surviving curve. The manifestation of a second or more shoulders assumed to be evidence that radiation damage must accumulate within the cell before a final event becomes lethal. This sublethal damage during the so called ”recovery interval” can be restored. The size of the shoulder depends on the repair capacity of cells and on the recovery interval, which usually ranged from minutes up to hours.

The *temperature* is also an important physical dose modifying factor. Experiments with dry spores of Bacillus megaterium shows that the sensitivity is constant between -268°C and -148°C, increasing temperature up to 20°C results an increased sensitivity by about 40%. The influence of temperature is similar for oxic and anoxic spores (14). Fully hydrated Bacillus megaterium spores equilibrated with oxygen the sensitivity increases by 16% on decreasing temperature from +18 to + 2,5°C. Further reduction in temperature down to -196°C decreases the level of radiosensitivity by about 45%. In contrast, for anoxic spores, the radiosensitivity increases slightly with decreasing temperature from +18°C to + 5°C. Reduction in temperature to -196°C results only a small decrease in sensitivity (15).

A number of investigations have reported that relatively mild doses of ionizing radiation sensitized bacterial spores (and many other microorganisms as well as viruses) very significantly to subsequent heat (16). Combined heat and radiation treatment of microorganisms yields a lethal effect greater than the additive rates of independent agents (17). Maximum synergism occurs at those conditions where heat and radiation are equally effective as destruction agents.

5.2 Chemical Dose Modifying Factors

Oxygen has received the greatest attention of all chemical agents known to modify radiation damage in cells. Oxygen has been found to increase the sensitivity to radiation of almost all type of cellular systems and even higher organisms, and this phenomenon has been generally known as the ”*oxygen effect*”.

The sensitizing effect of oxygen can be expressed by the Oxygen Enhancement Ratio (OER), which is the ratio of dose required under anoxic condition to that under condition of air to produce the same level of effect.

Gray (18) considered at first the possibility that the action of oxygen is mediated through reaction with products of the radiolysis of water. These reactions can be presented in a simplified form as follows,

$$H_2O \rightarrow HO^{\bullet} + H^{\bullet}$$

$$H^{\bullet} + O_2 \rightarrow HO_2^{\bullet}$$

$$e_{aq}^{-} + O_2 \rightarrow O_2^{\bullet -}$$

Secondary $HO^{\bullet}$ radicals can be produced from superoxide anion and hydrogen peroxide through the Haber-Weiss reaction (19).

Later Howard-Flanders proposed that the irradiation creates two types of damage (3)

$$R \xrightarrow{radiation} R''(lethal)$$

$$R \xrightarrow{radiation} R'(potentially\ lethal)$$

$$R' + O_2 \rightarrow R''O_2(lethal)$$

The potentially lethal damage is not lethal to the cells unless it reacts with oxygen. Later three distinct responses obtained by altering the gaseous environment (20, 21). (Figure 4.)

Class I. damage is seen when the cells are in anoxic condition during and after the irradiation. This damage is independent of oxygen.

Class II. damage, oxygen dependent and it is called as ”immediate oxygen dependent damage”. It occurs when oxygen is present during and after irradiation. It is believed to result from interaction of oxygen with short-lived radicals.

Class III. damage, is the post irradiation oxygen dependent damage, which occurs when the cells are in anoxic condition during irradiation and oxic condition after the irradiation. This damage is known to occur as the result of interaction of oxygen with long-lived free radicals, formed by irradiation in absence of oxygen.

The oxygen dependent sensitization is quite similar in the cellular radiobiology. The OER values are varied between 2-4.

For the radiation chemical mechanisms of oxygen effect two hypothesis have been proposed (22). Namely, the ”oxygen fixation” and the ”activated oxygen” hypothesis.

