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Drugs Radiosterilization: Parameters influencing the Selection of the Irradiation Dose

Abstract
Radiosterilization is now being recognized as an attractive method for drug sterilization. The irradiation dose must be sufficient to reach sterility, taking into account the sensitivity of microorganisms contaminating the product, but should not be too high in order to avoid excessive degradation of the pharmaceutical compound. This review summarizes the current guidelines concerning the selection of the irradiation dose and the effects of ionizing radiation on microorganisms as well as the mechanisms leading to their radioresistance. The radioresistance of microorganisms as a function of the environmental conditions is also critically reviewed.

Keywords
Radiosterilization, microorganisms, radioresistance, irradiation dose.

1. Introduction

Sterility in its general sense refers to the absence of viable microorganisms. However, as the inactivation of microorganisms is exponential, it is theoretically impossible to reach a complete elimination of microorganisms so that a different definition is needed. Therefore, in the pharmaceutical field, sterility is defined as a probability [1] called the sterility assurance level (SAL) which represents the probability of a viable microorganism being present on a product unit after sterilization. A product may be qualified as sterile when its probability of contamination equals or is below a given SAL value. The recommended SAL is 10^{-6} [2,3]. Terminal sterilization methods are now recommended, which involves that sterilization should be carried out on the drug in its final container in the last stage of production to avoid any further contamination. Sterilization is only required for some drugs such as parenteral injectable drugs, implants, ophthalmic preparations and sterile powders.

The European Agency for the Evaluation of Medicinal Products (EMEA) has published guidelines for the selection of the most appropriate sterilization method depending on the drug form [4]. For dry powders, non-aqueous liquids or semi-solids, the first choice method is thermal sterilization by dry heat. For thermosensitive solid-state drugs, radiosterilization (sterilization by exposure to ionizing radiation either gamma rays or high energy electrons) is deemed as the first choice method since it is a terminal method.

The method of choice for the sterilization of aqueous products is moist heat. The recommended sterilization cycle in an autoclave is 15 minutes at 121°. According to these guidelines, thermosensitive solutions should be sterilized by aseptic filtration, which is not a terminal method. Radiosterilization is not currently considered for the sterilization of aqueous solutions since a high degradation of the drug solute is generally observed after exposure of solutions towards ionizing radiation [5]. Two types of irradiation sources are available for the irradiation of pharmaceuticals: isotope and electron beam sources. Due to their high penetrating power, only gamma rays emitters are suited for the sterilization of products. The dose rate of gamma sources is limited by the specific activity of the radionucleide and is generally between 1 to 15 kGy per hour. Electron beam generators are able to produce high energy electrons (typically from 5-10 MeV for sterilization applications). Electron beam machines have less penetrative power than gamma rays but offer much higher dose rates up to 10^9 kGy per hour.

The aseptic methods which imply each production step to be carried out under aseptic conditions are pricey and do not provide a sufficient SAL (estimated around 10^{-4}) [6]. Therefore, they should only be used in the last resort for the production of sterile solid or liquid pharmaceutical forms.

Sterilization methods using alkylating agents such as formaldehyde or ethylene oxide should be avoided since these gases are mutagenic, carcinogenics and are still found in traces after sterilization. These methods are no longer considered in the current guidelines.

The development of analytical tools suitable for the analysis of irradiated drugs has allowed a better understanding of the mechanisms underlying drug degradation following

irradiation. Recent researches [7] pointed out the possibility to perform radiosterilization on drugs frozen aqueous solutions without significant loss in drug purity.

However, it seems fundamental in order to ensure sterility of drugs when irradiation is performed under various environmental conditions to examine more thoroughly the parameters influencing radiation sensitivity of those microorganisms as well as the current guidelines for the selection of the irradiation dose.

2. Selection of the irradiation dose

The dose of 25 kGy is recommended as the reference sterilization dose by the EMEA, the European Pharmacopoeia and the US Pharmacopeia [2,3]. This sterilization dose can be lowered according to the ISO 11137 guideline [8] which specifies the requirements for the development, validation and routine control of radiation sterilization process for medical devices although it may also apply to other products such as drugs [3].

When determining the radiation dose needed to sterilize pharmaceuticals, two approaches may be followed. The first approach, called traditional approach, involves treating the product with a minimum dose of 25 kGy. Historically, this dose has been considered as an effective sterilization dose almost universally since it was chosen according to the radiation resistance of the bacterial spores of *Bacillus pumilus* (*ca.* 3.1 kGy), considering a population of 100 CFU. However, it has now to be substantiated since international and European standards require evidence to demonstrate the effectiveness of the irradiation dose. The sterilization dose must be validated in order to allow parametric release of the drug batches.

The second approach, based on ISO 11137, relies on the determination of the number and possibly of the genuine radiation sensitivity of the microorganisms found in the product. The dose required to achieve a predetermined SAL is then predicted from these data. This allows a more rational selection of the irradiation dose as it is adapted to the microbiological quality of the industrial production and therefore, should be specific to a product and a manufacturing process.

