STERILIZATION BY IONIZING RADIATION

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IONIZING RADIATION

VOLUME II

ISBN
0-919868-02-9

2155 Pie IX Blvd.
Montréal, Québec HIV 2E4, Canada

STERILIZATION OF MEDICAL PRODUCTS BY IONIZING RADIATION

INTERNATIONAL CONFERENCE, VIENNA, AUSTRIA
April 25-28, 1977

SPONSORED BY

Johnson & Johnson

Editors: E. R. L. Gaughran and A. J. Goudie

MULTISCIENCE PUBLICATION LIMITED
MONTRÉAL, QUÉBEC, CANADA

Acknowledgement

Johnson & Johnson wishes to express its sincere appreciation to the United Kingdom Panel on Gamma and Electron Irradiation and to all others who assisted in planning the program and suggesting participants.

PREFACE

The International Conference on the Technical Developments and Prospects of Sterilization by Ionizing Radiation held in April of 1974 provided a forum for the exchange of information on radiation sources, dosimetry and the effect of radiation on the materials of which medical devices are constituted or are packaged. It was impossible to include the equally important microbiological and regulatory aspects of radiation sterilization. The application of radiation sterilization to biological products and medical products, other than devices, was intentionally omitted.

The Second Conference on the Sterilization of Medical Products by Ionizing Radiation held in Vienna, Austria on April 25-28, 1977 addressed these areas. The opening lecture presented the basic aspects of the mechanism of cell death due to ionizing radiation, thus providing an introduction to the First Session, which was devoted to microbiological considerations. Here the steps necessary for the commissioning of a new facility, the role of bioburden, and the methods of defining the degree of sterility assurance were examined. Discussed also in this session were the effect of dose rate on microbial kill and the role of water as a modulator of radiation damage to microorganisms.

The Second Session was directed to the effects of ionizing radiation on biological tissues, such as bone, tendon, cartilage, heart valves, etc.; on drugs and cosmetics; and the use of ionizing radiation in the preparation of vaccines and antigens.

The Third session was devoted to a review of the various national, international and compendial viewpoints on the regulation of sterilization by ionizing radiation.

We hope that this volume, along with the Proceedings of the First Conference, will have accomplished our objective to provide an authoritative reference in the field of radiation sterilization and to convey our optimistic feeling for the future of this method of sterilization.

New Brunswick
New Jersey

E. R. L. Gaughran
A. J. Goudie

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Conference President

I. W. Sizer

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Dr. W. J. Haines

Johnson & Johnson, New Brunswick, New Jersey

It is indeed a pleasure to be here this morning to welcome you to the Second International Conference on the Sterilization of Medical Products by Ionizing Radiation. It is of interest to note that the list of participants includes representatives from 30 nations. This, in itself, speaks to the importance and interest in this method of sterilization.

Whereas English has been chosen as the "official publication language" of the Conference, simultaneous translation is being provided in English, French, German and Russian for use during oral presentations and discussions. We hope that whatever slight "language barrier membranes" remain will be fully penetrated by the high dose level or irradiation technology which will be developed at this Conference. The resulting high titre of satisfaction of all participants will then be directly correlated with the essential "conference components" of collaboration, communication and scientific fellowship which we believe will prevail throughout this meeting.

In deference to and in recognition of our host city, Vienna, I would like to stray ever so slightly from "the common language of the Conference" and say just a few words in my uncommon form of the local language, which of course is German.

Im Namen meiner Freunde und Kollegen in Wien, Habe ich die Ehre alle Teilnehmer an dieser Konferenz herzlich willkommen zu heissen. Es ist mein aufrichtig er Wunsch, dass die Zeit, die sie auf dieser Tagung verbringen und die sie auf diese schöne Stadt aufwenden wollen, nützlich und schöpferisch sein wird.

(Speaking thusly on behalf of my friends and associates in Vienna, I am most pleased to bid a hearty welcome to all participants in this Conference and to express the sincere wish that your time at this meeting, and in this fair city, will be both productive and enjoyable.)

Let me now say just a brief word about the company, which is sponsoring this Conference. My remarks are essentially equivalent to the break in radio or television programs when the announcer says, "We pause briefly for the identification of your local stations and for a commercial message from one of our sponsors." These next few words, which are intended primarily to be explanatory, constitute the full "Johnson & Johnson commercial message" associated with this conference.

As you may know, the Johnson & Johnson Worldwide Family of Companies is made up of 187 corporate organizations in 53 countries. We have 145 companies in 43 nations of the world which manufacture as well as market our products. As an aside, it is interesting to note that in our worldwide operations, we utilize about 13 languages.

In view of our extensive multinational manufacturing operations, the Johnson & Johnson Family of Companies is one of the largest users of Ionizing Radiation for the sterilization of medical products. As such, we feel that it is both our responsibility and our obligation to foster the study and

improvement of this technology. This is the principal objective of this conference which Johnson & Johnson is indeed pleased to sponsor. As is already obvious, each of you is a member of a very select group in that the number of participants has been limited deliberately in order to provide ample opportunity to exchange ideas and to promote discussion.

It is our sincere hope that you will enjoy your stay in Vienna and that you will find this meeting stimulating, not only from the point of view of the formal presentations and discussions, but also from the informal exchanges of ideas with your scientific colleagues from around the world.

It is now my special pleasure to introduce to you the President of the Conference, Dr. Irwin Sizer. Dr. Sizer is Dean Emeritus of the Graduate School of the Massachusetts Institute of Technology, where he continues as a consultant for Resource Development. He also serves as President of "The Health Sciences Fund."

He has long been involved with the problems of sterilization of medical products, having been associated in a consulting capacity with three Johnson & Johnson companies for more than 25 years.

Dr. Sizer has served as a Trustee of Rutgers University, Leslie College, The Boston Museum of Science and the Boston Biomedical Research Institute. He has been adviser and consultant to the National Institutes of Health, The Ford Foundation, The Massachusetts State Board of Higher Education and the Rutgers University Institute of Microbiology.

It is indeed an honor to introduce the President of this Conference and my personal friend of almost 20 years, Dr. Irwin Sizer.

Welcome by President of Conference

I. W. Sizer

Massachusetts Institute of Technology,
Cambridge, Massachusetts, U.S.A.

As President of the International Conference on "Radiation Sterilization of Medical Products" it is my pleasure and privilege to welcome you to Vienna. Many of you participated in, or at least read the monograph of a similar conference held three years ago in Vienna on "Sterilization by Ionizing Radiation" (1). It should also be pointed out that a closely-related conference was held in New Jersey in 1976 with the title "First Johnson & Johnson International Kilmer Memorial Conference on Sterilization of Medical Products" (2). These three international conferences attest to the commitment of this company to the production of clean sterile medical products. Much of the initial credit for this performance goes to Dr. Fred B. Kilmer who for 45 years, beginning in 1888, was its scientific director. That same year Johnson & Johnson published the book, "Modern Methods of Antiseptic Wound Treatment," and by 1890 was using dry heat to sterilize cotton and gauze dressings. In 1897, sterilization procedures were extended to catgut sutures (3), and now, eighty years later, hundreds of sterile products are manufactured by the Johnson & Johnson family of companies.

You may well ask what is an old-fashioned enzymologist like me doing at a conference on sterilization. I can only say that I come by an interest in this field legitimately having been brought to the Massachusetts Institute of Technology back in 1935 by Dean S. C. Prescott whose interest in sterilization was paramount. It was Prescott and W. L. Underwood who developed a time and temperature relationship which could insure the sterilization of canned goods, and thereby laid the basis for the canning industry in the United States. Later one of the early contributors to the field of radiation sterilization was S. A. Goldblith (4), who as a student took my first course in biology at M.I.T. Another student who studied heat inactivation of enzymes of thermophilic bacteria with me was E. R. L. Gaughan (5), an authority on industrial sterilization who helped organize and edit these three international conferences. I should finally say that for the past 27 years I have been a consultant for the Johnson & Johnson family of companies. It was my special privilege to be involved around 1956 when C. Artandi (6) and his "team of daring young men at Ethicon" (quote from ref. 7) succeeded in commercially applying the electron beam to the sterilization of surgical sutures.

In the first Vienna Conference on "Sterilization by Ionizing Radiation" emphasis was placed on physical considerations which are of importance in the design and use of the "hardware" employed in irradiation. Although reference was made to the original work by Minch in 1896 on the killing of bacteria by X-rays, the attention of the conference was centered on sterilization by electrons and gamma rays. High voltage electron accelerators found their first industrial application in 1956 when Artandi and colleagues (6), first used electrons to sterilize sutures. On the other hand, cobalt-60 was first used by the Westminster Carpet Co. in Australia in 1960 to destroy the anthrax bacillus in goat's hair

(6). In the past 20 years the field of sterilization by ionizing radiation has undergone remarkable growth and has now become the method of choice around the world for many different medical products. At this conference considerable attention was devoted to dosimetry, since in the use of both electrons and gamma rays there is often a thin narrow line between effective sterilization dose and a dose which will damage the medical product or the packaging material. With reference to the latter, the destructive role of free radicals induced by the radiation in altering small and polymeric molecules was emphasized. When it came to a discussion of the mechanism of killing microorganisms by ionizing radiations, there was a dearth of reliable information, although many guessed that DNA must somehow be involved as the sensitive and fragile, yet important, molecule in living organisms.

At the Kilmer International Conference in 1976 (2) a broader approach to sterilization was taken with an emphasis on ethylene oxide and steam, as well as radiation as the major techniques employed. The conference focused not only on the hardware, but also on biological aspects, such as environmental control, role of the bioburden in the sterilization process and the use of biological indicators to measure its efficacy. Consideration was given to toxicological problems related to trace harmful chemicals which might be left behind as residues in the sterilized medical products. This led to a discussion of the various procedures used around the world for insuring sterility and for keeping contaminants below toxic levels in products designed for medical use.

In this second Vienna Conference of 1977, we focus once more on recent advances in the field of *Radiation Sterilization of Medical Products.* Session I seems to take a "microbe's eye view" of sterilization in the real world which utilizes manufacturing processes. The need for environmental control and concern for the bioburden all relate to the role of the microbiologist in defining the degree of sterility assurance in the final product. Also examined are the roles of dose rate and moisture in determining the effectiveness of radiation damage. Session II gets down to basic and applied problems, and examines in detail radiation sterilization of biomedical materials, including bone, tendon, cartilage, heart valve, vaccines and antigens, blood components, transfusion devices, pharmaceuticals and cosmetics. Session III takes a global view of the sterilizaton of medical products with special reference to regulatory problems as handled internally within countries, or cooperatively by international agencies and councils. Finally, Session III attempts to look into the crystal ball and foretell the future of irradiation sterilization.

In conclusion, I should like to say that despite the tremendous progress which has been made in the field of sterilization of medical products, there is much yet to be done at the basic level, the development of new technologies and of improved methods of testing and insuring sterility of the final product. As a biochemist, I believe there is much to be learned about the mechanisms involved at the cellular and molecular levels in the killing of microorganisms. If DNA is indeed the "Achilles heel" of microorganisms, we need to know exactly what happens to it during the injury and repair process after irradiation. Our ignorance of what occurs is especially serious in the case of certain viruses which may not be destroyed by the usual methods of sterilization. In particular, the strange, very slow-growing viruses which infect the nervous system and cause fatal diseases, such as kuru and scrapie, seem especially resistant to many methods of sterilization. On the other hand, some of these slow-growing viruses may be susceptible to dry heat or hypochlorite. Now that we have entered the strange and wonderful, yet frightening, world of recombinant DNA, we can expect some surprises here as well. If it is possible, by recombinant DNA techniques, to produce bacteria resistant to antibiotics, we can rest assured that some of these recombinant organisms may turn out to be refractory to certain classical methods of

sterilization. The one thing we can be certain of is that there will be no dearth of challenging problems to be discussed at the next international conference.

Although most of us have devoted our lives to the interaction and cooperativity between molecules and living organisms, we now devote ourselves to interaction and cooperativity between scientists from different countries of the world in solving the problems of sterilization. Now let's get on with the show, which we shall all find stimulating, challenging and exciting.

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Mechanisms of Cell Death Due to Ionizing Radiation

Tikvah Alper

Gray Laboratory of the Cancer Research Campaign, Mount Vernon Hospital, Northwood, England.

Abstract: *Biological macromolecules, irradiated extracellularly, respond as 'one-hit detectors', that is, biological or biochemical function is destroyed by single energy deposition events. The predictions of classical target theory for such detectors have been confirmed by experiment. It could therefore be expected that cellular targets for the cytocidal action of radiation should respond similarly. However, two radiobiological phenomena have led to a rather general belief that multi-hit action is involved in cell killing: the manifestation of 'shoulders' in cell survival curves, and the generally observed increase in the effectiveness of radiation as ionization density increases, whereas with one-hit detectors it should decrease.*

Those phenomena may be reconciled with the evidence from irradiation of model 'targets' if the cell's capacity for repairing damage is taken into account. One model for accommodating shouldered survival curves postulates a repair mechanism that becomes less effective with increasing dose, so that sensitivity is apparently considerably less at the outset of irradiation than subsequently. Increased effectiveness, with increasing ionization density, could be expected if two potential targets, in close juxtaposition, could effect mutual repair as long as one of them retained its structural or biochemical integrity. It is proposed that those requirements are met by cellular DNA and the membrane to which it is attached. There is independent evidence also for cellular membrane as a target much more likely than DNA to be sensitized to radiation by the presence of oxygen.

It was suggested to me that, although I should deal with my theme in a general way, the members of this audience would be most interested in the topic as it relates in particular to the killing of microorganisms by radiation. This makes my task easier, because most of my own experimental research work has been with bacteriophage, bacteria and yeast. I believe it would be fair to say that most of the important radiobiological phenomena of wide generality were discovered and investigated by the use of microorganisms. Nevertheless, the radiobiology of higher cells, and particularly mammalian cells, currently gets more attention, because it is seen as more relevant to radiotherapy and protection, the two broad fields in the context of which most radiobiological research is funded.

It is a reasonable assumption, widely adopted, that the lethal effect of radiation on cells is attributable primarily to the deposition of energy quanta in critical structures, or 'targets'. The status of target is conferred on a macromolecular cell component not by its peculiar sensitivity, but rather by the crucial role it plays in cell proliferation. That basic concept is sometimes mistakenly alluded to as 'the target theory', which is described on occasion as being 'out of date' or as having been disproved. Target theory is in fact a system by which the sizes and even the shapes of targets may be calculated, and it

works very well when it is applied to subcellular entities, like viruses or enzymes. That it cannot at present be specifically applied to the conceptual targets for cell killing by radiation is due to our want of information on the operation of intracellular repair, and on the significance of the relationships between neighbouring structures in the cell — for example, between the DNA and the membrane to which it is normally attached.

The simplest application of the theory is to targets that have a high probability of being inactivated by a single energy deposition event: so-called single-hit action. The target volume is defined as that volume within which a single event of adequate magnitude will cause loss of the function under test. If the whole of the macromolecule in question is the target itself, there should be good agreement between the target molecular weight, calculated from inactivation dose, and the molecular weight of the macromolecule as estimated by independent means. However, that agreement can be expected only if the conditions of radiation are such as to give a probability near to one of inactivation by a single event: for example, the absence of oxygen, during irradiation of dry enzymes and viruses, has been found to reduce that probability, presumably because, if the damage is not promptly fixed, the radical engendered by the energy deposition can regain its lost electron.

It is well known that single-hit action should result in exponential inactivation curves, described by

$$\text{f(surviving fraction)} = e^{-\lambda D} \text{ or } e^{-D/D_0} \qquad [1]$$

where λ is the 'inactivation constant' and D_0 the 'inactivation dose', that is, the dose required to deliver, on average, one inactivating event per 'target'.

The equation may be written $\ln f = -\lambda D$ or $-D/D_0$. Methods for calculating target size from the value of D_0, for a radiation of a given quality, were revised and refined by Lea (1). Over many years my colleagues and I have exposed a variety of subcellular model targets to bombardment by fast electrons (about 6 MeV) from the Medical Research Council's Linear Accelerator at Hammersmith Hospital. All the test systems were exposed as freeze-dried preparations, with oxygen present. Using Lea's method of calculating molecular size from D_0, as in the case of single-hit action by radiation of low Linear Energy Transfer (LET), we have been able to compare the 'target molecular weights' with the best determinations by other means. Figure 1 shows rather good agreement over several powers of 10 in molecular weight.

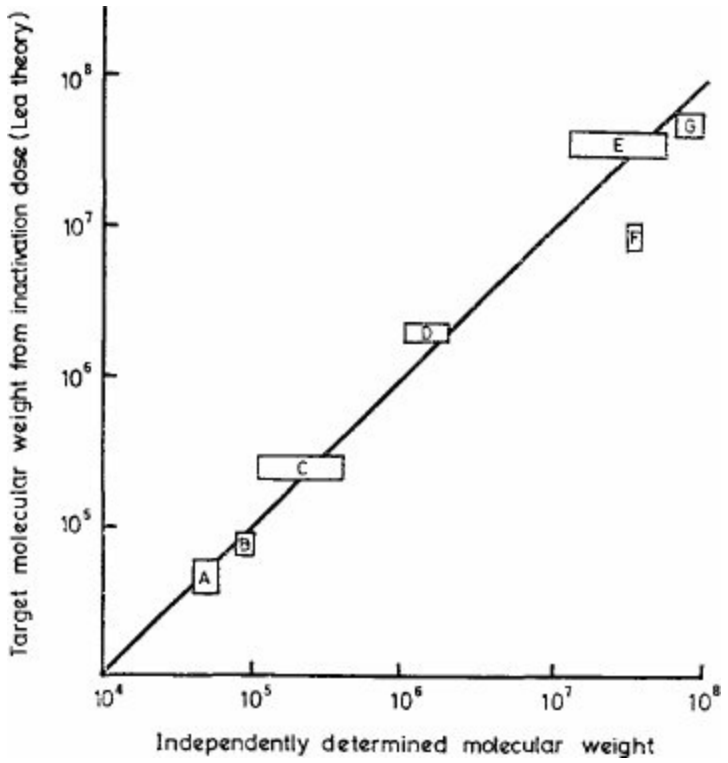

Figure *1*. Correlation between 'target' and independently determined molecular weights. Heights and lengths of rectangles give respectively 95% confidence limits for M.W's calculated from inactivation doses and 95% confidence limits, or quoted ranges, for independent determinations.
A. Target MW, combining power of Welch type A antitoxin/MW of γ-globulin Fab' fragment.
B. Target MW, urease/MW, urease sub-unit.
C. Target MW, glutamate dehydrogenase/MW, GDH sub-unit.
D. μ bacteriophage (RNA).
E. Yellow fever virus (RNA).
F. T3 bacteriophage (DNA).
G. Herpes simplex virus (DNA).