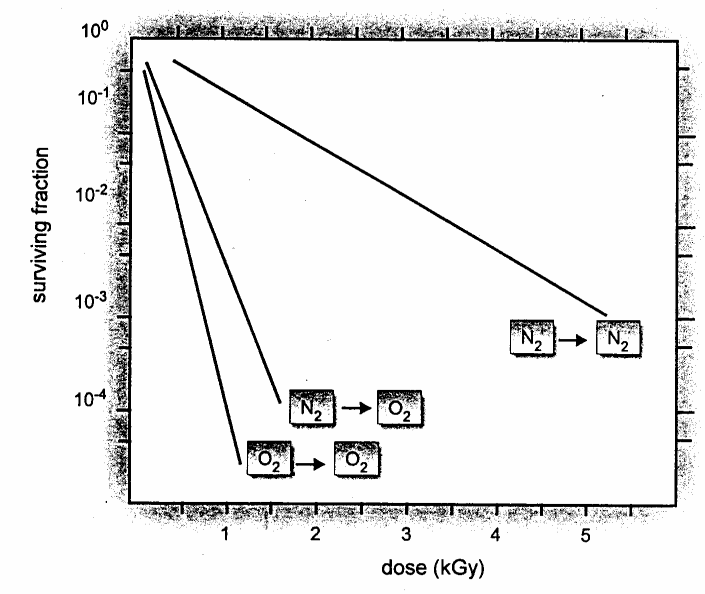

Figure 4. Surviving curves of bacterial spores irradiated under different gaseous conditions

The *chemical radiosensitizing agents* have practical value in the treatment of cancer with radiation. Regarding to the whole natural environment, many chemicals can enhance the radiation response. Within

the last decades, various classes of chemical agents have been found to increase the efficiency of radiation induced cellular damage. These include inorganic and organic chemicals with various properties.

a) Sensitizers specific for hypoxic cells:

- electron affinic agents
- membrane-specific agents

b) Analogues of DNA precursors:

- incorporated into DNA
- non-incorporated into DNA

c) Radiation-activation cytotoxic compounds

d) Agents which modify cellular regulatory processes:

- inhibitors of repair
- DNA-binding and intercalating compounds
- inhibitors of natural radioprotectors

From practical point of view the electron affinic sensitizers and inhibitors of natural radioprotectors play an important role in the cellular radiobiology. A large number of radiation sensitizing compounds of "electron affinic" class have been developed and tested in vitro and in vivo (23). The electron affinic agents are good scavengers of hydrated electrons when increases the yield of OH radicals, such a reaction between N_2O and e^-_{aq}

$$N_2O + e^-_{aq} + H_2O \rightarrow N_2 + OH^- + HO^\bullet$$

Scavenging the hydrated electron into OH radicals by electron affinic sensitizers prevent the reaction,

$$e^-_{aq} + HO^\bullet \rightarrow OH^-$$

which in absence of sensitizers converts the radical into harmless OH^-.

Natural thiols, mainly represented by glutathione, can also influence the radiation sensitivity (24). Glutathione can modify the radiation induced damage by scavenging radicals of the radiolysis of water and by hydrogen transfer to target radical. Glutathione may also involve in enzymatic repair processes by glutathione reductase, glutathione peroxidase and number of thiol-disulfide exchange enzymes (25). Inhibitors of natural radioprotectors, namely thiol reactive agents can decrease the actual glutathione content enhancing the radiation response (26).

Protective agents are chemicals, which reduce the lethal effect of radiation. The most remarkable group of protectors are the sulfhydryl compounds, which were discovered many years ago (27). Agents such as cysteine, mercapto-ethyl-amine and amino-ethyl-isothiuronium were among the most effective. Favoured hypothesises are the hydrogen donation from the -SH (as a reaction competing with damage fixation) and the ability to quench free radicals and their products (28).

The *water content* of the microbial cell at the instant of irradiation is also known to affect greatly its radiation response. For spores in N_2, progression from the wet to dry state causes a lessening in response of radiation, whereas for oxygenated spores, a similar progression results an increase in response (29). Similar overall water effect has been recognized in vegetative bacteria and mould spores.

5.3 Biological Dose Modifying Factors

Effect of radiation on cells can be modified not only by agents present during irradiation, but also by biochemical processes occurring over a much longer time. Profound changes in radiation response may be altered to progress of cell through different phases and stages of growth. These may be associated with changes in the intercellular environment. The literature on the radiation response of bacteria in different growth phases reveals some contradictory results (30). Unfortunately a general rule concerning the influencing effect of growth phase is not available. Sometimes survival curves are deeper in exponential than in stationary phases, sometimes the reverse is true. Shoulder of curves may be seen when microorganisms are in stationary phase, but not when the cells are growing exponentially or vice versa. Differences may be attributed to individual biological nature of the strains used.