According to ISO 11137, the selection of the irradiation dose may be based on three distinct methods:

- The first method only considers the bioburden (population of viable microorganisms on a product), which is determined by measuring the number of colony forming units (CFU) per drug product. It is assumed that the response of the product bioburden to radiation is greater than that of a population with the standard distribution of radiation resistance determined by Whitby and Gelda [9]. Each fraction of the standard population is characterized by its radioresistance (represented by the D_{10}, the dose needed to achieve one log reduction of the population) and its frequency. The resistance of the overall population consists in the sum of the individual curves of the different species. Figure 1 represents the radiation resistances of the different species and the resistance of the overall standard population for an initial bioburden of 1CFU

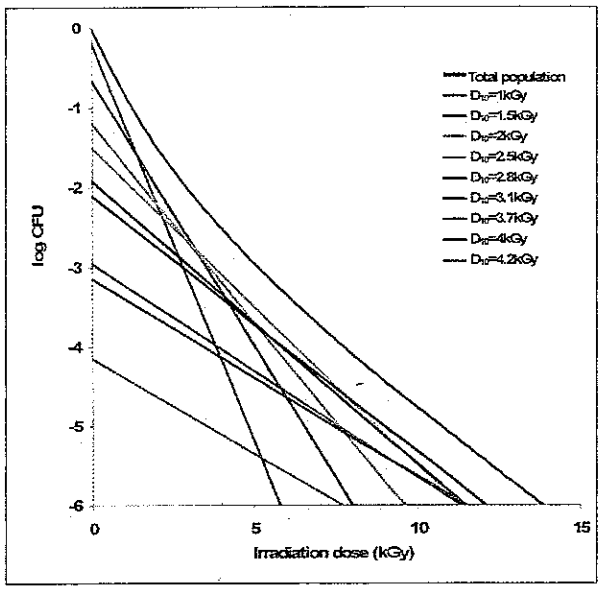

Figure 1. Probability of occurrence of a survivor as a function of the absorbed dose for the standard distribution of resistance for initial bioburden of 1 CFU. Survival of various subpopulations with different D_{10} values and survival of the total population (from D_{10} values and frequencies of the ISO 11137 reference population).

- For the second ISO method, the radiosensitivity of microorganisms as they occur on the products needs to be determined. Sterility tests are performed on product samples exposed to incremental irradiation doses to estimate the dose at which one in a hundred product unit is expected to be non-sterile (S.A.L. of 10^{-2}), which represents the verification dose. An estimate of the D_{10} value is made and extrapolated for SAL below 10^{-2} to determine the sterilization dose. This method allows the determination of the real radiation sensitivity of the microbial bioburden so that irradiation doses are often lower than those obtained with method 1 could be validated. However, it requires many more tests than the first method.

- A third method, called VD_{max} 15 or 25, has been added in the last revision ISO 11137, published in 2006 and which should be implemented before 2009. This method is based on the substantiation of the dose of either 15 or 25 kGy. The dose of 15 kGy is thus now also recognized as a reference dose although it may only be applied if the product bioburden is less than 1.5 CFU [8], which is achievable in circumstances where microorganisms contaminating products originate only from the manufacturing environment.

Regardless of the method chosen for the selection of the irradiation dose, periodic audit of the sterilization process

is needed to detect any changes in the bioburden that would require an adaptation of the irradiation dose.

Considering the advances in the GMPs in industries which contribute to provide a low bioburden, a 15 kGy dose could generally be sufficient to ensure a S.A.L. of 10^{-6}. Moreover, if the bioburden is kept low, then, the variety of microorganisms could be limited to a few species.

3. Microbiological quality of pharmaceuticals

An optimal sterilization dose should be determined depending on the contamination of the products, the radiosensitivities of the germs and the required S.A.L. The most common germs found in pharmaceuticals are those from human commensal flora, especially from cutaneous flora, such as *staphylococci*, *Corynebacterium ssp.* or fungi [13]. *Pseudomonas ssp.*, which are frequently isolated from water, are commonly found in liquids. Pharmaceuticals are usually poor culture media so that microorganisms with little alimentary requirements such as *E. coli, staphylococci, bacillus, streptococci*, gram negative bacteria and yeasts are favored [14].

The number and the type of germs present in pharmaceuticals depend on the origin of the raw materials and on the manufacturing process. Raw materials obtained by chemical synthesis posses the lowest contamination level [14]. Fecal microorganisms might be present in materials originating from soils.

Drug batches with very high contamination levels that would necessitate high irradiation doses should be excluded from the production instead of being radiosterilized since endotoxins and pyrogens cannot be destroyed by irradiation [15]. For example, the toxin of *C. botulinium* was found to have a D_{10} value around 21 kGy [16]. The initial bioburden should be reduced whatever sterilization method is used since most sterilization methods (except dry heat at 220°C [2]) cannot remove pyrogens. Sterilization must not be considered as a substitute for Good Manufacturing Practices (GMPs).

In order to decrease the bioburden of pharmaceutical raw materials, irradiation can be performed for decontamination purpose. There are no specific guidelines applying to the decontamination of pharmaceuticals by ionizing radiation. Low irradiation doses in the range of 5-10 kGy should be applied, such as it is the case for the decontamination of foods [17].

4. Effects of ionizing radiation on microorganisms

Many authors have described the sensitivities of germs towards ionizing radiation, especially those irradiated in food media [17]. However, there are less data on the radiosensitivities of germs irradiated in pharmaceuticals [18].