That satisfactory agreement, together with the exponential nature of the inactivation curves, provides good support for the hypothesis that inactivation of enzymes and of nucleic acid targets results from single-hit action. The theory of single-hit action predicts also that the effectiveness of radiation should decrease, as the LET (or density of ionization) increases. If one energy-deposition event is sufficient to inactivate a target, two or more such events within the same target will be no more effective, and some of the dose will therefore be 'wasted'. This prediction has been fulfilled by studies on enzymes and viruses, including double-stranded DNA bacteriophages (Figure 2). The latter point is worthy of emphasis, as, indeed, is the fact that survival curves for double-stranded DNA viruses are exponential (Figure 3), in view of the hypothesis that cell death is a consequence of a double-strand break in the DNA, and that, at low LET, an event must occur in each strand (i.e. there must be a dose-squared component) if a double-strand break is to be achieved.

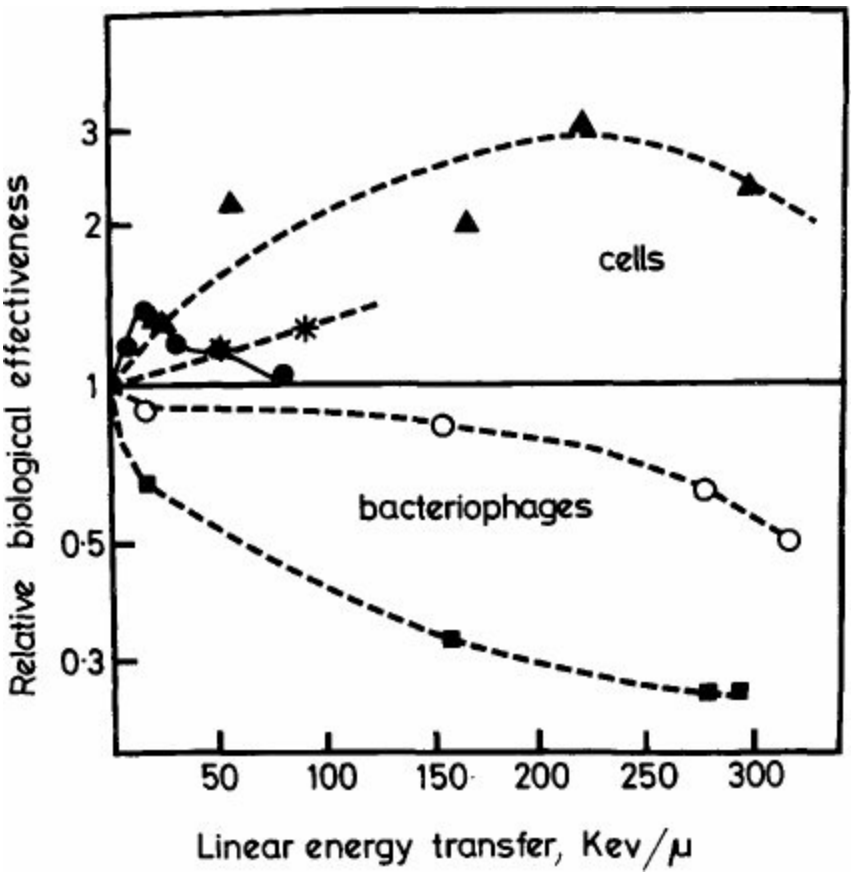

Figure *2.* Relative biological effectiveness vs. LET.

cells: ▲ human kidney cells (25). RBE values extrapolated to high dose values.

● *Shigella flexneri* (26). RBE values constant, all doses.

★ *Chlamydomonas rheinhardii* (27). RBE values extrapolated to high dose values.

bacteriophages: (RBE values constant all doses).

○ T_1 (double-stranded DNA) ■ ØX 174 (single stranded DNA) (28).

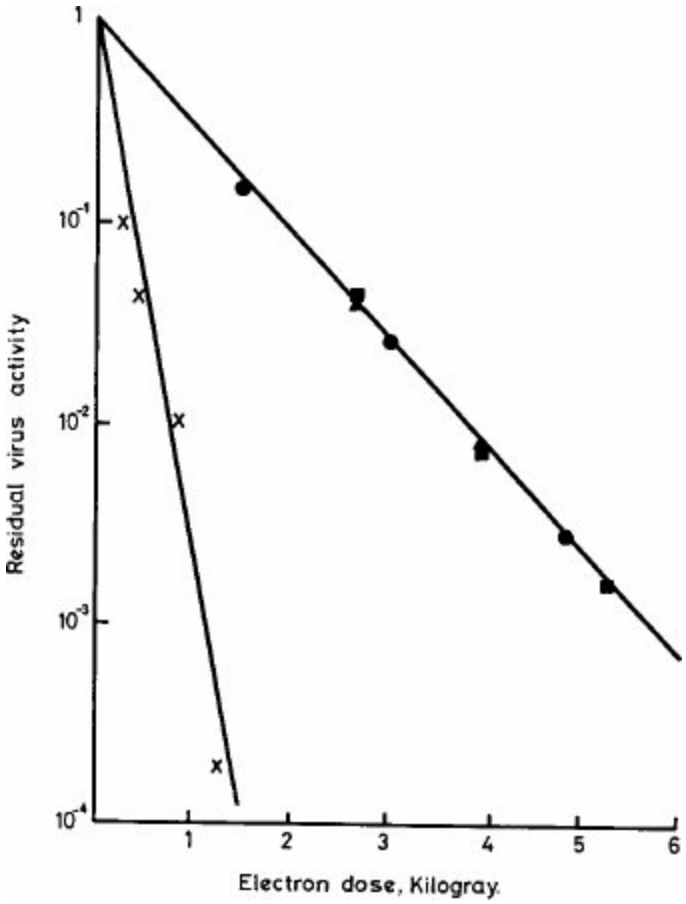

Figure *3.* Exponential inactivation curves, double-stranded DNA viruses.
(one Kilogray = 100 Kilorads)

x-Herpes simplex.

▲, ■, ●-T_3 bacteriophage. Different symbols for different host cell strains.

It seems to me plausible that the results obtained on model cellular targets should be relevant to the processes by which intracellular targets are inactivated, i.e. to the mechanisms of cell killing. Loss of biological function by nucleic acid targets evidently occurs by single-hit action. Since cellular DNA is attached to cell membranes, and the initiation of DNA synthesis is probably at the site of attachment, at any rate in bacteria, certain cell membranes (the nuclear membrane, in eukaryotic cells) would also seem likely to satisfy the criterion that their integrity is critical for cell viability. Unfortunately no system has so far been developed for investigating dose-effect relationships for damage to membranes in such a way as to test directly for single-hit action. However, the effects of neutrons and X-rays in damaging lysosomal membranes were compared by Watkins and Deacon (2), who found the radiation of higher LET to be the less effective. This is diagnostic of single-hit action, and the result would support the concept that biological detectors are no different from physical detectors in responding primarily to one-hit events (3).

It is unlikely that intracellular targets of radiation killing can involve macromolecules or structures other than enzymes, nucleic acids or membranes, and, as we have seen, the evidence supports inactivation of all of those by single-hit action, when they are irradiated extracellularly and tested for loss of biological or biochemical function. It is implausible, therefore, that inactivation of intracellular targets should require other than single-hit action: except that the operation of biochemical repair mechanisms within the cell might, in some circumstances, impose the need for the occurrence of more

than one energy deposit in the same target macromolecule if it is to be inactivated.

It might, therefore, be expected that cells should be killed by the exponential mode, and this is certainly true of many strains of bacteria and, indeed, of some lines of higher cells. Figure 4 shows an example of a reasonably exponential survival curve, through 11 decades, for *Brucella abortus.* The work was done in collaboration with M. Sterne of the Wellcome Foundation, who was interested in the possibility that immunogenic properties might be retained by bacteria that had been killed by radiation. Survival could be reduced to very low levels because I was provided with bottles containing 10^{12} each of freeze-dried organisms.

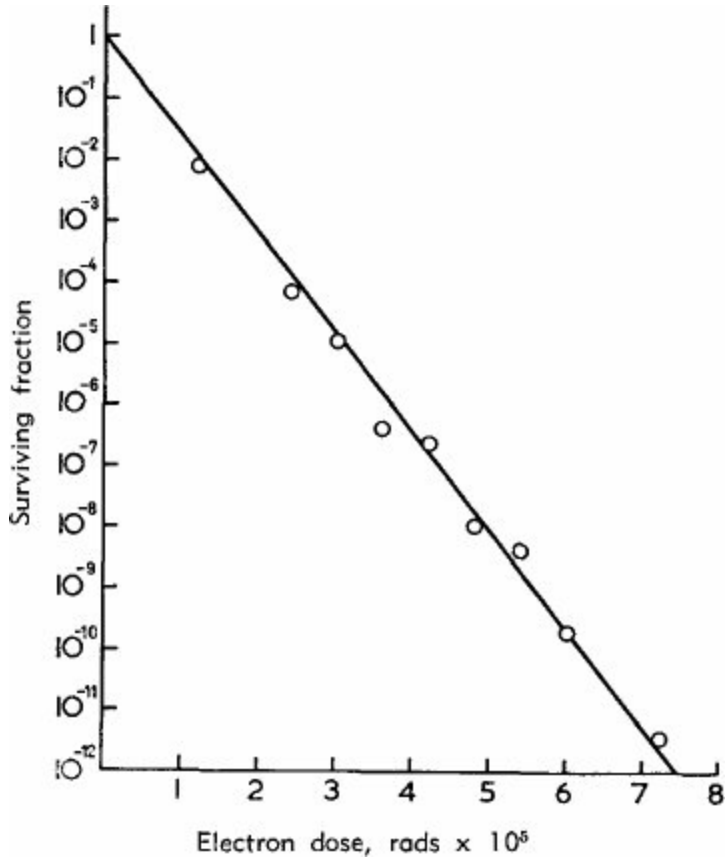

Figure *4.* Survival of *Brucella abortus,* irradiated as freeze-dried preparation.

Exponential killing of cells, represented by the algebraic expression in $f = -D/D_0$, might be thought of as demonstrating the existence of one target per cell, of such a size that the dose D_0 would give, on average, one inactivating energy deposit per target. However, various phemomena suggest the likelihood that there are several targets per cell, the inactivation of *any one of which* would result in cell death. If we write $\ln f = -\lambda D$, the inactivation constant λ may, therefore, have several components, so that it would be more accurate to write $\ln f = -D(\lambda_a + \lambda_b + \lambda_c \ldots)$ [2] Thus D_o or $1/\lambda$ of Equation [1] could in fact be the reciprocal of $(\lambda_a + \lambda_b + \lambda_c \ldots)$. That form of equation has been used for many years by Powers (4) in analysing effects of irradiation on *Bacillus* spores.

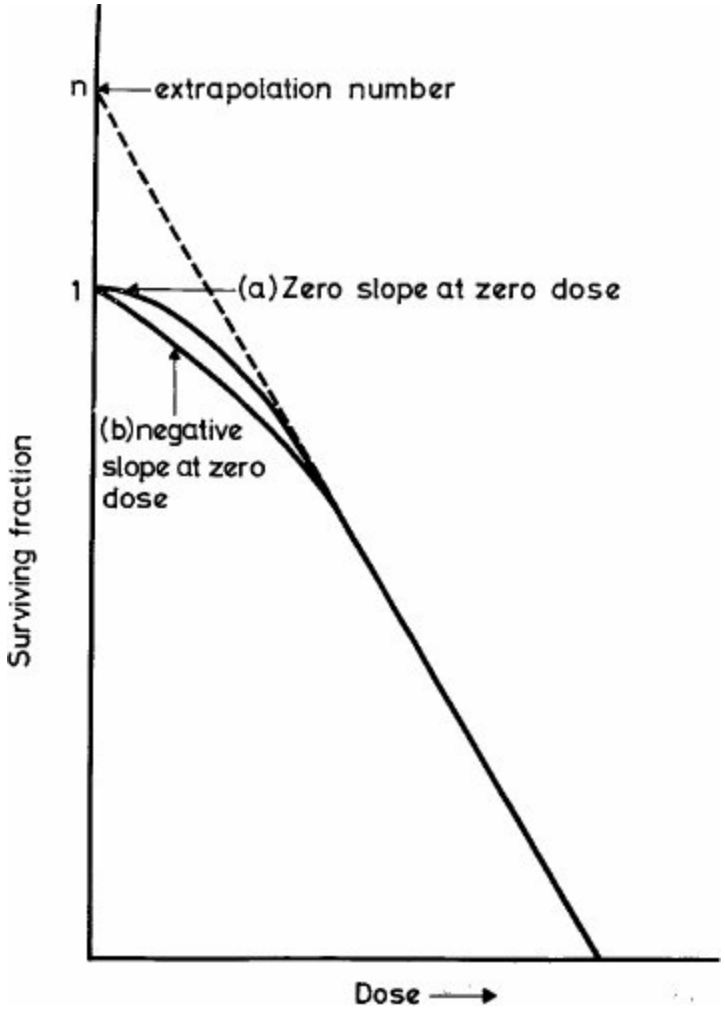

Figure 5. Hypothetical shouldered survival curves.

While there are many examples of accurately exponential survival curves, particularly for bacteria and bacterial spores, these are in the minority with eukaryotic cells, which commonly yield so-called 'shouldered' curves (Figure 5). Survival by that mode is commonly observed also with particularly resistant bacterial strains, like *Micrococcus radiodurans,* and is not uncommon with many other strains. The shape of the survival curve often depends on the stage of growth of the organisms, and on the conditions of culture (e.g. Figure 6).

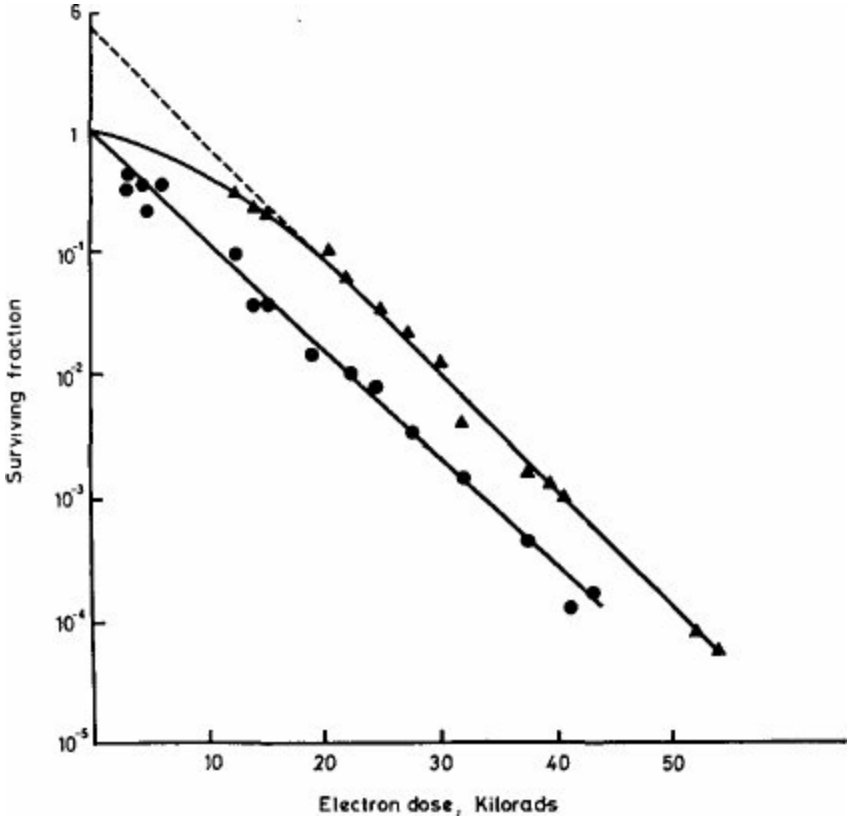

Figure *6.* Dependence of extrapolation number on culture medium before *irradiation, Shigella flexneri.*

● broth

▲ broth + added glucose.

Proponents of certain models to account for cell killing have specifically excluded bacteria as organisms to which their considerations apply (e.g. 5, 6). But the assumptions commonly made about critical targets are no different for pro- and eukaryotic cells; and most basic radiobiological phenomena, for example protection and sensitization, are common to all cells. It seems to me more profitable to test hypotheses that are generally applicable than to restrict these to certain classes of cell, at least until we know enough to take second order differences into account.

The evidence of single-hit action on putative targets for cell killing, therefore, requires to be reconciled with the observation that many survival curves have shoulders, whether the cells are pro- or eukaryotes.

One assumption that has been widely invoked is that of target multiplicity: not in the sense described previously (Equation [2]), but almost the opposite. A shouldered survival curve will be observed if the cell contains two or more like targets, *all* of which must be inactivated if the cell is to lose viability; in other words, the survival of any one target is sufficient to ensure the cell's viability. This is what would be expected, for example, if cells were multinucleate, provided each nucleus could independently divide, so that every cell with one surviving nucleus could give rise to a clone of cells. Survival would then be described by a modification of Equation [1], namely

$$f = 1 - (1 - e^{-\lambda D})^n \qquad [3]$$

At high dose this expression would approximate to

$$f = ne^{-\lambda D}$$

$$\text{or} \qquad \ln f = \ln n - \lambda D \qquad [4]$$

Thus if survival is plotted on a logarithmic scale, with dose on a linear scale, the high dose region would be a straight line of slope $-\lambda$, extrapolating to n on the zero-dose scale. With survival curves that do approximate to straight lines at high dose, n is commonly referred to as the extrapolation number: if the survival curve really does represent single-hit inactivation of multiple targets, n gives the number of targets per cell — or the arithmetic average of that number, if the irradiated population is heterogeneous in that respect (Figure 5).

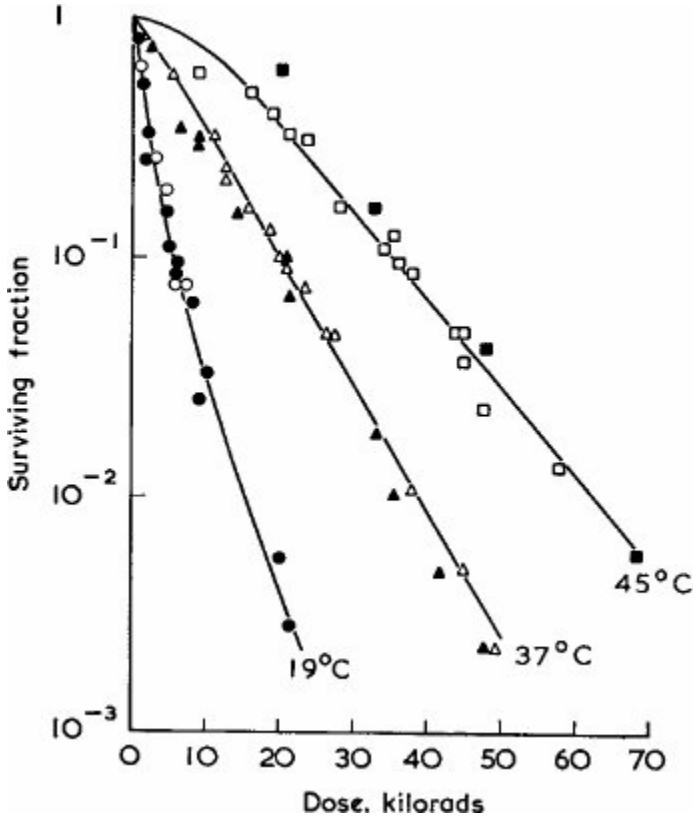

Figure *7.* Survival of *Escherichia coli,* strain B, irradiated in the absence of oxygen. Incubated for about 2 hours, immediately after irradiation and plating,

at □ 45°
△ 37°
○ 19°

Open symbols: several runs, 250 Kvp X-rays.
Filled symbols: several runs, 8 MeV electrons.