Some data are available concerning the effect of post irradiation cultivation conditions. The medium can influence the post-irradiation recovery. Alper and Gillies (31) reported that suboptimal growth conditions were best.

In this paper we tried to describe the extent of the radiosensitivity of microorganisms and factors, other than genotype of microbes influencing radiosensitivity.

6. DOSE CALCULATION

The most critical part of the inactivation of bioterrorism agents is the calculation of sterilization dose. The sterilization dose depends on three parameters, namely the initial microbiological contamination (bioburden), the radiosensitivity of microorganisms and the assurance of sterility required (32, 33). For the pure culture the following calculation should be used:

$$DS = D_{10}(\log N - \log SAL)$$

where DS is the sterilization dose, N is the number of microorganism and SAL is the sterility assurance level. SAL is the expected maximum probability of an item or unit being non-sterile after exposure to a valid sterilization process. The recommended Sterility Assurance Level of health care products is 10^{-6}, which is the expectation of 1 non-sterile item out of 1 million.

7. SUMMARY OF THE NATO ARW ON "RADIATION INACTIVATION OF BIOTERRORISM AGENTS (BUDAPEST, 2004)

The NATO Advanced Research Workshop on Radiation Inactivation of Bioterrorism Agents was held, 7-9 March 2004 in Budapest, Hungary. The conference was hosted by the Frédéric Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary and organized by Dr. L.G. Gazso and Dr. C.C. Ponta. The conference was in part an outgrowth of the co-organizers' forward thinking in this area and previous recommendations on the use of ionizing radiation for biological weapon agent inactivation (VI. Int. Symposium on Protection Against Chemical and Biological Warfare Agents, Stockholm, 1998 and Symposium on Nuclear, Biological and Chemical Threats in the 21st Century, Helsinki, 2000) [32., 33.]. Wisely, the conference brought together experts from across a number of professional disciplines and geographic boundaries from the private sector, government, scientific research, and international regulatory agencies.

The conference papers cover many of the factors essential to the successful application of ionizing radiation to biological agent inactivation. [34.] Consideration of international law and treaty issues and defining what constitutes various kinds of attacks are reviewed, which are likely to be important if there is need for a multinational response. Because the most efficient application of radiation requires the total

dose be well matched to the sensitivity of the microorganism(s) of concern, there were several valuable reports detailing progress on precise, accurate, rapid, and field-ready diagnostics to assay the type of microbial contamination. A strength of this conference was the inclusion of facility operators and experts on process control, safety, and dosimetry. Their operational knowledge, detailed information on the current state of the art, descriptions of facility capabilities, explanation of dosimetry standards, and presentation of available technology and emerging techniques provide a strong technical base. Only from such a technical base is it possible to consider what resources are available, determine those that could most effectively be used in any particular situation where there has been the illicit use of biological agents, and provide high degree of assurance of the effectiveness of the decontamination effort. Also addressed was the radiation sensitivity of several types of agents of concern, including bacteria, bacterial spores, and viruses. Furthermore, factors that could alter an agent's radiation sensitivity were discussed. Several conference participants presented information on the U.S. response to the mail contamination, the approach that was taken, and some of the lessons learned. This conference also provided a forum for radiation experts on a broad regional basis to meet one another or become reacquainted. Potentially, this may be one of the most important facets of the conference. An important aspect of the U.S. response was rapidly making the needed connections and coordination among the appropriate scientists, private sector facility operators, and regulatory officials.

The conference recommendations were encapsulated in a formal memo to the International Atomic Energy Agency. In brief, the memo made following recommendations: (a) there is a need for a comprehensive assessment of the potential use of ionizing radiation for the destruction of biologically hazardous materials, (b) a need to assemble a committee of experts to develop and maintain a database on the use of radiation technology for biological agent defeat and to identify critical areas that still need to be addressed, (c) consider organizing an experts' meeting to advise the Coordinated Research Project on possible future Member States' actions, and (d) compile a list of radiation sources and locations capable of contributing to biological agent inactivation.

This workshop is a valuable basic reference for the use of radiation decontamination technologies against bioterrorism agents. The conference and its proceedings also provide a template for future highly cooperative and productive meetings to facilitate international interactions among those concerned with preparing responses to biological agent attacks.

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