There is a growing interest in the use of ionizing radiation for the inactivation of microorganisms. Disposable medical devices have been sterilized by ionizing radiation since many years. The interest for the application of radiosterilization on drugs has gradually increased, mainly due to regulatory pressure to adopt terminal sterilization processes. New applications have also emerged. For example, in the United States, postal mail destined to some federal administrations is now irradiated by high energy electrons at doses up to 50 kGy to prevent any biological attack [19].

Like all other sterilization methods, irradiation represents a compromise between the inactivation of microorganisms and the destruction of the product. For sensitive drugs such as liquid solutions, a high irradiation dose should be avoided since it will lead to a decrease in drug purity [5]. For solids, high doses can lead to color and odor changes [20]. The dose of 25kGy is thus not always adequate for the sterilization of radiosensitive materials such as some pharmaceuticals. Therefore, the irradiation dose should be carefully selected in order to provide a sufficient sterility assurance level as well as avoiding the destruction of the drug. In order to justify the use of lower irradiation doses, it is necessary to keep a low bioburden but also to have a more precise insight on the sensitivities of contaminating microorganisms towards ionizing radiation.

4.1. Dose-effect

In the context of the exposure to ionizing radiation, the term survival does not distinguish living from non living cells but rather refers to their capability to reproduce themselves. This means that a cell that remains alive after irradiation but can no longer divide itself is considered dead.

The relationship between the survival and the irradiation dose follows an exponential so that it is often plotted on semi-logarithmic axes. The D_{10} or decimal reducing dose is defined as the dose required to kill 90% of an homogenous microbial population (one log reduction of cell population) where it is assumed that the death of microorganisms follows first order kinetics. Therefore, the inactivation dose should correspond to D_{10}(logN-logSAL) where N is the number of microorganisms.

As seen on figure 2, the relation between the effect of ionizing radiation on cell survival and the absorbed dose can be represented by different types of survival curves which illustrate the relationship between the number of organisms that survived and the radiation dose delivered [21].

The most simple case (2) consists in an exponential curve that can be fitted by $S=e^{-kD}$ where S is the survival fraction after the absorbed dose D and k the slope of the curve on a semilogarithmic plot. This is encountered when a single homogenous population of microorganisms is irradiated.

When more than one hit is needed for the inactivation of the microorganisms, the curve has a sigmoïdal shape with a shoulder (1). This is encountered for very radioresistant microorganisms.

In the case of a mixture of subpopulations of microorganisms with different sensitivities, these populations taken separately would follow an exponential dose effect curve so that the

dose-effect curve of the mixture (3) consists of the sum of each separate survival curve. This case is encountered in pharmaceuticals that contain many microbiological strains with different sensitivities and was already shown on figure 1 that represented the radiation resistance of the ISO standard population.

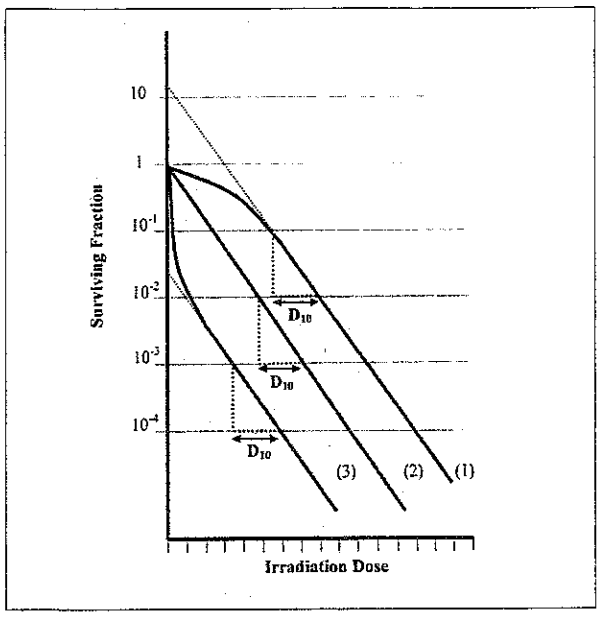

Figure 2 Dose-effect curves

Damages induced by ionizing radiation can be classified from their effects on the cell [22] as:

- Lethal: irreversible and non-reparable, lead to cell death
- Sublethal: can be repaired under normal circumstances unless additional sublethal damages are added
- Potentially lethal: can only be transformed into lethal damage if certain conditions are met such as the presence of oxygen, chemicals,...

4.2. Cellular damages induced by ionizing radiation

For the killing effect of ionizing radiation on cells, it is generally assumed that nucleic acids are the primary targets since the D_{10} values tend to correlate with the genome size [23].

It is estimated that the irradiation of a living cell at one gray induces 1000 single strand breaks, 40 double strand breaks, 150 cross-links between DNA and proteins and 250 oxidations of thymine [24]. Figure 3 shows some examples of radiation-induced lesions on DNA.

4.2.1. Strand breaks

The indirect effect plays a major role in strand breaks. Single and double strand breaks should be distinguished [22, 25]. Single strand breaks involve the breakage of the phosphate-deoxyribose bond. A large proportion of these breakages are due to hydroxyl radicals [22]. Following single-strand breakage, the two strands separate due to the penetration of water molecules within the breach and the disruption of hydrogen bonds between the bases.