Equation [3] should result in a survival curve that has zero slope at zero dose, as shown in the hypothetical survival curves (Figure 5a). While this is sometimes observed, a negative slope at zero dose (Figure 5b) is a more frequent experimental observation, and some authors have chosen to retain the 'multiple-target' concept, but to obtain a better fit to the data by assuming that there is also a 'single-hit' component of damage the effect of which is most obvious at low doses. Such models give the approximation at low dose

$$\ln f = -\lambda_1 D \qquad [5]$$

where $\lambda_1 < \lambda_1$ and at high dose the approximation is that given by Equation [4].

It is a basic assumption of Equation 3, and its modifications, that all n targets in the cell must be inactivated, if the cell is to be killed; so the inactivation of the first (n — 1) targets involves damage that is customarily referred to as 'sub-lethal'.

While multi-target models incorporate the assumption of unit action on cellular targets, several other models have been proposed as descriptions of shouldered survival curves. They all employ the concept that there is an accumulation of sub-lethal damage before the final event that kills the cell. However, none of these models, nor that represented by Equation [3], can easily accommodate the many observations showing how easily the extrapolation number n may be modified — particularly when the modification comes from post-irradiation culture conditions. This phenomenon is particularly well illustrated by results with *Escherichia coli* strain B. Figure 7 shows how the shapes as well as the slopes of survival curves depend on post-irradiation culture conditions — in this case, different incubation temperatures for a short time after irradiation.

Striking changes in the shapes of survival curves may occur also after irradiation by ultra-violet light. There is good evidence that the lethal effects of UV in the germicidal wavelength range are attributable to photoproducts engendered in nucleic acid, which specifically absorbs light in that range. *E. coli* B/r demonstrates a survival curve with a wide shoulder, when it has been grown before irradiation and plated thereafter on nutrient medium; but if the irradiated cells are plated immediately on minimal medium, survival is exponential (Figure 8). The question that suggests itself is, how can conditions imposed *after* irradiation reduce the number of targets per cell, or the number of hits per target that would have been calculated from the survival curve with the widest shoulder?

Clearly our ideas on the mechanisms by which cells are killed must take into account the reasons for the appearance of shoulders in some survival curves. These are also of practical importance, because of the intimate relationship between the magnitude of survival curve shoulders and the effects of fractionating radiation dose, or of changing the dose rate. This is particularly relevant in radiotherapy, and in setting standards for protection against the hazards of ionizing radiation; but it could have a bearing also on the effectiveness of sterilization by radiation. The connection is through the phenomenon that I shall call Elkind recovery.

In the hypothetical survival curves of Figure 5, we note that the fraction of cells killed, per unit dose, is much less in the initial, shoulder region than at higher doses. But if the irradiation is stopped, and the irradiated population is allowed a radiation-free interval, the survival curve for the survivors of the first dose will be a repetition of the original one (Figure 9): the fractionation interval has, so to speak, 'restored the shoulder' to the survival curve. This form of 'recovery', first observed with a green alga (7), and soon afterwards with mammalian cells in tissue culture (8), has been shown to be repeatable through several fractionation intervals (9).

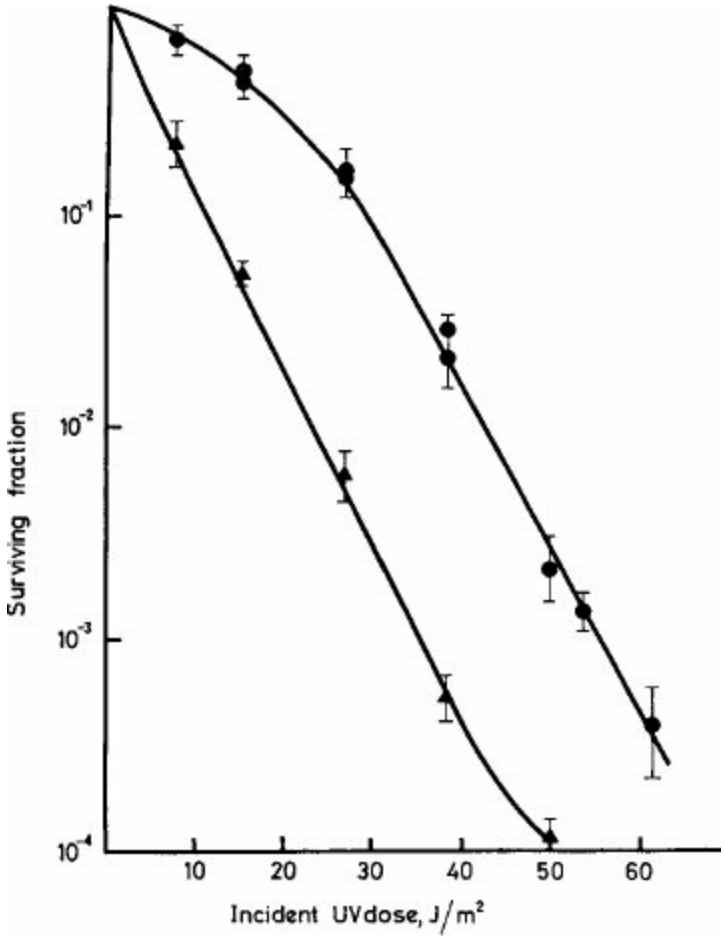

Figure *8.* Survival of *Escherichia coli* strain B/r after UV irradiation at 254 nm. Bacteria grown in nutrient broth, harvested while in logarithmic phase, plated after irradiation on

● nutrient agar

▲ salts-glucose agar.

Radiotherapists have known for a very long time that there is a considerable sparing of tissues if doses are delivered in small periodic fractions, or at low dose rates, and the known facts of Elkind recovery account very well for those observations. Radiation delivered at a low enough dose rate may well enable the maximum amount of this type of recovery to occur during the course of irradiation, so that survival becomes exponential, the slope being determined by the dose rate. If that is low enough, and if there is a non-zero slope to the survival curve, at zero dose (Figure 5b), the minimum slope at sufficiently low dose rate will be equal to the initial slope (Figure 9). This important consequence of the shape of a survival curve has been extensively studied with mammalian cells in tissue culture, and free-living eukaryotic cells like algae and yeasts, but much less with bacteria, perhaps because the time for division is so short that it is technically difficult to follow the time course of Elkind recovery; or perhaps because bacterial survival curves are so often exponential in the conditions in which many experiments are done.

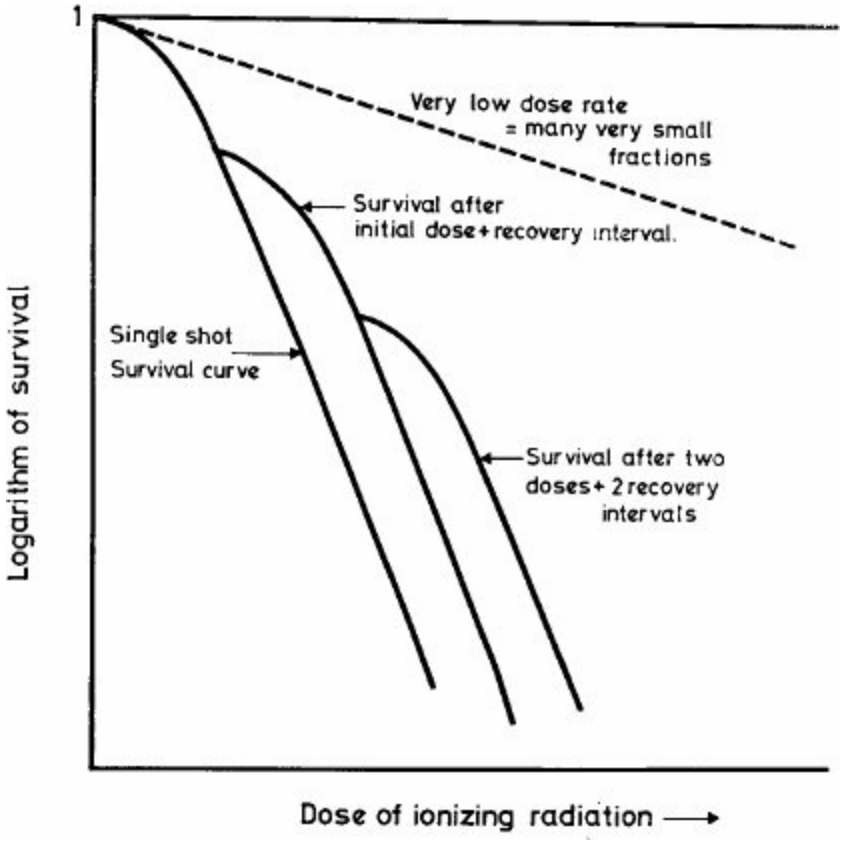

Figure *9.* Hypothetical shouldered survival curves for single and fractionated doses and very low dose rates.

The models so far mentioned for shouldered survival curves are based on the concept that cell death requires the accumulation of lesions that are not individually lethal until the last one has been inflicted: or, as they are commonly described, sub-lethal lesions. Since survivors of a dose of radiation will recover all their capacity for accepting lesions as sub-lethal, Elkind recovery is rather generally regarded as synonymous with repair of sub-lethal damage. Evidence of that repair can be achieved *only* in experiments in which doses are separated in time, or in which effects of high and low doses are compared. This point requires emphasis: it would be inconsistent to regard the shoulder as giving evidence that repair takes place, and at the same time to describe it as 'repair of sub-lethal damage'.

Although there is no support, other than common acceptance, for the attribution of survival curve shoulders to cumulative damage, comparatively little attention has been paid to a completely different explanation, which, as far as I know, was first advanced by Powers (4). He suggested that the extrapolation number, n [Equation 3] "represents a pool of chemical compounds that can reverse part of the radiation effect after it has been induced by X-irradiation…. This will continue until the source is exhausted and at that point the radiations appear to become more effective."

This model, designed to accommodate shouldered survival curves, as well as Elkind recovery (considered by Powers to be the process of replenishment of the 'pool' during a radiation-free period) was formulated algebraically by Orr, Wakerley and Stark (10) and Laurie, Orr and Foster (11). The 'repair' concept for the shoulders of survival curves was used also by Ginsberg and Jagger (12) and Haynes (13) in discussing the effects of germicidal UV on 'wild type', i.e. comparatively resistant strains of *E. coli;* but those authors invoked the operation of specific enzymic repair mechanisms, rather than the 'pool' suggested by Powers. In all the different forms of the model, the common assumptions are a) that a proportion of the incipient lesions initially engendered by the radiation are repaired and b) that

the effectiveness of the repair gradually decreases as dose (or the total number of incipient lesions per cell) increases, until there is effectively no repair, and the inactivation constant corresponds with that for unrepaired lesions. The general principles of repair models are illustrated in Figure 10.

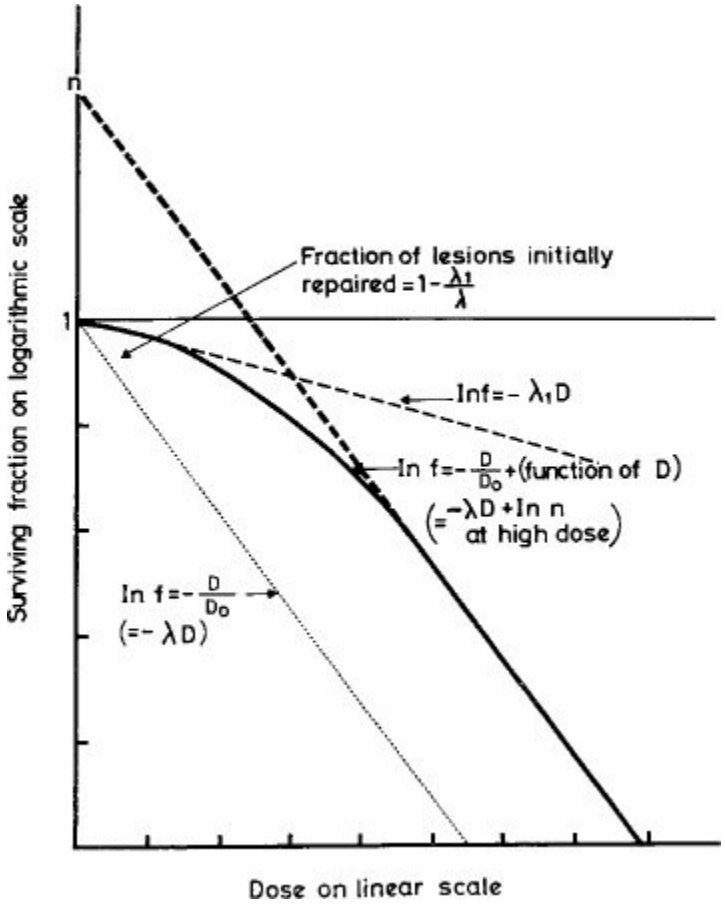

Figure *10.* Features of 'repair model' for shouldered survival curves.

Provided a shouldered survival has a non-zero initial slope, it is characterized by that slope, the final slope and the extrapolation number: exactly the same parameters as those of Equation [5]. Thus the curve may be algebraically expressed as

$$\ln f = \lambda D + (\text{a function of } D) \qquad [6$$

provided that the function approximates to ln n, at high dose, and to $(\lambda - \lambda_1)$ D at low dose, i.e. the low and high dose approximations must be respectively

$$\ln f = -\lambda D + \lambda(\lambda - \lambda_1)D = -\lambda_1 D, \text{ like equation } [5]$$

and

$$\ln f = -\lambda D + \ln n$$

just like the approximation for Equation [3] whether or not this is modified to allow for a non-zero initial slope. Those requirements are met in the various algebraic forms of the repair model that have been proposed.

Since repair models in their simplest form invoke only single-hit killing processes, they are more compatible than multi-sublethal lesion models with the evidence that the likely targets for cell killing behave like 'one-hit detectors'. Furthermore, several well-established radiobiological phenomena, mostly observed with mammalian cells, are more plausibly accommodated in the conceptual framework of repair models. Therefore, although direct supporting evidence is wanting, I propose to proceed on

the assumption that, in general, shoulders in survival curves are accounted for by a repair process that becomes less effective with increasing dose. Clearly there are some organisms that are genuinely multinucleate, and multitarget inactivation may then be combined with repair of the type described. But we know so little about the mechanisms of radiation damage that we are justified in confining our attention initially to less complicated modes of inactivation.

According to the simple model illustrated in Figure 10, the final exponential region of the shouldered survival curve is parallel with the exponential that would be observed if the postulated repair mechanism were not operating. A specific case was exemplified by Figure 8. But we have already seen (Figure 7) that post-irradiation treatments can change that slope as well as the extrapolation number of the survival curve. In some cases there is accurate 'dose-modifying' repair: final slopes are decreased, but there is no change in extrapolation number, as in 'liquid holding recovery' in yeast (14). Photoreactivation is similarly dose-modifying. There is, therefore, need to postulate also repair mechanisms that continue to operate effectively throughout the dose range used.

As we have seen, the type of repair mechanism first proposed by Powers (4) allows us to reconcile the 'one-hit detector' nature of biological targets with the manifestation of shoulders in survival curves, and affords also a more acceptable framework than multi-hit or multi-target models in which to account for the great variability in extrapolation number with conditions of culture before or after irradiation. However, multi-hit models, in particular, seem to offer a better means of accounting for the important generalization that the effectiveness of radiation in killing cells increases, as the LET (or density of ionization) increases, whereas the reverse must be true of 'one-hit detectors' and is indeed true of model targets irradiated extracellularly (Figure 2).

This apparent failure of the single-hit action hypothesis can, however, also be explained away by invoking repair mechanisms. Let us consider, first, the probable nature of cellular targets. On the face of it, *'the'* target for cell killing is obvious. Cells cannot be expected to be viable if the genome is damaged, which may seem to justify the frequent assumption that there is no need to consider anything other than cellular DNA as the main target within which an unrepaired incipient lesion will be fatal. But, as yet, little account has been taken of the fact that the DNA is not a free floating isolated entity within the cell (or the cell nucleus, in eukaryotes). It is very firmly attached to a membrane, at which point, indeed, DNA synthesis is initiated, at least in bacteria, so it would be surprising if energy deposited in those crucial membranes did not contribute to the lethal effect of radiation.

As it happens, there was, it seemed to me, distinct inferential evidence of an important target, chemically different from DNA, many years before those functions of cell membranes were described. One pointer was the observation that subcellular nucleic acid entities, like bacteriophage or Transforming Principle, failed to be sensitized to radiation by oxygen, when they were in suspension (15, 16). Indeed, oxygen was found to act as a radio-protector of phage in dilute suspension (17). Radiation sensitization of cells by oxygen is, however, a very general phenomenon. Furthermore, with some strains of bacteria the extent of sensitization of cells by oxygen present *during* irradiation depended on the cells' capacity for repair damage to DNA, even when this was changed by *post*-irradiation conditions. The greater the extent of repair, the less important is the contribution of that damage to overall killing, and the higher the oxygen enhancement ratio (18–20). Thus sensitization by oxygen was inferred to act mainly through its effect on the non-DNA target.

There are valid chemical reasons for expecting a lipid-containing structure to experience greatly enhanced damage if oxygen is present during irradiation; an additional pointer towards membranes as

the postulated 'non-DNA' target.

It has now been shown experimentally that damage to model membrane systems is much more highly sensitized by oxygen than is ever the case for cell killing (21, 22). This is to be expected, if oxygen enhancement ratios for the latter are some kind of average of the intrinsic ratios for all the targets, and if the intrinsic sensitizing effect of oxygen on DNA is small, or indeed non-existent. Figure 11 represents a table showing ranges of dose ratios required to give the same biological or biochemical effects on various wet systems irradiated without and with oxygen present. The ratios are lowest when damage to nucleic acid alone is responsible for the effect; in fact, RNA preparations, as well as bacteriophage in dilute suspension, are protected by the presence of oxygen (23, 24). The highest dose ratios in Figure 11 refer to systems in which the effects are directly attributable to energy deposition in membranes.