Double strands breaks involve the breakage of the two DNA strands either following simultaneous breakage, which implies a high energy transfer, or from the combination of two single strand breaks in the complementary strands (the breakages should not be separated by more than 15 base pairs). Double bond breakages are either called homologous if occurring on the same base pair or otherwise heterologous, which are more frequent. Most prokaryotic and eukaryotic organisms cannot withstand more than five double strand breaks without reduced survival [26].

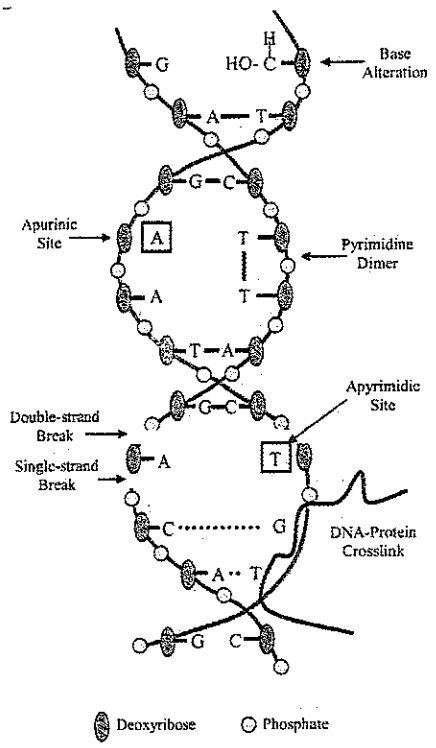

Figure 3 Radiation-induced DNA damages

4.2.2. Alteration of bases and sugars

DNA bases can be partially destroyed or chemically modified e.g. by hydroxylation. Thymine is the most radiosensitive base, followed by cytosine, adenine and guanine [27].

Alteration of deoxyribose is not very common and involves oxidation and hydrolysis of the sugar with cleavage of the base. The attack of the sugar by the hydroxyl radical produces a carbon centered radical that leads to the breakage of the furane ring with the liberation of the base.

4.2.3. Cross-links

Cross links in the spiral, links between two parts of the same strand (intrastrand links) or between the two strands (interstrand links) can occur. Cross-links between DNA molecules and proteins are also possible since DNA is surrounded by bounded proteins.

4.2.4. Oxidation of proteins

Apart from DNA, the other targets are proteins that can be oxidized by radicals from water radiolysis and sustain lethal damages.

4.3. Direct vs indirect effect on DNA

Radiation induced inactivation of living cells occurs through a series of complex steps involving physical, chemical and biological processes. Two distinct mechanisms are involved in the radiolysis of DNA [22,25,27]:

- The indirect effect where the energy is absorbed by water molecules, the radiolysis products of water diffuse and react with molecules generating chemical modifications.
- The direct effect which implies excitation and ionization of target molecules such as DNA within the cell followed by scission of molecular bonds.

There is much controversy on the relative importance of the direct and indirect effects [25,28,29]. The significance of the indirect effect depends on the environment as it is highly influenced by cellular water content or by the presence of some additives [29]. It was observed that, even for the same microorganism, the indirect effect might be either absent or predominant depending on the experimental conditions [29,30,31]. Some authors even question the importance of the indirect effect since the access of water radicals to DNA may be difficult due to its structure [25]. The sensitive sites of DNA need to be accessible to the reactive species resulting from water radiolysis. For example, the sites located inside the helix are not readily accessible.

DNA contains bond water. As the diffusion of radicals generated from the radiolysis of this water shell is very limited, they react with closely associated DNA and therefore, could be considered as a part of the direct effect [29]. The effect of irradiated bond water molecules is also called semi-direct effect and is sometimes considered as being part of the direct effect [29].

The radiosensitivity of DNA depends on its structure [32] which varies with the state of the cell. During cell replication, as DNA is less condensed, the accessibility of water radicals to DNA is increased [33].

4.4. Repair mechanisms

Living organisms have developed different strategies to recover from losses of genetic information caused by DNA damages. Damages to DNA alter its spatial configuration so that they can be detected by the cell.

In the case of single strand breaks (see figure 4 below), the damaged DNA strand is excised and its complementary strand is used to restore it. Efficient and accurate repair of the damages can take place as long as the integrity of the complementary strand is maintained. Radiosensitivity is highly influenced by the capability of the strain to repair single-strand breaks. Strains that lack this ability are far more radiosensitive than the others [21,22].

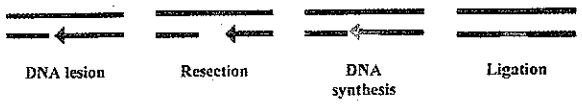

Figure 4 Repair mechanism for DNA single strand breaks

Double strand breaks are far more hazardous since they can lead to genome rearrangements. Two distinct mechanisms have been described for the repair of double strand breaks: non homologous end joining and recombinational repair [34].

- For non homologous end joining, the free ends are joined by simple ligation which may result either to perfect reparation or to genetic mutation if sequences are not homologue.
- Combinational repair (see figure 5 below) necessitates the presence of another copy of the genetic material within the cell since an identical DNA sequence is used as a template. This last mechanism cannot be achieved by all bacteria since some only possess one copy of genetic material per cell [35].

Figure 5 Repair mechanism for DNA double strand break by combinational repair

If DNA damages are extensive, the SOS repair mechanism may be triggered. The SOS response leads to the induction of SOS genes which are responsible for the production of a less accurate DNA polymerase that can insert mismatched bases at the level of the lesion, which greatly increases the frequency of mutation.

4.5. Radiosensitivities of microorganisms

Radiation sensitivity is not a constant for a given microorganism. It depends on its repair capabilities, its environment and its physiological state at the time of irradiation. Due to the very different experimental conditions used in the literature, and considering the effect of the irradiation conditions on the sensitivity, which will be discussed in more details in the next section, it is quite difficult to make absolute comparisons between the radiosensitivities of microorganisms.

The majority of the studies on radiation sensitivity of bacteria have been performed in foods which contain lots of scavenging substances. The sensitivities in regulated aqueous solutions should be higher [36]. If radiation sterilization was to be applied more widely, the sensitivities of the microorganisms found in pharmaceutical industries should be evaluated when irradiated in drug media. The type and the sensitivities of microorganisms

encountered in drug media could be very different from those encountered in medicinal devices and foods. Specific guidelines applying to drugs and that take into account the specificities of pharmaceuticals should be developed.

Figure 6 shows the theoretical inactivation curves for some microorganisms. As a general rule, it may be assumed that radiosensitivity will increase with the size of the genome [22]. However, there are many exceptions amongst microorganisms.

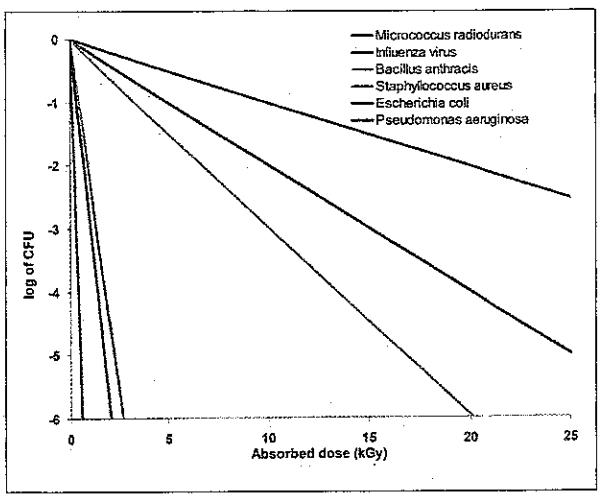

Figure 6 Theoretical inactivation curves of some microorganisms

4.5.1. Bacteria

For bacteria, radiation sensitivity depends highly of the bacterial type. Gram negative bacteria are more sensitive than gram positive species (D_{10} values of most germs range from 180 Gy to 890 Gy for non-sporulated gram positive versus D_{10} from 29 to 240 Gy for gram negative). Mycobacteria are very radiosensitive [21,21].
Due to their low water content, spores are far more resistant than vegetative species. For examples, the D_{10} value of *B. megaterium* vegetative cell is around 0.6 kGy whereas in the same condition, D_{10} of spores is 1.5 kGy.

4.5.2. Fungi

Radiation sensitivity of fungi is influenced by the number of cells in a spore and the number of nuclei per cell. The majority of fungi have D_{10} values between 100-500 Gy. Dematiaceous fungi, that are found in soils and rotten woods but normally not in pharmaceuticals, are highly radioresistant with D_{10} values from 6 to 17 kGy [37].

Yeast are more resistant than other fungi. *Candida albicans* for example was found to be quite radioresistant with D_{10} of 1.1 to 2.3 kGy.

4.5.3. Viruses

In general, it is observed that viruses are less sensitive towards ionizing radiation than bacteria and fungi. As seen in table 3, D_{10} values for most viruses range from 3 to 5 kGy [38], which is far more than bacteria. Radiation sensitivities of single stranded DNA viruses is higher than those of double stranded ones.

Viruses should not normally be found in pharmaceuticals, except in those originating from biotechnological processes. Biological products are submitted to specific guidelines [39] and the use of higher irradiation doses may be validated for the elimination of viruses. Inactivation with a sufficient S.A.L. ($<10^{-9}$) of viruses such as HIV or hepatitis in grafts necessitates high doses from 60 to 100 kGy [40].

4.6. Resistance mechanisms

Radiation resistant bacteria can be found in the environment, and are mostly gram positive and spore forming bacteria although some non-spore forming species such as *Micrococcus radiodurans* and *Deinoccoccus ssp.* are also radioresistant [41]. Amongst gram negative bacteria, *Moraxella ssp.*, *Acinetobacter*, and *Pseudomonas radiora* are also able to sustain large irradiation doses [42].

Although it was first believed that less damages were induced to the DNA of radioresistant bacteria, it was later demonstrated that the amount of DNA damage caused by a given irradiation dose is similar for sensitive and resistant bacteria [43]. The mechanisms responsible for the resistance towards ionizing radiation imply a complex DNA repair system that is able to repair radiation-damaged DNA.

It has been suggested that some pigments synthesized by microorganisms may play a role in their resistance towards ionizing radiation. For example, carotenoids synthesized by *Exiguobacterium acedicum* were found to be responsible for its radioresistance [44]. Fungi that synthesize pigments such as *Curvularia geniculata* (melanin) or other Dematiaceous fungi that contain melanin and carotenoids have higher D_{10} values [37,45]. These pigments appear to be involved in both photo- and radio-protection[45]. It was also discovered that a higher amount of Mn^{++} in some radioresistant bacteria may partly explain their resistance due to the decrease of protein oxidation in presence of higher concentrations of Mn^{++}.