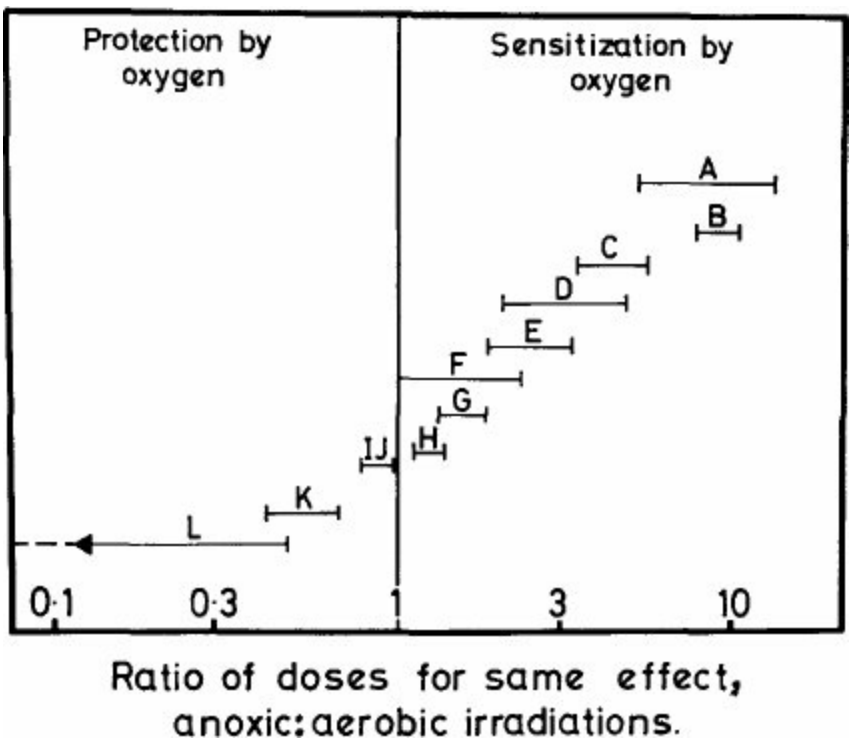

Figure *11*. Ranges of oxygen enhancement or protection ratios, various systems irradiated in suspension, then tested for loss of biochemical or biological function.

A. Lysosomal enzyme release.
B. Inhibition of DNA synthesis by DNA membrane complexes.
C. Inhibition of enzyme induction in bacteria when a permease is required for transport of inducer.
D. Killing, repair-proficient bacteria.
E. Killing, mammalian cells.
F. Killing, repair deficient bacteria.
G. Bacteriophage irradiated in the presence of a high concentration of -SH compound.
H. Bacteriophage irradiated within host cell, immediately after injection of DNA.
I. Transforming DNA irradiated in broth.
J. Bacteriophage irradiated in broth.
K. RNA, various biological activities.
L. Bacteriophage, dilute suspension in buffer or with various added solutes.

If it is correct to regard those membranes to which DNA is attached, as well as the DNA itself, as

critical targets for cell killing, it is reasonable to speculate that both structures are capable of mutual repair. A primary lesion in DNA might well be capable of repair as long as the element of membrane to which it is attached retains its integrity; and, conversely, a lesion in the membrane might not affect the proliferative capacity of the cell if the associated DNA has not experienced an energy deposit. But, as ionization density increases, the probability also increases that an event will occur simultaneously in each element, with fatal results to the cell, so the relative biological effectiveness (RBE) will also increase. The hypothesis that mutual repair capacity between membrane and DNA can account for increasing RBE gains some support from observations on various strains of bacteria, including some known to be deficient in capacity to repair damage to the DNA (Table I). While the effectiveness of neutrons is greater than that of photons, in killing repair-proficient bacteria, the sensitive, repair-deficient strains show little or no increase in RBE, as well as considerably reduced oxygen enhancement ratios.

Table I. — Correlation between pair proficiency, oxygen enhancement ratio and RBE, neutrons/X-rays

Bacterial strain	Proficiency in repair of damaged DNA	o.e.r.* X-rays	Effectiveness of neutrons relative to X-rays
E. coli B strains			
B-H	Proficient	3.4	1.4
B/r	Proficient	3.2	1.4
B:			
Condition a.	Proficient	3.2	1.3
b.	Reduced	2.5	1.2
B_{s-1}	Much reduced	1.8	1.1
B_{s-12}	None	1.0	1.0
E. coli K_{12} strains			
AB1157	Proficient	3.5	1.5
AB2463	Much reduced	2.1	1.1

*Oxygen enhancement ratio

Clearly the interpretation of the cytocidal action of radiation depends on much more knowledge of repair mechanisms than is currently available. Effectively all the research in this field has been directed towards repair of DNA, and much more is known about the repair of lesions inflicted by germicidal UV than by ionizing radiation. The former exerts its lethal effects on cells mainly by inducing dimerization of adjacent pyrimidine bases, and two well documented enzymic system are known that can eliminate the dimers. One is the photoreactivating enzyme, which monomerizes dimers when activated by light; the other is the well-known system for ‘excision repair’. The extent of repair correlates well with observations on cell killing or survival. But very much less is known either about

the nature of the critical lesions induced in DNA by ionizing radiation, or about their repair. DNA strand breaks are induced, and their repair has been intensively studied, but sound evidence is wanting for associating strand breaks with cell killing. As regards membrane damage and its repair, this is effectively an untouched topic. We must hope that, by the time the next of this series of conferences is convened, considerably more insight will have been gained into mechanisms of damage by ionizing radiation both of cell membranes and of DNA, and of their repair.

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FIRST SESSION

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Environmental Control and Bioburden in Manufacturing Processes

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Abstract: *The practice of effective environmental control in the manufacture of sterile medical devices should reduce radiation sterilization costs and result in better estimates of the probability of sterility.*

Contamination control for plants molding and assembling devices consists of two key elements for a reasonably clean environment. First, components and completed devices should be protected from exposure to plant air for extended preiods. Molding, assembly and packaging should be completed expeditiously and if storage is necessary in the process, closed containers should be used. Second, manual manufacturing processes which result in product or component handling are an excellent source of bioburden on devices. A maximum degree of automation is a key element to ultra clean products. Methods are described for calculation of radiation doses to achieve product sterility, as well as to yield a defined probability of contamination. These approaches use an estimate of the product bioburden or are based on the qualitative dose required to sterilize the natural contamination, combined with biological indicators for estimation of maximum probability of contamination.

Introduction

Environmental control in the manufacture of sterile medical devices directly affects the safety of the user, as well as production costs. Environmental control has a material affect on the numbers of microorganisms on the products, and therefore, influences the sterilization treatment required to result in a safe, effective product. In my viewpoint, minimization of presterilization counts permits safe reduction of radiation dose. It is the purpose of this discussion to illustrate several important points of control to reduce presterilization counts on devices, and then to show the use of bioburden data in establishing minimum sterilization conditions. It might be noted that minimum exposures to radiation to achieve a safe device is consistent with current trends for minimizing contamination from environmental factors. While irradiation is not known to leave harmful by-products, the current trend is to obtain added assurance of purity by reducing exposures to a minimum.

Microbiologic Response to Radiation

Before discussing contamination control and the approach to use of bioburden data for setting radiation doses, it would be of value to review briefly some principles of radiation sterilization. These

help to establish microbiological criteria for manufacturing controls.

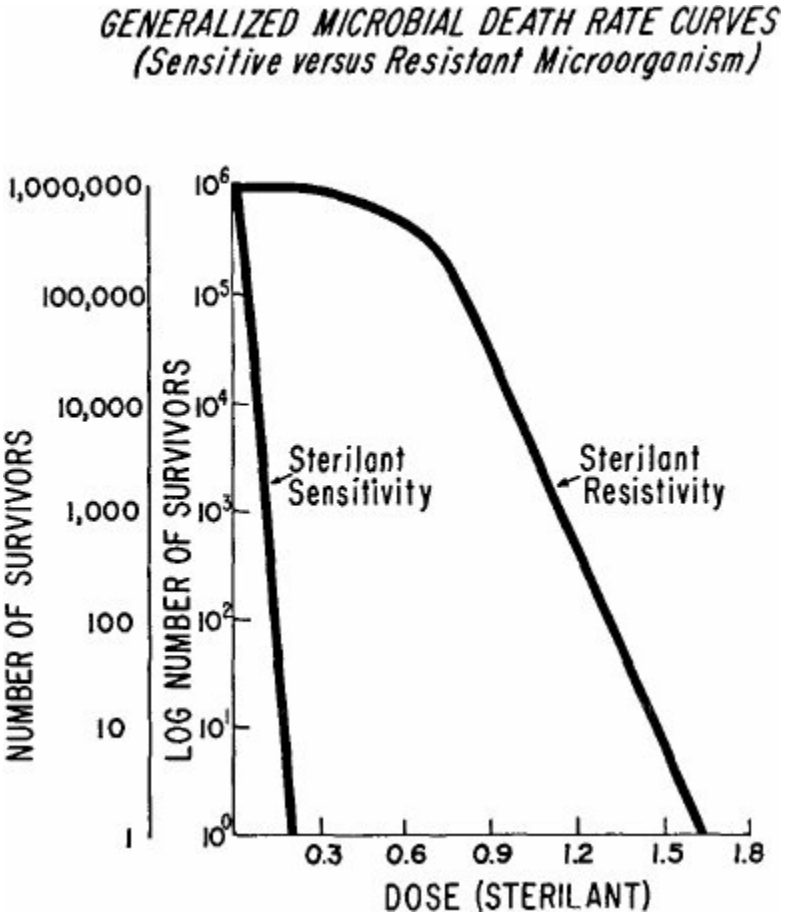

Figure *1.* Generalized microbial death rate curves for sensitive and resistant microorganisms.

When exposed to a lethal environment, two typical dose-response curves of microorganisms can be demonstrated (Figure 1). Both can show exponential kill, but the one that reflects the response of most organisms shows sensitivity to the deleterious environment, as in the first curve. Some organisms are more resistant to the mode of sterilization used and respond as in the second curve. Both of these survivor curves are seen with microorganisms exposed to radiation. Naturally, we are more concerned with sterilant-resistant organisms than with the sterilant-sensitive organism. However, we are also concerned with the total microbial load. The import of these points is that although the microbial flora on a product is mixed, and there will be a mix of survival curves from those organisms exposed to a mode of sterilization, tight control on contamination of a product during manufacturing will limit numbers and somewhat limit the range of sterilant-resistance exhibited by the flora.

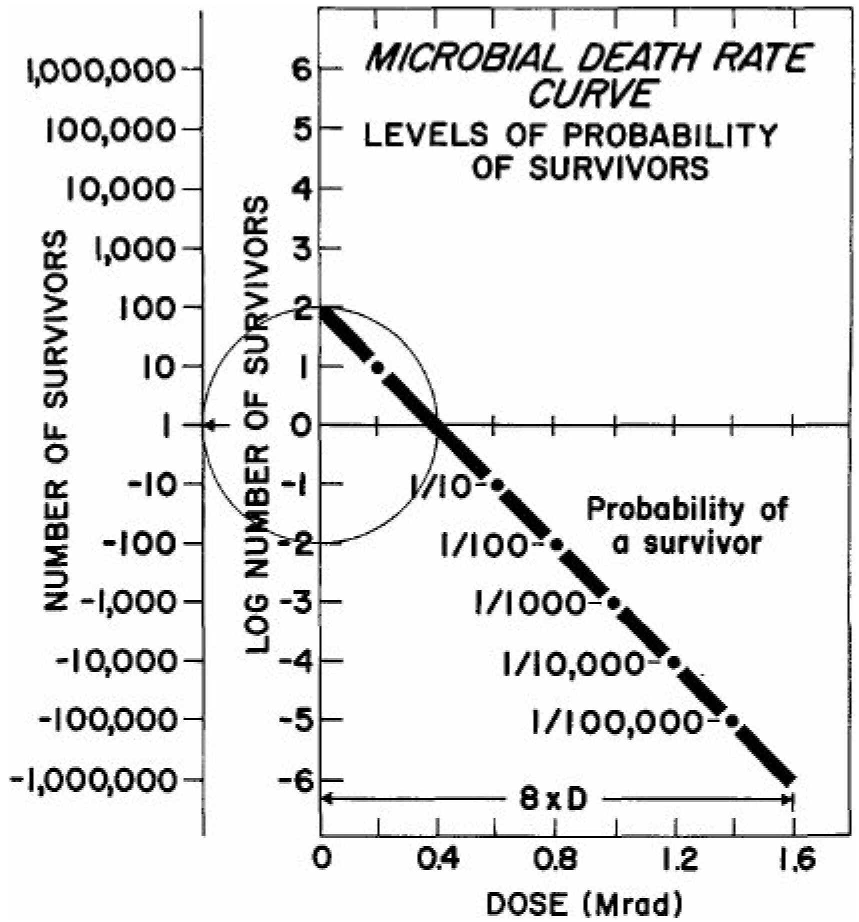

Figure *2.* Microbial death rate curve showing levels of probability of survivors.

Radiation-resistance can be associated with the D_{10}-value, i.e. the dose of radiation required to reduce a microbial population by 90%. It will be noted in Figure 2 that a device with a microbial load of 100 organisms will have a count of 10 after exposure to one D_{10} unit. Only one live organism will theoretically be present after exposure to two D_{10} units. Exposures to additional D_{10} units will provide assurance that the probability of contamination is reduced as shown. Thus, the commonly stated goal for sterilized medical devices of less than one chance in one million of a contaminant is achieved by exposure to six D_{10} units of radiation beyond the calculated sterilization point.

In the food processing industry, where a challenge population of biological indicator organisms is added to the normal population, one finds the 12D concept. In that industry, the level of sterility to be achieved by ionizing radiation was required to provide a minimum sterilization effect equivalent to the accepted 12D thermal sterilization process used by canners. However, the 12D concept is based on the assumption that the food item could have 1×10^{12} organisms per gram and that all of the organisms have maximal resistance to radiation. In hospital sterilization, an 8D concept is sometimes proposed for sterilization of medical products, even though one rarely encounters such a high count (1×10^{8} organisms), and such a high count would normally be evidence of inadequate, improper, ineffective cleaning procedures.

The sterilization requirements, based on these uses of the D-value, are intended to insure that not one single organism from the inflated and unrealistic contamination levels will survive. However, there are no provisions made for the effects of the mode of sterilization upon the product, or for control over bioload, or for resistance to sterilization by the bioload. The approach implies that personnel will only

have the minimal training required to push a button, and that by using a high dose of sterilant, one can dispense with care and concern for the product and for the user of the product. It can be an expensive approach. An alternate technique is possible under tightly controlled and monitored production systems wherein the presterilization level of bioload rarely exceeds 100 organisms/product. The concept of basing sterilization doses on a level of probability of a survivor can make economic and microbiological sense. Again using D-values, but relating D-value to the resistance to sterilization of the specific bioload in question, one can provide an adequate margin of safety in radiation sterilization of medical products. The positive benefits are to minimize product degradation caused by high levels of radiation, lower cost of sterilization, and to gain knowledge of the adequacy and efficacy of the sterilization process.

To insure sterilization of product with minimum affect on the material, a primary consideration for an effective radiation sterilization process should be a low presterilization count, that is, the number of organisms on the article to be sterilized should be minimized. If the presterilization counts are sufficiently low, i.e. not greater than 100 organisms per unit, a dose of 2.5 megarads (Mrad) should be *more* than adequate; it should be excessive.

The criteria for adequate and effective Cobalt-60 (Co-60) sterilization of medical products can be a) to keep the presterilization counts to a minimum, b) to monitor that they are indeed at a minimum, c) to test the efficacy of the Co-60 radiation sterilization process, using the radiation resistance of organisms commonly encountered on the package and device, d) to monitor this radiation resistance periodically, and e) to avoid post-radiation contamination during storage and handling of the sterilized product.

Contamination Control

We have extensively investigated presterilization microbial load on products to be sterilized. This is motivated by our responsibility for product safety and also by the concept that sterilization processes, and therefore, manufacturing costs, can be affected by the contaminant counts on products. Our studies indicate that counts on products resulting from the manufacturing environment can be controlled, and can be altered. The results of only a few key studies are summarized here to illustrate the effects of certain practices on the manufacturing floor on product contamination levels.

One of the most effective ways to cause large microbial numbers on products is by extended exposure to plant air during the manufacturing process. In one study, a plant producing sterile disposable syringes was utilized to evaluate the effects of storing product parts before assembly and packaging. For this test, an exaggerated exposure to the environment of over five weeks was employed. For controls, syringes were assayed that had been produced and immediately assembled and packaged by the normal production processes, without abnormal exposure to the plant environment. Normal manufacturing consists of a totally automated process where component exposure to room air is minimized and where personnel do not handle product except for final boxing after packaging.

Table I. — Effect of open storage on viable counts of unsterilized syringes

	NORMAL	STORED
Average Count/Syringe (CFU*)	0.8	167 (est.)

Maximum Count/Syringe (CFU)	10	TNTC
Number of Syringes with No Detectable Counts	60%	0%

* Colony forming points

Contamination data are shown in Table I. The assays of syringes which had not been stored, but were removed from the production line and immediately tested, indicated a maximum count on any syringe plus the inner side of its packaging of 10 organisms. The average count per sample was 0.8. Further, on 60% of the syringes, no organisms were found. This is a normal observation for the devices made in the plant. In fact, other presterilization assays have indicated, on occasion, up to 90% of the units to be sterile *before* sterilization.

In the test group of syringes, purposely exposed for five weeks to the plant environment, it was found that 46% of the product assayed had more than 300 organisms per product. In other words, the assay plates were too numerous to count (TNTC). If a figure of 300 is used for TNTC plates, then the average count found was 167 organisms per product, or 200 times higher than on syringes assembled and packaged automatically in a timely manner. None of the devices were free of organisms.

Numerous other studies of presterilization load have been completed in the plant in question. Similar results for non-stored components were obtained. For example, in one of these, lots of syringes with their packaging from the normal automated lines were assayed over a period of 30 weeks. The results obtained are summarized in Table II.

Table II. — Presterilization counts of syringes collected over a 30 week period

Average organisms/Syringe	0.89
Maximum organisms/Syringe	46
0 — 10 organisms/Syringe	98.5 of those tested
11 — 46 organisms/Syringe	1.5% of those tested

The average count was 0.89 organisms per packaged syringe. The maximum count observed on any syringe throughout the test period was 46, and only 0.1% of the units tested had this count. The majority of syringes, 98.5%, had counts of 0 to 10 organisms. About 1.5% had viable counts of 11 to 46 organisms.

The excellent state of syringes from the normal production, from a microbial contamination standpoint, can be ascribed in our view to two factors. First, as indicated above, syringes and their components are not held in storage during production and exposed to fall-out from ambient air for significant periods of time. Parts are covered during any delay in the molding, assembly, and packaging processes. It should be noted that the plant air supplies in this case consist of forced air with dust filters only. There is no exotic air treatment with ultra high efficiency filters. It is probable that the manufacturing activities result in more airborne dust than is introduced in the air supplies because of the clean external environment

The second factor which results in low bioburden levels is minimization of human contact. High sporadic contamination levels on products can be expected by handling of parts by workers. It has consistently been our experience that the less handling by humans the better. Automation of

manufacturing processes results in lower contamination levels on the average, but also eliminates the extreme peak counts one sees with manual processes. Such variability is illustrated in Table III which shows a series of counts on devices packaged by manual and machine modes. The remarkable difference in counts and count variability is apparent.