The most radioresistant bacteria ever found, with D_{10} values up to 10kGy is *Micrococcus radiodurans* (also called *Deinococcus radiodurans*). *D. radiodurans* is a gram positive non-spore forming bacteria which was first isolated from irradiated meat [46] and was later found to originate from desert soils [48]. In addition to its radioresistance, *D. radiodurans* is also able to withstand extreme heat, dessication, oxygen peroxide and acids [43]. The resistance of this bacterial strain has been attributed to its capability to repair its genetic material and recover from extensive DNA damages such as hundreds of double strand breaks [45]. It was first suggested that the origin of the resistance was an adaptation to environmental radiation. However, the natural radiation sources present on earth do not generate the acute doses to which these bacteria show resistance. The radioresistance was later found to be a consequence of its ability to resist prolonged dehydration, which also induces DNA double strand breaks [45]. The mechanisms responsible for this phenomenon that have been identified so far include homologous recombination of DNA as this bacterium possesses multiple genome copies per cell, degradation of damaged DNA and its export outside the cell to prevent mutations [43].

4.7. Influence of the irradiation conditions on radiosensitivity

As seen in the previous sections, the irradiation conditions may play a major role in the sensitivity of the germs. It is therefore necessary to investigate the influence of the irradiation conditions and the environmental conditions on the radiosensitivity of microorganisms.

4.7.1. Influence of the dose rate

Radiosensitivity of bacteria towards gamma irradiations has been widely studied. For different activities of ^{60}Co sources, no significant difference in the radiosensitivity of microorganisms was observed [22]. There are less data concerning sensitivities towards high energy electrons. The comparison between the effects of gamma rays and high energy electrons for the inactivation of bacteria irradiated in the same conditions shows that the majority of bacterial strains have lower D_{10} values when irradiations are carried out with high energy electrons [46,47,48]. This effect can be attributed to the higher dose rate which generates a larger number of lesions in a short time. The decrease in survival observed with high energy electrons is due to the accumulation of sublethal events that cannot be repaired during the short irradiation length. The longer irradiation times needed to achieve gamma irradiations allow the repair system to remain effective so that sublethal lesions can be repaired [22]. The effect of the dose rate may vary from one cell to another, depending on the contribution of sublethal damages to cell death (capacity of the repair system). Due to their higher penetration, gamma rays are still useful for decontamination of bulk materials whilst electron beam irradiators are more suited for high throughput sterilization and thus for industrial applications.

4.7.2. Effect of additives

The evaluation of the radiosensitivity of bacteria as a function of the addition of radical scavengers is quite difficult since many experiments have been carried out either on isolated DNA, which does not take into account the effects within the cell. For experiments carried out on bacteria, the concentration of the scavenger within the cell was assumed to be equal to that of the extracellular media, which is generally not the case. The effects of different scavengers cannot be compared since the intracellular concentration of the scavenger is unknown [49]. To be effective, a scavenger must be concentrated in the immediate vicinity of the cellular target molecules [30].

It was shown that the protection of bacteria against ionizing radiation in the presence of hydroxyl radical scavengers was highly dependant of the irradiation conditions. When *E. coli* was irradiated either in aerated or N_2 saturated solutions in presence of t-butanol, a very efficient $^{\bullet}OH$ scavenger, no protective effect was observed; A slight protective effect of t-butanol was only observed in 1% O_2 solutions [50]. Scavengers are unable to prevent semi-direct effect due to the hydroxyl radicals from the bound water since the water lattice around DNA does not possess any solvent power [31]. Therefore, scavenging of the radicals from the bound water by an exogenous protector is almost impossible. It was observed that thiols are able to repair DNA damaged sites before a breakage occurs [33].

The effects of additives do not always occur through radical scavenging but also through the modulation of DNA structure that could decrease the sensitivity of DNA by reducing the accessibility of hydroxyl radicals [33].

The lethal effect of irradiation on microorganisms was also found to increase with the addition of some chemicals. For example, the addition of 60 ppm of vitamin K5 was found to decrease the survival of *Micrococcus radiodurans* [51]. Although vitamin K5 has an antibacterial activity, the increase in radiosensitivity is not due to this effect but might be due to the formation of very short lived intermediates that are toxic to the cell, to changes in cellular permeability or to the formation of intermediates between vitamin K5 and cellular constituents. The lethal effects of vitamin K5 combined to ionizing radiation was found to be greater under nitrogen atmosphere for most bacteria. Compounds similar to vitamin K5 such as 4-amino-1-naphtol or 1-amino-2-naphtol were also found to exhibit greater effects [52]. N-ethyl maleimide also increased radiation-induced damages [53].

In the absence of oxygen, some molecules such as nitric oxide have been shown to increase radiosensitivity of bacteria, even at low concentrations (10 µM). This capability might depend on the presence of unpaired electrons within these molecules.

4.7.3. Effect of water content

The water content within the bacteria is of fundamental importance. A decrease in water content results in a lower sensitivity towards ionizing radiation since the proportion of the indirect effect decreases. For this reason, spores, that contain less water, are far less radiosensitive. This explains the higher D_{10} values observed for spore forming bacteria such as *Bacillus ssp.* In addition, DNA conformation may vary with cellular water content which could influence the radiosensitivity [29].

4.7.4. Effect of oxygen

Oxygen is able to sensitize organisms to ionizing radiation. The presence of high oxygen concentrations may affect radiosensitivity of cells up to a 2 or 3 fold factor. However, for an air saturated solution, all the available oxygen in the solution is consumed after a 0.5 kGy dose so that there is a shift to anaerobic conditions at higher doses, provided oxygen cannot be readily resupplied [21,22].

When radical lesions are formed in DNA, some are only converted into lethal lesions when oxygen is present. The affinity of the oxygen molecule for unpaired electron is explained by the electronic structure of molecular oxygen which is a biradical molecule as two unpaired electrons are present.

4.7.5. Effect of temperature

Temperature plays a major role in the radiosensitivity of microorganisms. As temperature decreases, water radicals become less mobile. As a general rule, microorganisms are less radiosensitive when irradiated at low temperatures [54]. For example, whilst sensitivity of spores from *Bacillus megaterium*

was constant between –268 and –148°C, an increase in temperature to 20°C led to a 40% increase in sensitivity. Effect of temperature was observed to be similar for oxic and anoxic spores [19].

Water cellular content decreases with temperature since water is frozen out. For example, 94% of internal water is lost at –33°C for a slow cooling and 71% water is lost at –72°C for fast cooling. Radiosensitivity at low temperature is therefore a function of cell hydration [31]. However, in cells dried or frozen to a point compatible with their viability, the bound water is not removed from the cell [31] so that reactions with hydroxyl radicals might still occur.

The indirect effect is partially abolished by freezing the solution. The highest decrease in sensitivity is observed between 0 and –15°C. For example, D_{10} value of *E. coli* irradiated in meat increased from 0.41 kGy at +5°C to 0.62 kGy at –15°C. For *S. aureus*, D_{10} at –76°C was 0.82 kGy instead of 0.48 kGy at +4°C [55].

Subfreezing temperatures offer less protection for spores than for vegetative species since they already have a low moisture content.

5. Conclusions

With the increased regulatory pressure to use terminal sterilization methods, the possibility to use radiosterilization has been reexamined. In order to promote the application of radiosterilization on solid pharmaceuticals, new regulations should be implemented, such as it was the case for foods that could now be irradiated without having recourse to complex tests and procedures. Irradiation of solutions at subfreezing temperature seems to minimize drug degradation [7,56] but one issue raised by the use of lower temperature is the potential lower sensitivities of the germs at these temperatures. To substantiate the irradiation dose for frozen products, it should be necessary to evaluate the exact sensitivities of microorganisms in drug media at low temperature since sensitivities are expected to be slightly lower than those at room temperature. Therefore, new guidelines applying specifically to the sterilization of drug products and taking into account the physical state of the product should be developed since ISO guidelines were not specifically designed for drugs.