Table III. — Counts of aerobes on syringes as function of manufacturing period

Syringe	Total aerobic count for syringe	
	Hand Packaging (1973)	Automatic Packaging (1975)
1	8	0
2	5	1
3	184	1
4	2	0
5	1	5
6	1	1
7	1	3
8	2	0
9	15	0
10	8	0
11	8	0
12	1	0
13	1	1
14	6	0
15	TNTC	0
16	1	0
17	3	1
18	0	2
19	0	0
20	0	0

Effect of Presterilization Load on Sterilization by Irradiation

There is a practical relationship between presterilization count levels and the radiation dosage required to sterilize. It was the object of our studies to demonstrate the nature of that relationship.

We have measured the relative resistance to irradiation of organisms on syringes which have been contaminated in normal, automated manufacturing (low counts) to those receiving extended exposure to the environment over a five week period (high counts). Table IV illustrates the numbers of syringes failing sterility tests following exposure to a graded series of irradiation doses.

Table IV. — Response after exposure of normal syringes and stored syringes to various doses of gamma radiation

Dose (Mrad)	Number Positive/Number Negative	
	Normal Syringes	Stored Syringes
0.01	10/10	10/10
0.05	1/10	10/10
0.10	0/10	10/10
0.20	0/10	10/10
0.40	0/10	6/10
0.80	0/10	0/10

Note that with the normal syringes, a dose increase of about 0.04 Mrads decreased the syringe positives by about 90% On the other hand, stored syringes, which had at least forty times more organisms, required over forty times the dosage to reduce syringe positives by 90%. As we would expect, the higher the contamination level, the higher the radiation dose required to achieve the desired level of sterility assurance.

Table V. — Effect of radiation dosage on hemodetoxification coils bearing natural and inoculated contaminants

	Dose (Mrad)	Product Response No. Positive/No. Tested
Trial 1	0.17	1/5(A)
	0.34	0/5
	0.51	0/5
	0.68	0/5
	0.85	0/5
Trial 2	0.13	2/2(B)
	0.21	2/2
	0.32	2/2
	0.51	2/2
	0.63	0/2
Trial 3	0.11	5/5(C)
	0.24	5/5
	0.42	5/5
	0.60	0/5

(A)Product with normal bioload. (< 100 CFU/device)

(B)Product inoculated with 5.0×10^5 spores *B. stearothermophilus* and 5.0×10^5 cells of *Micrococcus* sp.

(C)Product inoculated with 5.0×10^5 spores *B. pumilus* E601 and 5.0×10^5 cells *Pseudomonas* sp.

Table VI. — Effect of radiation dosage on syringes bearing normal and abnormal contamination levels

	Dose (Mrad)	Contamination	
		Normal (A)	Abnormal (B)
		No. Positive/No. Tested	
Trial 1	0.01	5/10	10/10
	0.05	1/10	10/10
	0.10	0/10	10/10
	0.20	0/10	10/10
	0.40	0/10	6/10
	0.80	0/10	0/10
Trial 2	0.01	8/20	20/20
	0.02	2/20	20/20
	0.10	0/20	20/20
	0.20	0/20	19/20
	0.40	0/20	8/20
	0.80	0/20	0/20

(A) Average aerobic count 1.86 CFU/device; <57% of devices with counts.
(B) Average aerobic count 65.2 CFU/device; 100% of devices with counts.

Further examples of the effects of bioburden on necessary sterilization dose are given in Tables V, VI, and VII. A range of product types were exposed to graded doses of radiation. These included hemodetoxification coils, syringes, and needles. For each type, normal production items were used which were manufactured under normal, clean conditions. Additionally, devices were exposed which had been purposely contaminated (coils) or which had been exposed to plant air for excessive periods of time (syringes) or had been collected from floor spillage (needles). In the latter cases, the indicator of greater bioburdens was an increase in the precentage of items with microbial counts. Our experience indicates that as counts per item increase, the percentage of exposed devices showing some counts also increases.

Table VII. — Effect of radiation dosage on syringe needles bearing normal and abnormal contamination levels

	Dose (Mrad)	Contamination	
		Normal (A)	Abnormal (B)
		No. Positive/No. Tested	
Trial 1	0.01	2/20	20/20

	0.05	0/20	20/20
	0.10	0/20	17/20
	0.20	0/20	8/20
	0.40	0/20	3/20
	0.80	0/20	0/20
Trial 2	0.01	4/20	20/20
	0.05	4/20	19/20
	0.10	0/20	20/20
	0.20	0/20	14/20
	0.40	0/20	8/20
	0.80	0/20	0/20

(A) Average aerobic count 0.03 CFU/device; 49% of devices with counts.
(B) Average aerobic count 4.86 CFU/device; 99% of devices with counts.

It will be noted that contamination level does affect the minimum dosage required to sterilize. For exaggerated levels, up to fifteen times more radiation is required. Also, note that all devices of all three types were sterilized at 0.8 megarads. Obviously, our worst case would have been sterilized at the normal dose of 2.5 megarads. However, our probability of contamination among sterilized products is far less with normal production from well controlled environments. For example, with needles, 0.1 Mrad sterilizes normal production, while 0.8 Mrad is required with exaggerated contamination.

The problem imposed by products which have high, sporadic counts including observations of TNTC counts is, of course, one of uncertainty regarding the real contamination patterns. This uncertainty results in the imposition of severe sterilization cycles, as indicated earlier. Such cycles are designed to handle virtually any degree of microbial load that can result from the manufacturing practices. Unfortunately, probably the vast majority of devices are over-exposed. Worse still, the margin of safety is not known. On the other hand, with a low level of contamination which is measurable, we have a real opportunity for basing our sterilization cycles on product data. Furthermore, there is a valid basis for stating the level of probability of contamination occurring among sterilized products. We feel confident that control over presterilization microbial load and the knowledge of that load are attainable goals which permit basing a sterilization cycle on product counts.

Radiation Cycles for Low Contaminant Levels

The final issue to be discussed is concerned with the advantages that could accrue to sterilization of devices produced under clean and controlled environmental conditions. The consistently low levels of counts on these items provide a means to use low radiation doses and, at the same time, result in an acceptable and measurable probability of contamination.

Two approaches can be employed to determine the appropriate radiation dose. One utilizes the computed D_{10}-value for products exposed to graded doses (see discussion of sub-process dosing in later paper of these proceedings). This is of value only when contamination levels are sufficiently high to

yield partial responses to graded doses. An example is shown in Table VII for abnormally contaminated needles. Using the Stumbo method (1), a D_{10}-value is computed. Counts on products are then used to calculate the number of D_{10} units required to achieve sterility and then to further provide the desired probability of sterilization as illustrated in Figure 2 and as discussed earlier. A simple example of this process follows:

Co-60 Dose (Mrad)	Device Sterility Tests No. Positive/No. Tested
0.01	10/10
0.05	5/10
0.10	1/10
0.20	0/10

D-value = 0.09 Mrad
Count per product = 100 CFU (colony forming units)
Sterilizing dose = 3D = 0.27 Mrad
Added Assurance (1×10^6) = 6D = 0.54 Mrad
Total radiation dose = 0.81 Mrad

It should be apparent that this approach requires a high degree of confidence in the levels of contaminants on products and on their relative resistance to irradiation. In establishing the sterilization cycles, it is necessary to make repeated observations on product lots over extended periods of time. It requires statistical techniques to define counts, means, and limits for bioburdens and for their resistances (2, 3, 4). It further requires a continuing monitoring program of product counts and resistances on an intermittent basis to assure that the process remains in control. Most of all, a program of this nature requires a consistent contamination control program in manufacturing. However, we believe that these controls eliminate the need for sterility release testing where radiation dosage measurements are routinely made.

The second method of determining a radiation dose for a particular product is simpler insofar as the determination itself is concerned. Much less background data on products is required. However, the resulting total radiation dosage will be generally higher, and a monitoring program on products to verify resistance is recommended.

In this case, the bioburden on devices before sterilization is not characterized. However, it is necessary to determine the radiation dose required to sterilize the products in the manner illustrated in Table VII. Referring to the column titled "Normal Contamination Patterns", it is clear that the contaminant resistance to radiation is so low as to make an estimation of product D_{10}-value impossible. Even at the lowest practical dose, 0.01 Mrads, few positives were obtained in the microbiological assays of products and lower partial responses were not obtained at higher doses. In an attempt to get summary data from these types of responses, we have examined procedures to linearize the plot of the response of contaminated product to increment-doses of Co-60 radiation through the use of a variety of transformations and analyses of data. Transformations have contributed little to better definition or responses of contaminants to graded doses. It is probable that these manipulations are beyond the content of the data and, in fact, are not necessary in light of the variation in response we can accept. The use of transformations with heavily contaminated products where more partial dose-responses are

obtained do, we believe, contribute materially to D_{10}-value estimations.

Returning again to Table VII, the next step is simply to record that radiation dose which consistently yields sterility of all products. This same dose also becomes a process control value for an intermittent test program. If, from product sterility tests following graded dose exposures, there is evidence of increasing resistance, the manufacturing process must come into scrutiny.

With the sterilization dose identified, a biological indicator is selected which has a D_{10}-value in excess of that number. For example, in Table VII, 0.10 Mrad sterilizes all needle groups. The use of *Bacillus pumilus* at a count of 1×10^5 spores per carrier (D_{10} = 0.20 Mrad) as a cycle determinant would be appropriate. Exposure of the lot to 1.2 Mrad will normally yield a sterile indicator and assure a sterile product which has a chance of less than one in one million of a contaminant. Superior control in this model would be obtained by a series of indicators at graded count levels. (5) Then, a precise statement of the new probability of contamination can be given, if complete indicator kill is not obtained.

For this second method of establishing a total radiation dose, it is again essential that a good control program be conducted. The essential elements of that program include: a sound environmental control effort; periodic product tests using graded dose levels to verify the sterilization point for normal contaminants; and cycle control by physical dosimeters and intermittently with a form of biological indicator.

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Ecological Studies of Radiation Sensitivity in Microorganisms at Some Enterprises of Medical Industry

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Abstract: *As evidenced from the experimental study of radiation sensitivity in microorganisms contaminating the products of the medical industry in various geographical regions of the USSR, the data of D_{10} for dried cultures of radiation sensitive and radiation resistant microorganisms were 10-120 krad and 120-350 krad, respectively. When aqueous suspensions of microorganisms were irradiated the data of D_{10} decreased by 3.0-4.5 and 1.3-1.6 times for radiation sensitive and radiation resistant microorganisms, respectively. No essential regional differences in radiation sensitivity of one and the same organism strain were observed. Radiation resistant microorganisms were found in the contaminating population with the frequency of 10^{-2}-10^{-4}. The data obtained in the study can be used for selecting the values of sterilizing and pasteurizing doses of irradiation.*

As follows from the known radiobiological mechanisms, the values of sterilizing dose of irradiation and doses for "radiation pasteurization" depend on the radiation sensitivity of microorganisms contaminating the irradiated items. The radiation doses selected on the basis of survival curves of highly resistant microorganisms can be overestimated if the level of radiation resistance of test cultures exceeds the radiation resistance of the industrial microflora. The data presented in the literature do not provide adequate knowledge about actual radiation sensitivity of the industrial microflora.

We made an experimental study on the radiation sensitivity of microorganisms contaminating the products from a number of enterprises of the medical industry at various geographical regions of the USSR (Moscow, Leningrad, Riga, Penza, Kurgan, Kharkov).

Table I. — Radiation sensitivity of dried microorganism cultures obtained at chemical-pharmaceutical enterprises in various geographical regions of the country.

Microorganisms	Place where microorganisms were obtained	The number of strains	% of strains, for which the values of D_{10} (krad) are:		
			10–100	101–200	201–400
Staphylococci	Kharkov	300	30	70	—
	Penza	300	25	75	—

	Kurgan	300	33	67	—
	Riga	300	27.7	72.3	—
	Moscow	300	31	69	—
Total		1500	29.3(1)	70.7(2)	—
Streptococci	Kharkov	50	26	46	28
	Penza	60	15	48.3	36.7
	Kurgan	55	25.4	45.5	29.1
	Riga	45	22.2	51.1	26.7
	Moscow	80	23.8	53.7	22.5
Total		290	22.4(1)	49.3(2)	28.3(3)
Gram-positive	Kharkov	75	8.0	44	48
spore-forming	Penza	100	9	43	48
microorganisms (spores)	Kurgan	135	11.1	45.2	43.7
	Riga	106	10.4	43.4	46.2
	Moscow	100	7	48	45
Total		516	9.3(1)	44.8(2)	45.9(3)
Gram-negative	Kharkov	68	45.3	54.4	—
non-spore-forming	Penza	41	46.3	53.7	—
microorganisms	Kurgan	36	41.7	58.3	—
	Riga	62	46.8	53.2	—
	Moscow	318	43.7	56.3	—
Total		525	44.4(1)	55.6(2)	—
Fungi	Kharkov	23	43.8	56.2	—
	Penza	18	38.9	61.1	—
	Kurgan	16	56.3	43.7	—
	Riga	14	35.7	64.3	—
	Moscow	50	46.0	54.0	—
Total		121	44.6(1)	55.4(2)	—

1. for regions in this group $P > 0.1$
2. for regions in this group $P > 0.25$
3. for regions in this group $P > 0.05$

Using the method of random selection, we isolated about 8,000 strains of gram-positive microorganisms (*Staphylococcus* sp., *Streptococcus* sp., *Bacillus subtilis* etc.), gram-negative microorganisms *(E. coli, Pseudomonas aeruginosa, Proteus* sp., *Enterobacter* sp., *Citrobacter* sp., etc.) and

various species of fungi. Determination of D_{10}-values under similar conditions (temperature: 20°C, relative humidity: 10-12%, dose rate: 40 rad/sec) did not indicate any essential regional differences in radiation sensitivity of the same strains of microorganisms (Table I). The most radiation sensitive were gram-negative microorganisms — not spore-formers: the D_{10}-values were 10-100 krad and not more than 150 krad for 41.7-46.8% for these microorganisms. Radiation sensitivity of staphylococci and especially that of streptococci were much lower. The D_{10}-values reached 250-300 krad for individual strains. But streptococci with a very high level of radiation resistance similar to fecal streptococci described by Christensen et al. (1, 2) were not isolated. The most radiation resistant were spores of gram-positive microorganisms including the spores of *Bacillus subtilis*. The D_{10}-values were 200-350 krad for about half of the strains of these microorganisms.

Since our studies did not indicate any essential regional differences in radiation sensitivity of one and the same strains of microorganisms, the results obtained in determining the values of D_{10} for various groups of microorganisms can be presented in a general form for all geographical regions (Table II). The values of D_{10} given in Tables I and II are determined on the dried cultures of microorganisms. Determination (in the same condition) of D_{10}-values on aqueous suspensions (distilled water, phosphate buffer, pH 7.0, isotonic solution of sodium chloride) showed a 1.3 to 1.6 fold decrease of these values for radiation resistant and radiation sensitive microorganisms, respectively. This shift in phenotypical manifestation of radiation sensitivity in microorganisms contributes to the fact that the necessary radiation doses can be rather small (3).

Table II. — Radiation sensitivity of dried microorganism cultures obtained at chemical-pharmaceutical enterprises.

Microorganisms	D_{10} (krad) $\bar{x} \pm 26$
Staphylococci	120 ± 45
Streptococci	160 ± 70
Gram-positive spore-forming microorganisms (spores)	205 ± 105
Gram-negative non-spore-forming microorganisms (including *E. coli* and *Pseudomonas aeruginosa, Proteus sp*.)	105 ± 50
Fungi	110 ± 50

The values of D_{10} listed in Table II and in the corresponding correction coefficients for the irradiation of microorganisms in water solutions, are convenient to use for selecting the values of sterilizing and "pasteurizing" doses of irradiation (on the basis of the qualitative and quantitative composition of the microflora contaminating the products). The sterilizing dose of irradiation can be determined from the known relationship between the values of D_{10}, initial contamination, reliability coefficient and dose (3). The "pasteurizing" doses can be estimated by radiation sensitivity of microorganisms predominating in the microflora, e.g., if the products are mainly contaminated by

gram-negative microorganisms for which the values of D_{10} are 105 ± 50 krad, the requirement to reduce microbial contamination by 10^3 times will be satisfied with the dose of not more than 500 krad.

Since the value of the sterilizing dose is greatly influenced by the presence of radiation resistant microorganisms as part of the contaminants, the ecological studies in this field acquire special importance. Theoretically these studies should better be performed on a random sample. But samples in this case should be large, resulting in much time-consuming work. Therefore, we studied the distribution of radiation resistant microorganisms in the contaminating population by the routine method of radiation selection. The mixed population were irradiated with a dose of 0.5 Mrad and the value of D_{10} was determined for survived microorganisms. Those microorganisms whose D_{10}-values exceeded 250 krad were considered as radiation resistant. It has been found that the frequency of radiation resistant microorganisms being present in the population of contaminants was 10^{-2} to 10^{-4}. The low frequency of highly radiation resistant microorganisms in the contaminating population indicates that practically those microorganisms will not determine the values of "pasteurizing" radiation doses. As for sterilizing doses of irradiation, their values are much dependent on the presence of radiation resistant microorganisms in the population of contaminants; e.g. if the frequency of these microorganisms in the microflora is 10^{-2}, they will fully determine the value of sterilizing dose of irradiation. This is due to very high demands on reliability made for the method of radiation sterilization. The reliability coefficient should not be less than 10^6.

In conclusion, it should be noted that the data on radiation sensitivity of contaminating microflora obtained from ecological studies at some enterprises of medical industry in the USSR can be used for selecting sterilizing and "pasteurizing" doses of irradiation.

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The Role of Microbiology in Commissioning a New Facility and in Routine Control

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Abstract: *Microbiological methods for evaluation of the quality level of radiation sterilized medical products are discussed.*

Total counts of the microorganisms contaminating the products prior to irradiation, examinations of the frequency of occurrence of microorganisms with high radiation resistance and intercomparisons of dose-response curves for test strains of bacteria in standard preparations are recommended in commissioning a new radiation facility and in routine control.

Some examples of evaluation of the radiation resistance of bacteria picked up under routine control are given.

The use of biological indicators in the daily routine control of radiation sterilization and of sterility tests on the finished products are not recommended. However, even these two methods can give useful information about the standard of quality control of radiation sterilized products.

Introduction

Sterilization of medical products is a tight-rope walk between an exposure harmful enough to the microorganisms to ensure that the probability of surviving organisms is acceptably small and an exposure that has an acceptably small damaging effect on the physical or chemical characteristics of the product.

The criteria for acceptability are not the same in all countries, and we can not expect them to be so in the near future. The background for the evaluations and estimates described in this paper is the quality norms for medical products in the Nordic countries.

Microbiological measurements made at the commissioning and under routine operation of a radiation plant for sterilization of medical products are the only details of the microbiological control system that are necessary for the manufacture of sterilized medical products. Several excellent papers and recommendations covering the manufacture of medical products in general have been published in recent years (e.g. 1, 2). I can therefore concentrate on radiation sterilized products, knowing that the general conditions for *lege artis* manufacture of medical products are well known.

I can sum up the principles that I assume to be accepted as follows:

1. Regardless of the methods used for the manufacture of sterile medical products, the same level of microbiological safety shall be aimed at.

2. Control of the raw products, of the production environment and processes, and of the end-product prior to sterilization shall be performed with due regard to the kind of product manufactured. Microbial measurements are, of course, only a part of the control system, and often not the most important.
3. Chemical and physical measurements shall be made of the parameters important for the antimicrobial effect of the sterilization procedure.
4. Microbiological standard preparations shall be used as an integrated measure of the efficiency of the sterilization procedure.
5. Quality assessment of the final product shall be performed, e.g. a sterility test.

To avoid misunderstanding it is necessary to point out that the importance of the various measures is not the same for all kinds of medical products and sterilization methods. Modifications and even omission of parts of this framework can be justified in relation to the manufacture of some medical products.

I do not wish to get involved in a discussion about *can be used* or *shall be used* in relation to microbiological methods for the control of radiation sterilization. The national health authorities must decide what minimum requirements are necessary for the quality control of medical products. It is the responsibility of the quality control managers at the companies to decide whether any measurements should be used to supplement these official requirements. The safety level decided upon will, of course, be the result of an evaluation of a number of factors, and these factors are not and can not be the same in all countries, as long as there are differences between poor and rich countries.

I can see no good reason for any differences in the microbiological examinations used at the commissioning of a new radiation plant and those used in routine operation. Of course, difficulties can be expected when examinations are performed for the first time, and there may also be a need for some basic information not already available when a new plant is built in an area where there is little or no previous experience with radiation sterilization. I shall mention such problems in connection with the various microbiological examinations.

Total counts of microbial contamination prior to sterilization

When the first version of the *Code of Practice for Radiation Sterilization of Medical Products* was published in 1967 (3) by the International Atomic Energy Agency, Vienna, it was a new idea to use the total counts of bacteria and fungi contaminating the medical products prior to sterilization for routine purposes. Today — 10 years later — such examinations are accepted in several countries as a necessary part of the microbiological control, not only for products to be sterilized by radiation but for all medical products regardless of the method of sterilization. Such counts are of much help when the hygienic standard of the production is to be evaluated. The average number of organisms per product unit, and the deviations from the average, can give an objective picture of the functioning of the precautions taken to avoid contamination.

In the majority of sterilization procedures carried out by means of heat or toxic gases, the minimum inactivating effect on the microorganisms — the microbiological efficiency at the location in the product where the microbicidal effect is lowest — is difficult to measure, and consequently the use of a large safety margin is unavoidable. However, in radiation sterilization the relationship between the absorbed dose and the inactivation of microorganisms is relatively well defined, and the minimum dose

can be measured with acceptable accuracy. Therefore the relationship between the number, the kind and the resistance of the contaminating organisms and the absorbed dose necessary to achieve a defined level of probability for surviving organisms has attracted much interest.

I shall try to give an estimate of the average radiation resistance of the bacteria and fungi contaminating medical devices in Denmark. The curve (Figure 1) is a summation of a number of examinations of dust samples from production areas and of the microflora collected from medical devices. The differences between two samples from the same factory, and between two factories producing the same kind of product, can be significant but are in most cases astonishingly small. Populations of microorganisms from environments where selection of microorganisms has caused a domination of special kinds of organisms are not included.

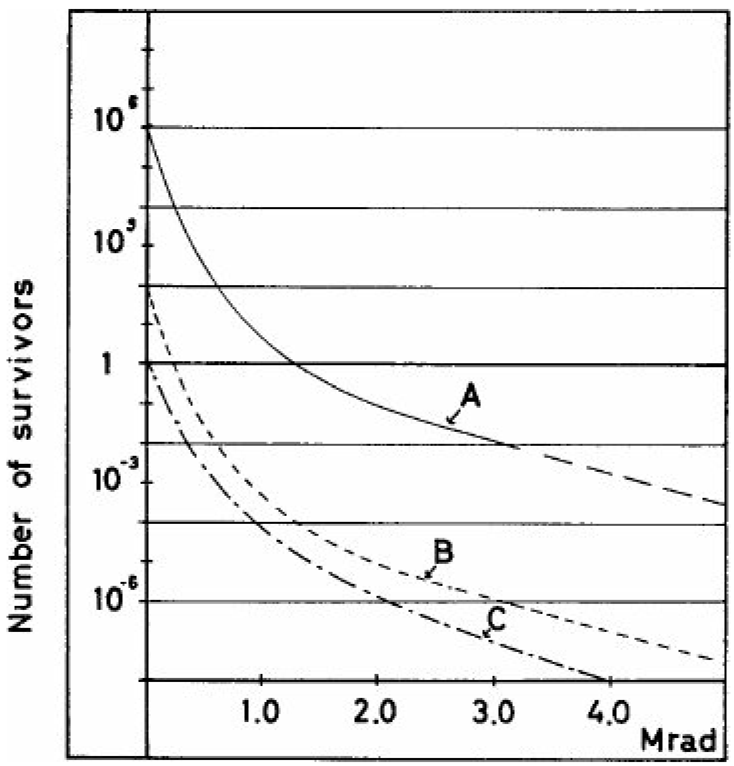

Figure *1.* The curve A is a summation of a number of examinations of dust samples from production areas and of the microflora collected from medical devices. The dotted line B is parallel to A, based on the assumption that the radiation resistance is independent of the density of organisms in the samples. The dotted line C is a coarse estimate of the radiation resistance of contaminants in clean rooms.

The figure demonstrates that the standard for sterilized medical products recommended by the health authorities in the Nordic countries: not more than one colony-forming unit per one million product units can be achieved with a minimum dose of 3.2-3.5 megarad, provided that the number of microorganisms prior to radiation is lower than about 100 per product unit. The figure also demonstrates that a minimum dose of 2.5 megarad gives a better result than needed when sterility tests of the finished product are used to define sterility. The difference between the levels achieved by 2.5 and 3.5 megarad is one to two logarithmic steps. A significant decrease in the number of contaminating organisms, and/or a decrease in the frequency of occurrence of the most resistant organisms, can justify a decrease in the minimum dose.

The idea that a number of contaminants higher than about 100 per product unit justifies an

increase in the minimum dose has been anything but popular. The health authorities in the Nordic countries recommend that products contaminated with less than 50 colony-forming units per product unit be irradiated with a minimum dose of 3.5 megarad. With counts between 50 to 500, a minimum dose of 4.5 megarad shall be used, and with counts between 500 and 5000, 5.0 megarad shall be used (4). The reason for this recommendation is *not* that Nordic manufacturers need a possibility for compensation for poor hygienic conditions. Also in the Nordic countries microbial contamination must be kept as low as good manufacturing conditions can produce. However, at any given time there will be some items where the initial counts are higher, e.g. new and perhaps complicated items, not yet manufactured with sufficient mechanization and protection and maybe under rapid development of production techniques.

The result of this recommendation has been that virtually only products with initial counts lower than 50 are radiation sterilized. The impact of this recommendation on the general level of hygienic conditions in factories is difficult to evaluate, but it should not be underestimated.

In the last few years a new type of problem can be seen in some countries. The controlled areas for the manufacture of medical devices are so clean that nearly all microorganisms in these areas originate from the human beings working in the area. The number of microorganisms per item prior to sterilization can under such circumstances be lower than one on the average. Provided it is accepted that the dose can be adjusted to a more favorable (or more unfavorable) microbial contamination, two questions arise from this new situation. How can this low level of contamination be measured? And how resistant are these microorganisms?

The answer to the first question should be easy to find. The conventional sterility test of the final products is at this level of sensitivity. Positive cultures will be found when the number of unsterile units in the batch is between 1 per 10 and 1 per 100 units. The number of colony-forming organisms from 10 or 100 product units can be counted on a membrane filter.

The second question is the difficult one. How can the radiation resistance of these few microorganisms be evaluated?

Evaluation of the radiation resistance of the contaminating microorganisms

As demonstrated on Figure 1 the dose response curve for a mixed population of microorganisms is not a straight line in the semilogarithmic system. A small fraction of the population with a relatively high resistance will be the important factor, when a high level of safety is to be ensured.

A priori, it cannot be excluded that a very resistant organism normally occurring in a number of one per 10^5 or 10^6 of the total number of colony-forming units in the environment could be the dominating contaminant. However, this remains a hypothetical possibility so far, as no example is known, but a less drastic change in the number of resistant organisms could still be serious enough.

When dealing with products contaminated with about 100 organisms per item, there are several possibilities for bacteriological examination. A reasonable number of organisms can be collected from the products, or from the environment, and screening tests can be performed for the frequency of the occurrence of organisms with unusually high radiation resistance.

When a membrane filtration technique is used for counting the microorganisms prior to sterilization, the filters from this examination give an excellent opportunity for such a screening

procedure (5, 6, 7).

Another possibility is the use of a sterility test technique on items irradiated with doses much lower than the routine dose (8, 9).

All — or nearly all — the data on the composition of contaminating microorganisms and the frequency of occurrence of highly resistant organisms in medical products prior to radiation sterilization have been collected in North Europe or in North America (6, 7). Investigations are needed when a new plant is built in an area where the organisms in the environment may have a different resistance against radiation. Is it likely that a subtropical or tropical climate could give another resistance pattern?

In my own laboratories we have been interested in the resistance of organisms from so-called clean areas for many years. Our interest does not only concern resistance against radiation, but heat and toxic gases as well. I can give an example from examinations of the radiation resistance performed by Hanne Kristensen and myself during the last two years (Table I). Microorganisms are collected from the air in various controlled areas by means of a slit-sampler. The samples are examined for the occurrence of colonies that survive 2.0 megarad or more. It was expected that resistant organisms could be picked up, because examinations of microorganisms from towels and underwear had revealed a number of resistant organisms, most likely originating from the human skin. The occurrence of about 1 per 100 was, however, more than expected.

Table I. — Radiation resistant organisms isolated from clean rooms.

Department	Number of organisms collected by means of slit-sampler	Colonies surviving = 2.0 Mrad	Isolated organisms	Inactivation factor 10^6 by dose in Mrad
I (various locations)	400	+	Gram-pos. rod	3.6 - 4.2
	200	+	Gram-pos. rod	about 4.5
	200	–	–	–
	200	–	–	–
	150	+	Gram-pos. rod coccus	about 4.5 about 3.0
	150	–	–	–
	100	+	diplococcus	about 4.2
	100	–	–	–
	50	–	–	–
	50	–	–	–
II	250	+	diplococcus	about 4.2
III	500	+	diplococcus	about 3.0
IV	400	+	diplococcus	4.2 - 4.8

V	450	+	coccus	about 3.0	
VI (various locations)	250	–	–		–
	100	+	Gram-pos. rod	about 3.5	
			Gram-pos. rod	about 3.5	
	100	+	Gram-pos. rod	about 3.0	
	100	–	–		–
	100	–	–		–
	25	+	Gram-pos. rod	about 2.5	
VII	250	+	diplococcus	about 5.0	
VIII (various locations)	300	+	diplococcus	4.5 - 5.0	
	300	–	–		–
	150	+	diplococcus	about 3.5	
	100	+	Gram-pos. rod	about 2.5	
	50	+	Gram-pos. rod	about 2.5	
	50	–	–		–
	50	–	–		–

Standardized microbiological preparations

Microbiological preparations can be used to demonstrate the microbiological efficiency of the doses delivered in a radiation plant. When the environmental conditions for the microorganisms before, during and after the irradiation are defined, the relations between the inactivation factors for the microorganisms and the absorbed doses in megarad are also defined. The dose-survival curves achieved after irradiation of the same standard preparation of microorganisms are therefore in principle the same after irradiation under similar conditions at different plants. Any significant differences can be explained either by dosimetry failures or by failures in the microbiological procedures, or — and this is not the most unusual case — by the unexpected influence of one or more factors either on the dosimetry or on the microbiological examination.

When the first version of the IAEA's Code of Practice for Radiation Sterilization in 1967 (3) was worked out, the microbiologists who participated in the working group felt that it was necessary to include measurements of the microbiological efficiency of the doses measured at all new plants. A difference of 10-15% in biological efficiency between doses given in electron accelerator plants and in Co-60 plants had recently been recorded, and other types of radiation plants might — as far as it was

predictable at that time — be invented before new and better "Codes" could be expected. It was known at that time that the differences between the effects of the same absorbed dose given in accelerators and in Co-60 plants were not always the same, but varied with the kind of microorganisms used in the examination. Therefore the use of endospores, non-sporeforming bacteria and virus was recommended.

Today, new types of radiation plants are not expected, and the differences in microbiological efficiency of the absorbed doses in electron accelerator plants and in Co-60 plants are not important. Therefore, a less complicated program can be used for such examinations. We have access to a number of very resistant and stable strains of bacteria, and one or more of these can be used as test strains.

Four inactivation curves are demonstrated in Figure 2. In all cases, dried preparations of microorganisms protected by small amounts of organic materials are used. Curve I is a *Micrococcus radiodurans.* This organism can obviously be used as monitor in the range from 3.5 to 6.0 megarad. Curve II is *Bacillus sphaericus,* strain C_1A. This strain can be used for about 1 to about 6 megarad. However, the accuracy of the measurement is not impressive because of the very high resistance and the nearly straight-line relationship between the inactivation factor and the dose. Curve III is a *Bacillus cereus* mutant (C 1/1-18). Because of the convex curve and a resistance very close to the resistance of a number of non-spore-forming bacteria, this strain is often used for measurements from about 2.0 to about 4.0 megarad. Curve IV is *Bacillus pumilus* E 601. Unless this strain is protected by a rather costly preparation, it can only be used for measurements of doses lower than about 2.5 megarad. It is a very stable and reliable test strain.

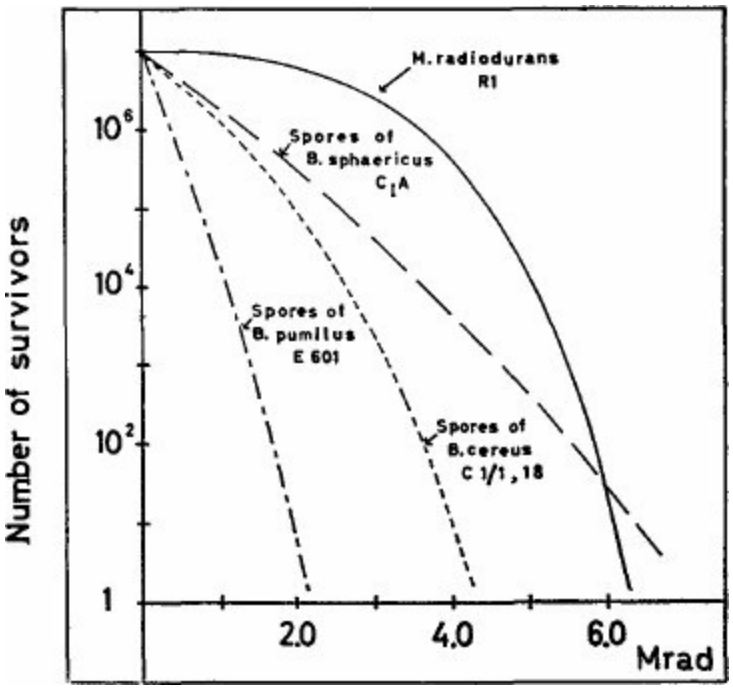

Figure *2.* Inactivation curves for four strains of bacteria irradiated in dried preparations. These strains can be used for examination of the microbiological efficiency of the absorbed dose at various dose levels.

Using a test strain such as the *B. cereus* mutant a test can be made of the dosimetry and the bacteriological skill at a new radiation plant. The amount of information can be multiplied by extending the examination to non-spore-forming strains. Figure 3 gives two examples only. Curve I is *Streptococcus faecium,* strain A_21, and Curve II is an *Acinetobacter.* The first of these strains is an

enterococcus, the other a Gram-negative rod. Several other strains could be used and because of the relatively short storage life-time of these non-spore-forming strains I would recommend the use of a strain familiar to at least one of the microbiological laboratories involved.

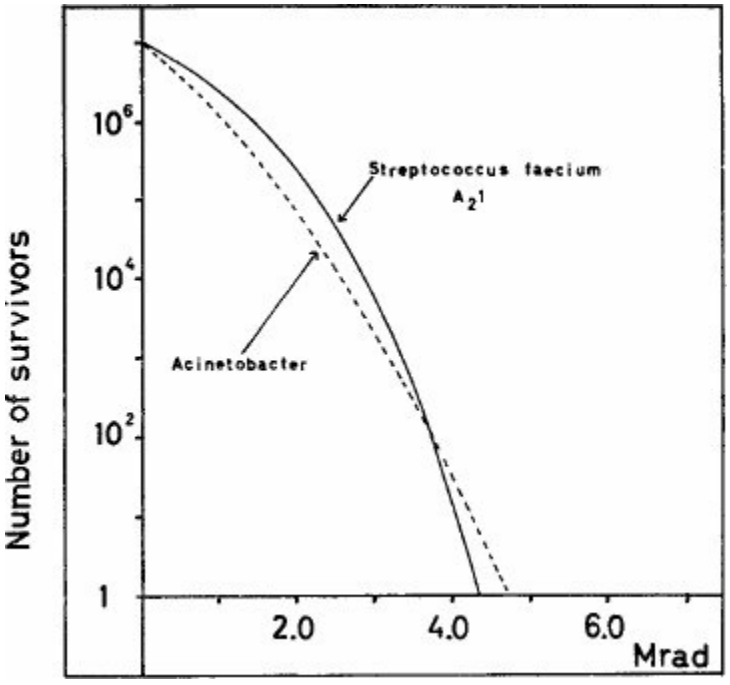

Figure *3.* Inactivation curves for two non-spore-forming strains of bacteria. Such strains can be used for examination of the microbiological efficiency of the absorbed dose.

The examination program could, of course, be extended to fungi and to viruses. Such examinations would perhaps be justified if a plant is built for very special purposes. It is, however, very unlikely that examinations of fungi or viruses would reveal information on the dosimetry and the microbial efficiency that was not already revealed by the dose-response curves for bacteria.

Figure 4 shows an example of a comparison between a dose-response curve from a research plant used by a national health authority as reference plant and dose-response data from a commercial plant on commissioning.

It must be mentioned that Nordic experience published in 1974 (10), showed that discrepancies could be demonstrated between inactivation curves from irradiation in commercial plants and from the reference plant in nearly all cases at the first comparative examination. Experience gained since this information was published only supports this conclusion. It should also be mentioned that the Nordic health authorities recommend at least one examination of the biological efficiency of radiation plants per year.