1 European Comity for Normalization. EN 556-2,. Sterilization of medical devices: Requirements for medical devices to be designated sterile. CEN, Brussels, **2003.**
2 European Pharmacopoeia, 5th Edition, Council of Europe, Strasbourg, **2005.**
3 United States Pharmacopeia National Formulary 28, United States Pharmacopeial Convention, Rockville, MD, **2005.**
4 European Agency for the Evaluation of Medicinal Products (EMEA). Decisional Trees for the Selection of Sterilization Methods (CPMP/QWP/054/98), London, **1999.**
5 C. Slegers, B. Tilquin. Theoretical approach to the destruction or sterilization of drugs in aqueous solution, *Radiat. Phys. Chem.* **2005**, 72, 363-365.
6 F.M. Nordhauser, W.P. Olson, 'Sterilization of drugs and devices, technologies for the 21st century', Interpharm Press, Buffalo Grove IL, **1998.**
7 A. Maquille, J.L. Habib Jiwan, B. Tilquin, *Eur. J. Pharm. Biopharm.* **2008**, 69, 358-363.
8 ISO 11137, Sterilization of health care products-requirements for validation and routine control – radiation sterilization. International Organization for Standardization, Geneva **2006.**
9 J.L. Whitby, A.K. Gelda, *J. Parent. Drug Assoc.* **1979**, 33, 144-155.
10 J.B. Kowalski, A. Tallentire, *Radiat. Phys. Chem.* **1999**, 54, 55-64.
11 J.B. Kowalski, C. Herring, L. Baryschpolec, J. Reger, J. Patel, M. Feeney, A. Tallentire, *Radiat. Phys. Chem.* **2002**, 64, 411-416.
12 A. Tallentire, *Radiat. Phys. Chem.* **2004**, 71, 399-402.
13 A. Gèze, M.C. Venier-Julienne, J. Cottin, N. Faisant, J.P. Benoit, *J. Microencapsulation* **2001**, 18, 627-636.
14 M. del Carmen de la Rosa, M. del Rosario Medina, C. Vivar, *Pharm. Acta Helv.* **1995**, 70, 227-232.
15 J.J. Previte, *J. Bacteriol.* **1968**, 95, 2165-2170.
16 A. Skulberg, *J. Appl. Bacteriol.* **1965**, 28, 239-241.
17 WHO (World Health Organization), 'High-dose irradiation: wholesomeness of food irradiated with doses above 10kGy', WHO Technical Report, Geneva, **1999.**
18 A.M. Dam, L.G. Gazso, S. Kaewpila, I. Maschek, *Int. J. Pharm.* **1995**, 121, 245-248.
19 S.L. Helfinstine, C. Vargas-Aburto, R.M. Uribe, C.J. Woolverton, *Appl. Environ Microbiol.* **2005**, 71, 7029-7032.
20 G.P. Jacobs, *Radiat. Phys. Chem.* **1985**, 6, 133-142.
21 L.G. Gazso. *NATO Science Series. Series I: Life and behavioural sciences* **2005**, 365, 59-68.
22 M. Tubiana, J. Dutreix, A. Wambersie, 'Introduction to radiobiology', Taylor and Francis, New York, **1990.**
23 R.J. Lowy, G.A. Vavrina, D.D. LaBarre, *Antiviral Res.*, **2001**, 52, 261-273.
24 ABCRI (Action biologique et chimique des rayonnements ionisants). Ed. B. Tilquin, Academia, Louvain-la-Neuve, **1999.**
25 J.F. Ward, *Radiat. Res.* **1985**, 104, 103-111.
26 M.K. Elkind, *Cancer* **1985**, 56, 2351-2362.
27 J. Cadet, T. Douki, D. Gasparutto, M. Gromova, J.P. Pouger, J.L. Ravanat, A. Romieu, S. Sauvaigo, *Nucl. Instr. and Meth. in Phys. Res. B* **1999**, 151, 1-7.
28 F. Hutchinson, *Science* **1961**, 134, 533-539.
29 S.G. Swarts, D. Becker, M. Sevilla, K. Wheeler, *Radiat. Res.* **1996**, 145, 304-314.
30 T. Sanner, A. Pihl, *Radiat. Res.* **1969**, 37, 216-227.
31 Y.N. Korystov, *Radiat. Res.* **1992**, 129, 228-234.
32 M. Spotheim-Morizot, M. Begusova, M. Charlier, *Actualité chimique* **2003**, 11, 97-102.
33 ABCRI (Action biologique et chimique des rayonnements ionisants), Ed. B. Tilquin, Nauwelaerts, Beauvechain, **2001.**
34 S. Broomfield, T. Hryciw, W. Xiao, *Mutation Res.* **2001**, 486, 167-184.
35 A. Kuzminov, *Microbiol. Mol. Biol. Rev.* **1999**, 63, 751-813.
36 N. Grecz, O. P. Snyder, A.A. Walker, A. Anellis, *Appl Microbiol.* **1965**, 13, 527–536.
37 Y.G.Saleh, M.S. Mayo, D.G. Ahearn, *Appl. Environ. Microbiol.* **1988**, 54, 2134-2135.
38 T.A. Grieb, R.Y. Forng, R.E. Stafford, J. Lin, J. Almeida, S. Bogdansky, C. Ronholdt, W.N. Drohan, W.H. Burgess, *Biomaterials* **2005**, 26, 2033-2042.
39 International Atomic Energy Agency (IAEA). International standards for tissue banks, IAEA, Vienna, **2004.**
40 D.G. Campbell, P. Li, *Aust. N.Z. J. Surg.* **1999**, 69, 517-521.
41 F.A. Rainey, K. Ray, M. Ferreira, B.Z. Gatz, M.F. Nobre, D. Bagaley, B.A. Rash, M.J. Park, A.M. Earl, N.C. Shank, A.M. Small, M.C. Henk, J.R. Battista, P. Kampfer, M.S. da Costa, *Appl. Environ. Microbiol.* **2005**, 71, 5225-5235.
42 S.C.J. van Gerwen, F.M. Rombouts, K. van't Riet, M.H. Zwietering, *J. Food Prot.* **1999**, 62, 1024-1032.
43 J.R. Battista, A.M. Earl, M.J. Park, *Trends microbiol.* **1999**, 362, 1566-1568.
44 D. Kim, H. Song, S. Lim, H. Yun, J. Chung, *Radiat. Phys. Chem.* **2007**, 76, 1213-1217.
45 V. Mattimore, J.R. Battista, *J. Bacteriol.* **1996**, 178, 633-637.
46 P. Dion, R. Charbonneau, C. Thibault, *Can. J. Microbiol.* **1994**, 40, 369-74.
47 G. Blank, D. Corrigan, *Int. J. Food Microbiol.* **1995**, 26, 269-277.
48 M. Miyahara, M. Miyahara, *Bull. Natl. Ins Health Sci.* **2002**, 120, 75-80.
49 C. Herskind, O. Westergaard, *Radiat. Res.* **1988**, 114, 28-41.
50. D. Billen, *Radiat. Res.* **1984**, 97, 626-629.
51 A.M. El-Tabey Shehata, *Radiat. Res.* **1961**, 15, 78-85.
52 G.J. Silverman, A.M. El-Tabey Shehata, S.A. Goldblith, *Radiat. Res.* **1962**, 16, 432-440.
53. C.K.K. Nair, D.S. Pradhan, A. Sreenivasan, *Radiat. Res* **1976**, 67, 382-385.
54 D.W. Thayer, G. Boyd, *J. Food Prot.* **2001**, 64, 1624-1626.
55 C.H. Sommers, B.A. Niemira, M. Tunick, G. Boyd, Meat science 2002, 61, 323-328.
56 H. Terryn, A. Maquille, C. Houée-Levin, B. Tilquin, Int. J. Pharm 2007, 343, 4-11.