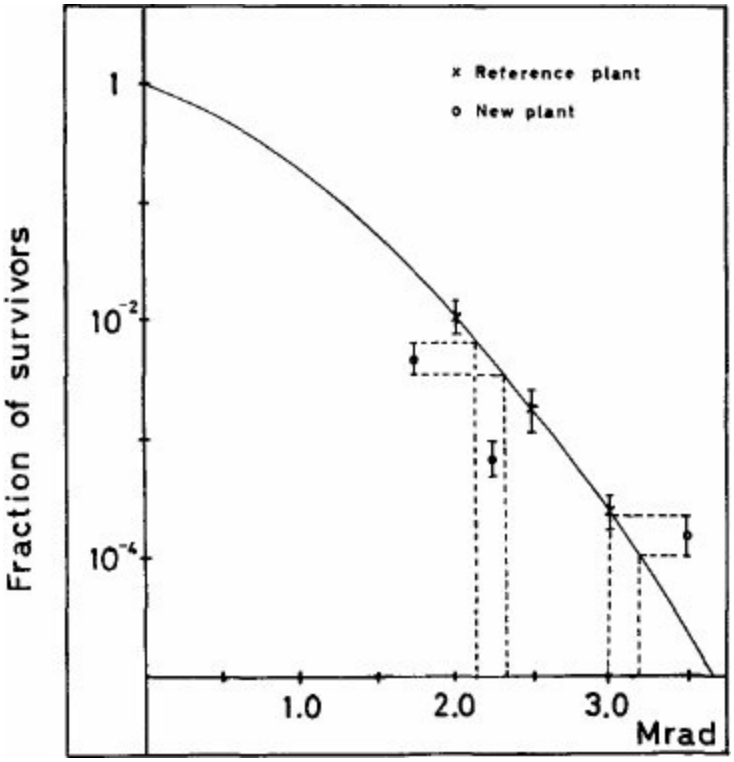

Figure *4.* Inactivation curve for a test strain irradiated at a research plant compared with dose-response data from a commercial plant.

For many years a condition for the radiation sterilization of medical products has been that the manufacturer or the radiation plant — should have access to a qualified dosimetrist and a qualified microbiologist. In microbiological efficiency measurements both dosimetrist and microbiologist are involved. If these people are qualified and have equipment of an acceptable standard, these measurements are not difficult and cause very few problems. Neither are the measurements very costly, if the laboratories are already there — and they should be. It is understandable if health authorities or quality control management, or both, wish to include some microbiological examinations in the program for commissioning a radiation plant for the sterilization of medical products. A few inter-comparisons of dose response curves each year might be justified also.

The kinds of standardized bacteriological preparation mentioned above could be used as dosimeters, giving an integrated measurement of the effect essential for the sterilization procedure — the ability to inactivate microorganisms. An inspector may not be very familiar with radiation chemistry and radiation physics and he or she may not be very familiar with radiation biology either. A few red perspex dosimeters and a few spore samples examined at a few different laboratories can give the inspector the evidence he needs for a positive (or a negative) decision.

Biological indicators

The recommendation in favour of the use of bacteriological monitors should not be understood as a recommendation of biological indicators in routine control of radiation sterilization.

Biological indicators are designed to indicate — with a positive warning — when the safety margin of a sterilization procedure is too narrow. Indicators based on resistant endospores are very useful in the safety system when sterilizing by means of saturated steam. Biological indicators are necessary when

toxic gases are employed for sterilization. However, it is, of course, a misapplication to use biological indicators in cases where temperature and time measurements could give the same information more accurately and at a lower cost. Numerous chemical dosimeters are available for routine dosimetry in industrial radiation plants, and these dosimeters can give the accuracy needed. Chemical dosimetry is accurate and faster and less expensive than the use of biological indicators.

However, should the use of a biological indicator in radiation sterilization be necessary for some reason or other, it is essential for the value of such a biological warning system that the resistance of the indicator is adjusted to the routine dose.

A quality control system should be able to tell in a variety of other ways if the efficiency of a sterilization process is far on the wrong side of the limit for acceptable sterilization. The biological indicator can be much more sensitive than these coarse warnings against too low a dose. I mention this point only because some proposals for biological indicators for radiation sterilization have been put forward in recent years in various working groups dealing with recommendations for sterilization.

Figure 5 illustrates some examples of the proposed biological indicators.

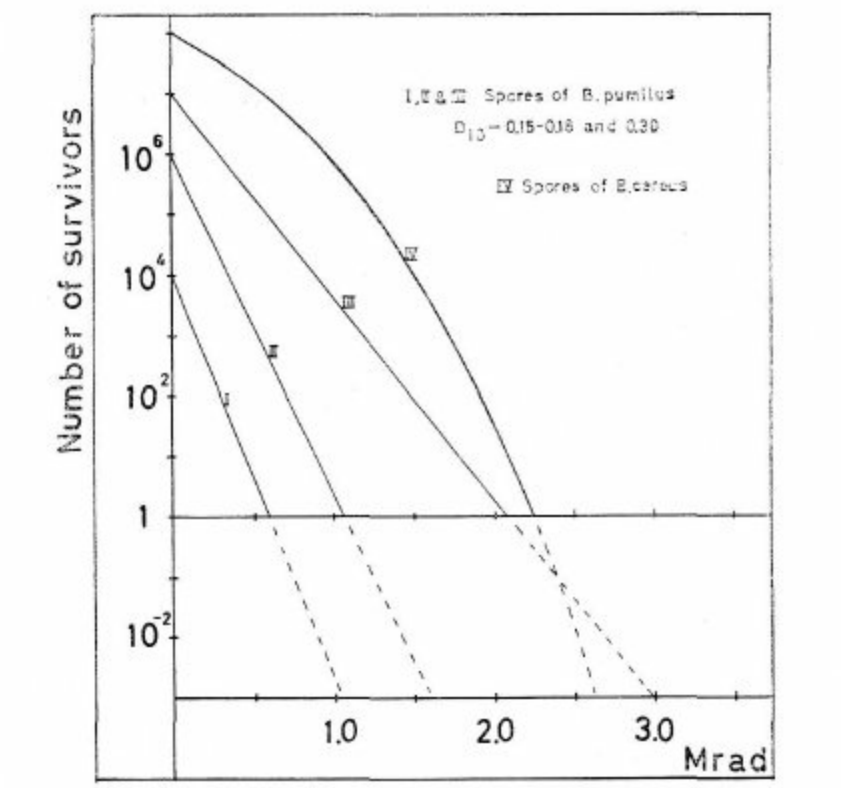

Figure *5.* Inactivation curves for various biological indicators proposed for control of a minimum dose of 2.5 megarad. Biological indicators with a radiation resistance about the level indicated by the curves III and IV can give information relevant for the quality control. The indicators with the inactivation curves I and II, could be used at dose levels about 0.7 and 1.2 megarad, respectively.

Curve I is based on washed spores of *B. pumilus,* Curve II on the same organisms, but with a higher number of spores per indicator. As these indicators are proposed for control of a minimum dose of 2.5 megarad they are not very useful for quality control. They could be valuable for other purposes, but these will not be discussed here. Curves III and IV are based on spores of *B. pumilus* and *B. cereus,* respectively; both preparations protected by organic materials. By means of one of these two indicators, a warning would be given if the dose was about 20% lower than the 2.5 megarad.

Sterility tests of the finished products

Sterility tests on sterilized medical products are still mandatory in several countries. It is, however, well known and demonstrated both theoretically and practically, also from routine manufacture of medical products, that sterility tests under optimal conditions can only reveal — with a reasonable probability — a frequency of contaminated product units higher than about 1%. Such a method is without much value when the required standard is less than one contaminated product unit per million.

However, this method is the only available one for a direct test of the microbial contamination of the finished products in the majority of medical products. Therefore it is still useful; for example, sterility tests of gas sterilized products are still recommended in Denmark. On products not sterilized in the final container, a sterility test should, of course, be mandatory. However, for radiation sterilized products manufactured with due regard to current quality control systems, sterility tests are without value in relation to microbiological safety. The test may be mandatory, in which case it then has its legal value.

If the control of contamination prior to radiation is neglected, if the dosimetry is inaccurate, and the frequency of the occurrence of radiation-resistant organisms is unknown, then sterility tests are needed, even if the required minimum dose is higher than 2.5 megarad.

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The Sub-Process Dose in Defining the Degree of Sterility Assurance

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Abstract: *A model relating the frequency of contaminated items and increasing radiation dose is described and its use for assessing the value of sub-process doses in defining sterility assurance is considered. Evaluations of the model are given for a range of doses assuming a) a Poisson and b) an Exponential distribution of the numbers of organisms on items prior to and during irradiation. Such evaluations show that direct extrapolation to process doses of data relating the proportion of contaminated items and radiation dose, generated at sub-process radiation doses, is not valid. Other possible means of extension of the data are examined. The basis of a new model which will allow investigation of the manner in which distribution of numbers of organisms on items affects the frequency of contaminated items at sub-process doses, is presented.*

The inactivation of microorganisms by radiation follows exponential law; there is, therefore, always a finite probability of a microorganism surviving, regardless of the dose applied. It follows that the sterility of any single item in a population of items subjected to an accepted radiation sterilization treatment cannot be guaranteed.

A working definition of the sterility of a population of irradiated items is that a certain low probability of the existence of a contaminated item in that population should be achieved. A generally accepted level of probability of sterility is one contaminated item in a population of one million items (1).

The probability of sterility of a population of items, defined in these terms, is what is meant by the degree of sterility assurance achieved by exposure of items of that population to a sterilization process.

The usual means of obtaining an estimate of this probability is by the use of "microbiological reference standards" (2). These are laboratory preparations of relevant, inherently resistant organisms present in an environment designed to simulate conditions likely to arise in practice that minimize the organisms' response to radiation. In practice, a measurement is made (a) of the extent of inactivation, by the process radiation dose, of a chosen reference organism, and (b) of the level of adventitious contamination normally found on items of the population destined to be given that dose. On the assumption that the response of all contaminants to radiation is equal to that of the reference organism, the probability of sterility (or the degree of sterility assurance) is predicted for that population of items at the particular process dose. Obviously, the validity of the prediction rests almost entirely on the relevance of the laboratory contrived conditions of the reference standards to those conditions occurring on manufactured items. Unfortunately there is no means at present by which this relevance

can be assessed.

Recognizing this uncertainty, Ley and Tallentire (3) in 1965 contended that a better estimate of sterility assurance would be obtained from measurements of inactivation of microorganisms present on naturally contaminated production items. They envisaged a test in which actual production items would be given doses of radiation that were only fractions of the process dose; these items would then be scored for the presence of viable microorganisms by a sterility testing technique, allowing a functional relationship to be drawn between the frequency of contaminated items and radiation dose. Assuming a precise form for the relationship established at sub-process doses, it was hoped to predict the probability of a contaminated item existing at the process dose.

The usefulness of this idea has been explored in wholly theoretical circumstances. A simple mathematical model has been devised to simulate the types and levels of contamination on items prior to irradiation and to observe changes in contamination with increasing radiation dose. The findings from evaluations of this model provide a basis for assessing the scope and limitations of the sub-process dose concept.

Relevant Evaluations of a Mathematical Model Describing the Microbiological Status of Items Undergoing Irradiation

The model itself

The model is based on the premise that prior to irradiation a population of items of finite size is contaminated with microorganisms of different types (i.e. types responding differently to irradiation) and that numbers of the microorganisms on the items are distributed around a known average number per item in a describable manner. Items within the population possessing no viable microorganisms are regarded as uncontaminated or 'sterile' and those with one or more viable microorganisms as contaminated. This hypothetical situation has been described before in mathematical terms as follows (4):—

The probability $q(m, i)$ that an item chosen at random from a population of X items contains m organisms of type i is given by

$$q(m, i) = f(m, i) \Big/ \sum_{m=0}^{t(i)} f(m, i) \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad [1]$$

where $f(m, i)$ is the frequency of items with m organisms of type i, $t(i)$ is the maximum number of organisms of type i per item,

and $\sum_{m=0}^{t(i)} f(m, i) = X$, the population of items.

In particular, the probability that an item has no organisms of type i is given by

$$q(0, i) = f(0, t) \Big/ \sum_{m=0}^{t(i)} f(m, i) \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad [2]$$

The probability $p(i)$ than an item possesses one or more organisms of type i is

$$p(i) = \sum_{m=1}^{t(i)} f(m, i) \Big/ \sum_{m=0}^{t(i)} f(m, i) \quad [3]$$

where

$$q(0, i) + p(i) = 1 \quad [4]$$

If a total or r types of organisms is distributed over the items, the probability that an item contains no organisms of any type is given by

$$Q = \prod_{i=1}^{r} q(0, i) \quad [5]$$

and the probability that an item contains one or more organisms of any of the r types is P, where

$$P = 1 - Q \quad [6]$$

Thus P gives the proportion of contaminated items in the population.

The probability that an item will contain m organisms of type i depends on the distribution of the particular type i throughout the items. For the purposes of the model, this distribution has been approximated by a frequency distribution $\Theta(i)$, characterized by the population mean (μ) and (s - 1) moments about the mean [$\mu(2)$, $\mu(3)$ . . $\mu(s)$], allowing the probability that an item possesses m organisms of type i to be written as

$$q(m, i) = \Theta(i)\,[\mu(i), \mu(2, i), \mu(3, i) \ldots . \mu(s, i); m] \quad [7]$$

In particular, the probability that an item has no organisms of type i is

$$q(0, i) = \Theta(i)\,\mu(i), \mu(2, i), \mu(3, i) \ldots . \mu(s, i); 0] \quad [8]$$

and the probability that an item possesses no organisms of the r types is

$$Q = \prod_{i=1}^{r} q(0, i) = \prod_{i=1}^{r} \Theta(i)\,[\mu(i), \mu(2, i), \mu(3, i) \ldots \ldots \mu(s, i); 0] \quad [9]$$

Equations [6] and [9] have been taken to represent a wholly general model for the calculation of the proportion of items contaminated (P) in a population of items, the model holding for all radiation doses.

In making such calculations, assumptions were made concerning the form of the distribution of numbers of organisms on items (Θ), the behaviour of these distributions with radiation dose and the mathematical expression describing the overall response of the contaminating organisms to irradiation. For convenience in computation it was preferred that the chosen distribution held at all dose levels and that its form was characterized by a single definable statistic. The statistic of choice was the distribution mean since this could be fixed initially (when D = 0) at any desired level and, with increasing dose, determined as a function of dose from a knowledge of the overall response of the contaminating organisms to radiation.*

Determinations of P over a range of incremented values of D have been made for different average initial numbers of organisms/item on populations of items having a single type of organism or two types with widely differing responses to radiation. To facilitate comparison, these evaluations are

presented in the form of curves relating *ln P* and *D*.

Distribution of numbers of organisms on items assumed Poisson

The grounds for choosing the Poisson distribution for the first evaluations of the model and the associated assumptions have been detailed elsewhere (4). Calculations of *Q*, the probability that an item in a population of items will have no organism of any of the *r* types at a dose *(D)*, were done employing

$$Q = \prod_{i=1}^{r} \exp\left[-a(o, i)\left\{1 - \left[1 - \exp\left(-k(i)D\right)\right]^{n(i)}\right\}\right] \ldots\ldots \quad [10]$$

in which $a(o, i)$ is the average number of organisms of type i on X items at $D = 0$ and $k(i)$ and $n(i)$ are constants characterizing the response of the organism of type i to radiation. Values of P, the probability that an item is contaminated, were then derived directly from Equation [6]. Generally, for this wholly theoretical treatment k and n values have been fixed so that organisms populating items respond to radiation in one of two ways; they have either a high sensitivity to radiation (designated as H sensitivity) or a low sensitivity (L sensitivity). While these levels of response are not intended to relate in an absolute sense to responses of microbial contaminants on actual production items, they serve to illustrate the general behaviour of P with increasing D for circumstances where organisms with different radiation responses are present on items at different average initial numbers per item, variations unquestionably relevant to the practical situation.

Figure 1, composed of some of our earlier findings (4), shows two sets of idealized curves relating frequency of occurrence of contaminated items and radiation dose that bear directly on the subject under consideration, namely, sub-process dose treatments and their value in determining the degree of sterility assurance achieved by a sterilization radiation dose. The principal general conclusions that can be drawn from these findings may be summarized as:—

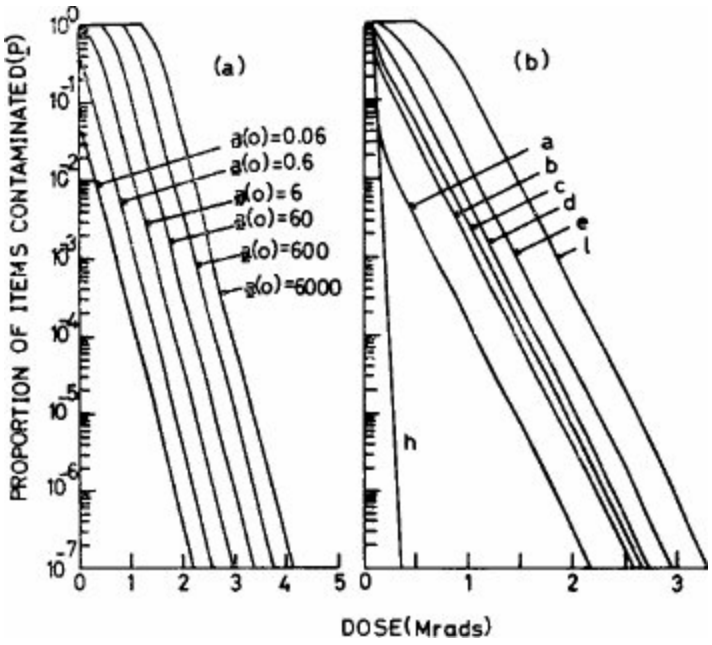

Figure *1.* Curves relating the proportion of contaminated items *(P)* and radiation dose *(D)* for items with numbers of contaminants distributed in a Poisson manner.

(a) Contaminants of a single type present at different average initial numbers/item, *a*(o).

(b) Contaminants of two types present either singly or together at a fixed average initial number/item of 60; the type designated L has a response to radiation which is 10 × lower than that of type H: Curves 'h' and 'l' are for items having only organisms of types H and L respectively, curves 'a', 'b', 'c', 'd' and 'e' are for items with the proportions of L to H types present at ratios 1:1000, 1:100, 1:60, 1:30 and 1:10 respectively.

(i) The sole influence of an increase in the average initial number of organisms of the same type/item is to displace the lnP/D curve to the right (Figure 1a).

(ii) The effect of populating items with organisms of reduced response to radiation at a given average initial number/item is to displace the curve upwards and to the right (Figure 1b, curves h and l).

(iii) Curves derived from items possessing organisms with heterogeneous responses to radiation (H and L sensitivities) at a given average initial number/item exhibit a pronounced concave form, the location of the 'tail' being determined by the proportion of organisms of L sensitivity (Figure 1b, curves a to c). When the proportion of organisms of L sensitivity is marked (curve e) no 'tail' is seen, since the influence of these organisms overrides any possible influence of organisms of lesser response to radiation.

The implications of these findings on interpretations based on data obtained from sub-process dose treatments are discussed below.

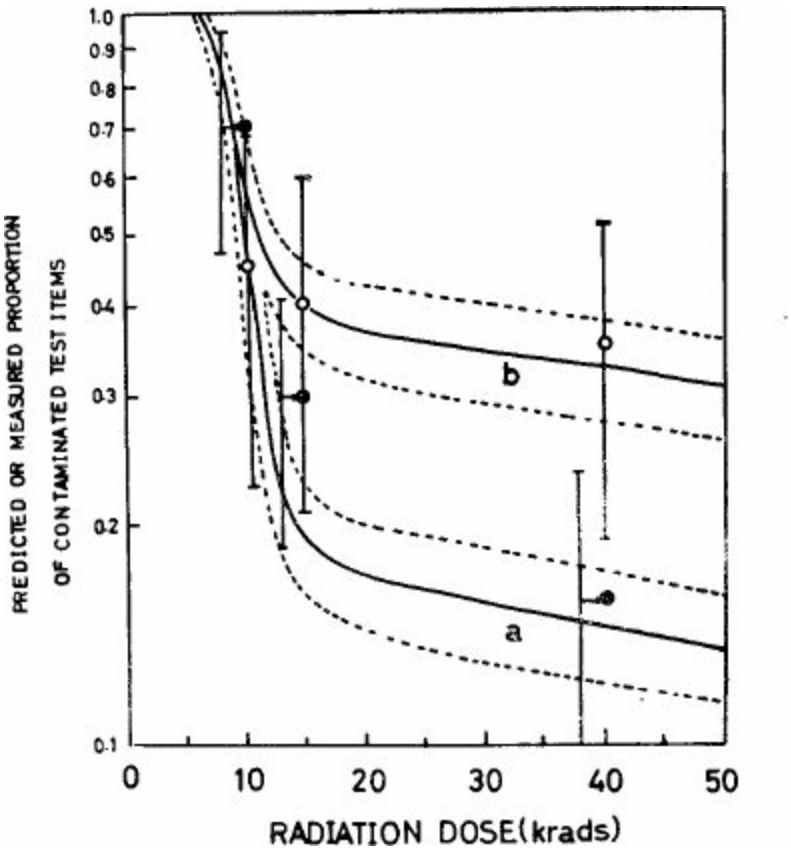

Figure *2.* A comparison of measured and predicted levels of contamination for irradiated test items possessing viable microorganisms of markedly different resistance to radiation. Curves are those predicted from evaluations of the model in the form described by Equation [10], appropriately substituted, and points are measured values. Dotted lines denote one s.e. on either side of a given curve. The error bars about the points are 95% confidence limits.

Curve (a) and solid points: Test items possessing initially on average 0.5 *B. pumilus* spores and 100 *S. marcescens* cells/item. Curve (b) and open points: Test items possessing initially on average 0.2 *B. pumilus* spores and 100 *S. marcescens* cells/item.

Recently we have performed a series of laboratory exercises aimed at testing whether or not the evaluations derived from the Poisson specified form of the model provide a reasonable description of events occurring in a known practical situation. A test system was devised on which it was possible to distribute, in a given manner and at a chosen frequency, particular species of microorganisms that respond in a highly predictable way to radiation. This system was used to simulate the microbiological contamination on items prior to irradiation (5). After exposure to a particular radiation dose, the presence of viable cells was recognized by a growth/no growth technique to give a *measurement* of P, the proportion of contaminated items. By employing graded doses, the behaviour of P (measured) was followed as a function of radiation dose (D). The conditions set initially for the test system were such that the model, in the form of Equation [10], mathematically defined the microbiological status of items at D=0. Subsequent evaluations of Equation [10] gave predicted values of P which were compared directly with values of P (measured) over a range of doses. Comparisons have been made for 'contaminants' of two types, having a fifty fold difference in response to radiation, present on simulated items either singly or together at various frequencies (6). Particularly interesting in the present context are the findings seen when the contaminant of low sensitivity is present at low

frequencies together with the contaminant of high sensitivity at a moderate frequency. These appear in Figure 2; clearly measured values of P agree well with predicted values in the range of P where meaningful comparisons can be made. This, in our view, makes credible the predictions of the model of a marked influence of contaminants of low sensitivity on the behaviour of P in circumstances where measurements cannot be made.

Distribution of numbers of organisms on items assumed Exponential

During the last few years an increasing emphasis has been placed on producing items for terminal sterilization under conditions that minimise the initial microbial contamination on items. This development, coupled with the fact that the environment in which organisms find themselves immediately prior to sterilization is generally biostatic, has meant that items in a population now have numbers of organisms/item distributed in a somewhat different fashion than previously (7) around significantly lower mean values. Typical recently acquired data are shown in Figure 3 (private communication). The form taken by the frequency distribution is characterized by a pronounced skew to the left and a continuously decreasing slope, a shape resembling, in general, the Exponential distribution. The expected behaviour of this frequency distribution with increasing dose is that as the mean number of organisms/item decreases the distribution skews progressively to the left. This property characterizes the Exponential distribution at low mean values (Figure 4).

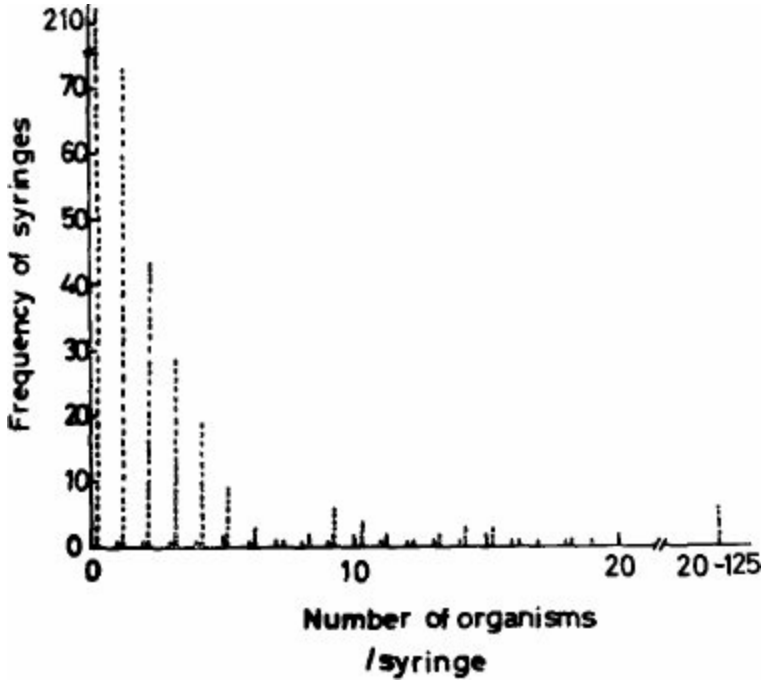

Figure *3.* Recent data on the initial numbers of organisms present on 420 syringes withdrawn randomly from production destined for radiation sterilization.

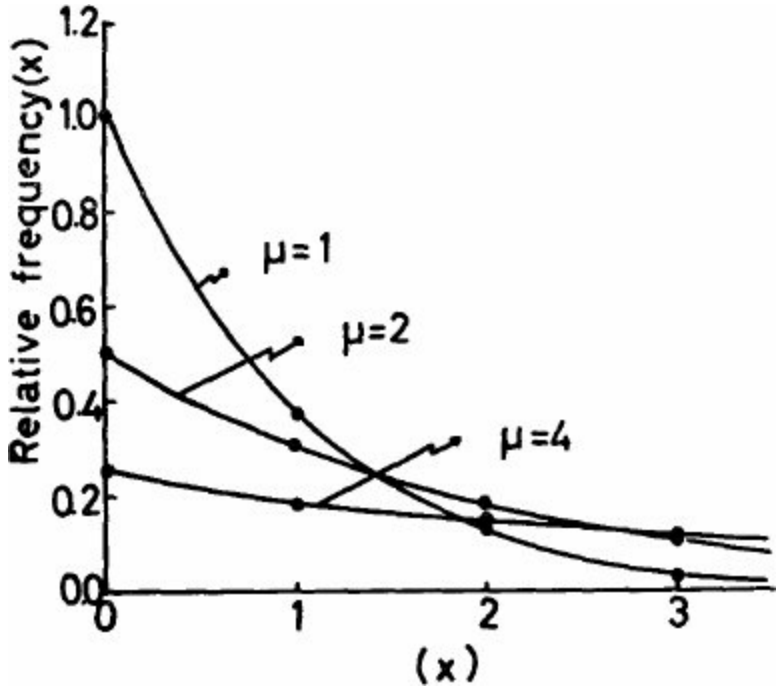

Figure *4.* Exponential distributions for low mean values.

On the above grounds, the Exponential distribution, appropriately modified for discontinuity, has been chosen as the second formal distribution to be used in evaluations of the model. The assumptions are that the distribution is maintained with irradiation and that there is independence of probabilities (i.e. Equation [5] holds).

Values of *P* have been derived on the basis of the following equation

$$Q = \prod_{i=1}^{r} 1 - \exp\left[-1/(a, o)\left\{1 - (1 - \exp\left(-k(i)D\right))^{n(i)}\right\}\right]. \quad [11]$$

in which characters take the same identity as stated above for Equation [10]. To permit strict comparisons to be made, *Q,* and hence *P,* have been evaluated for conditions identical to those employed for the Poisson distribution. Relevant plots of *ln P vs. D* are given in Figure 5. Clearly, for chosen levels of contamination the findings are generally similar to those for the Poisson distribution depicted in Figure 1, the trends in displacements of curves being maintained.

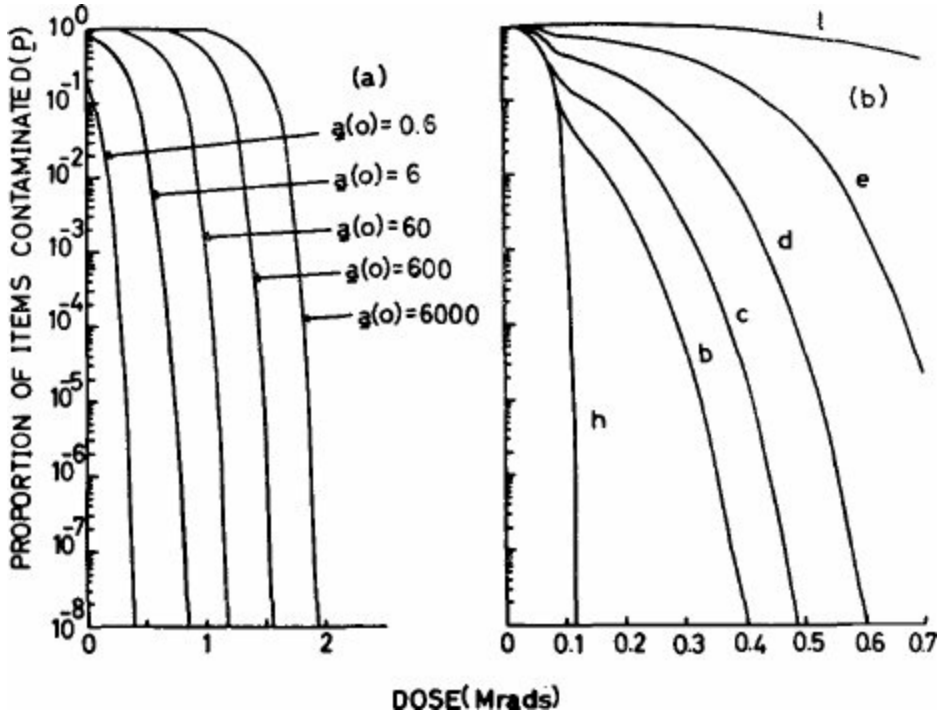

Figure 5. Curves relating the proportion of contaminated items *(P)* and radiation dose *(D)* for items with numbers of contaminants distributed in an Exponential manner.
(a) Contaminants of a single type present at different average initial numbers/item, *a*(o).
(b) Contaminants of two types present either singly or together. The designation of types of organisms and conditions on items are the same as those given in Figure 1.

Practical Implications of Evaluations of the Model

Experience has shown that for a given population of items, moderately contaminated with adventitious microorganisms as is likely to be found in practice, real estimates of P (the proportion of items contaminated) can only be measured at relatively low radiation doses. For example, in the exercise of Ley et al. (8) on disposable plastic syringes (average initial numbers of contaminants per syringe about 50), it was noted that the highest dose yielding measurable values of P was 0.5 Mrad and for cotton rolls (average contamination per roll about 1900 organisms), White (9) found this dose to be 0.55 Mrad. The reason for this restriction becomes evident from a consideration of the expected general form of a curve relating P (measured) and dose *(D)* over unlimited values of D. This curve, depicted by the solid line in Figure 6, will fall as dose increases up to a dose, denoted D_m, where P approximates to 10^{-3}, the frequency of observation of 'false positives' in Tests for Sterility conducted under the best known conditions. Due to this inherent limitation of sterility testing, further increase in dose will not give a reduction in P (measured), so that the curve continues parallel to the D-axis. Thus, *actual* values of P are only seen at doses $<D_m$; these are the levels of dose thought of as 'sub-process' doses in our original idea. Faced with such a situation, the all important question is can a knowledge of P acquired at sub-process doses be used to obtain an estimate of the probability of a contaminated item occurring at the process dose D_p?

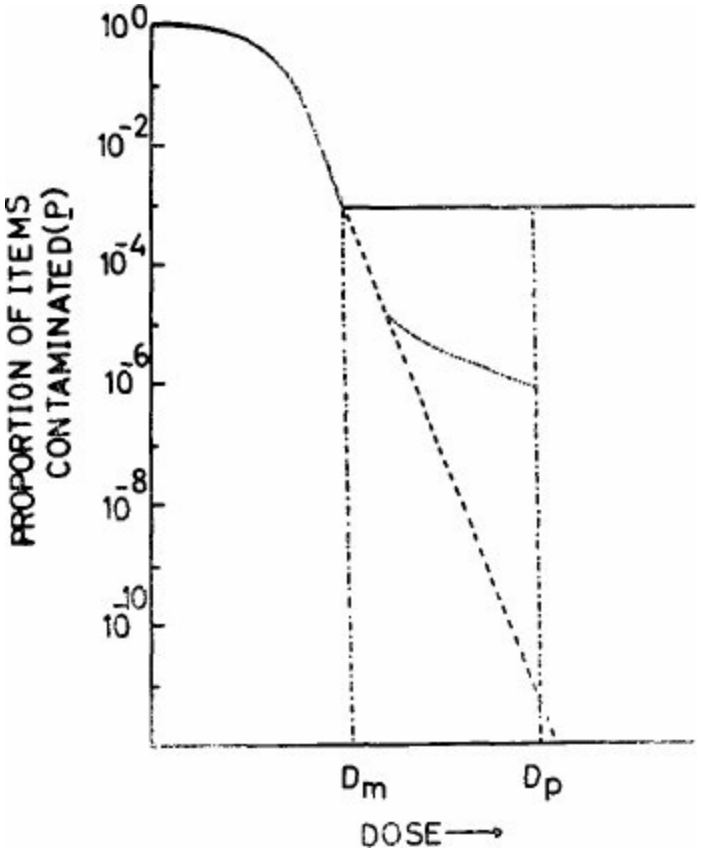

Figure *6.* Hypothetical curves, referred to in the text, relating the logarithm of the proportion of contaminated items *(ln P)* and radiation dose *(D).*

The simplest imaginable way of using sub-process data is to extrapolate the curve relating *ln P* and *D* beyond D_m to D_p (angled dashed line, Figure 6). Clearly the correctness of such an action is wholly dependent on the maintenance of the functional dependence of *P* on *D,* established experimentally for doses $<D_m$, over doses falling between D_m and D_p. In so far as the evaluated forms of the model (Equations [10] and [11]) provide a reasonable description of microbicidal events taking place on a population of items undergoing irradiation, the curves given in 1b and 5b provide information that bear upon this dependency. They reveal that in situations where items possess low numbers of contaminants of low sensitivity (L sensitivity) together with a majority of high sensitivity (H sensitivity) the dose/*ln* probability curves can take on distinct 'tails', and that with organisms of L sensitivity in sufficiently low numbers the 'tail' occurs at doses $>D_m$ (depicted hypothetically by the dotted line in Figure 6). As all available evidence says that production items are generally populated by organisms of heterogeneous responses to radiation (10, 11), the simple extrapolation of experimentally determined curves is categorically excluded.

If direct use of sub-process data is to be made, then other means of extending the *ln P*/*D* curve have to be sought. One basis for extension is the continuation of the curve from the point of discontinuity *($P = 10^{-3}$, $D = D_m$)* along a course that would be taken if all organisms populating items after exposure to D_m were of a particular type. Necessarily, this type would be one with a response characteristic of a known, relevant, highly resistant organism. For example, the response could be that of the type or types of organisms responsible for the measurement of *P* at or around D_m (i.e. an organism surviving the radiation dose D_m) or of a type used as a reference organism in radiation processing (2). In other words,

for purposes of assessing sterility assurance, D_p, the process dose, can be regarded as being made up of two components, a component D_m for which the effectiveness is truly known (it renders sterile 99.9% of items), and component *(D_p* minus *D_m)* whose effectiveness is estimated by reference to the response of a laboratory organism. The appeal of the treatment is that the estimate of P at D_p approaches the real value.

The principle of extension of the *ln P/D* curve in the way outlined above has now found application in practical situations. White (9) has used it in estimating the sterility assurance of a process sterilizing dose from results of sterility tests on items given a "substerilizing dose" of one level only. This dose, referred to as the *challenge* dose, fell between D_m and D_p. In effeect, the treatment described by White was to put the point ($P = 10^{-3}$, D = *challenge* dose) as the location of the discontinuity on the *ln P/D* curve and to extend the curve from there to D_p on the basis of a known response of a radiation resistant organism. White gives a simple expression, relating P and D over the range D_m to D_p, for the chosen organism of known radiation response. Recently Forsyth (12) has used the principle in a reverse operation, i.e. knowing D_m for a particular production item and after fixing an acceptable level of P at D_p, the process dose has been calculated. This operation, like White's, requires a judgement to be made about the relevance of information gained in the laboratory to a particular production situation for the extension of the lower portion of the *ln P/D* curve. While ideally judgement decisions should have no part in the definition of sterility assurance, the best that can be done at present is to limit them to a partial involvement.

Obviously, the conclusions drawn from evaluations of the type depicted in Figures 1 and 5 apply in a practical situation in as much as the assumptions made in deriving Equations [6], [10] and [11] are valid. Of these assumptions, the one relating to the distribution of numbers of microbial contaminants on items initially and the manner in which this distribution behaves with dose is probably the most arguable. Properly, the Poisson and Exponential distributions were chosen after due consideration had been given to available detailed data, but it should be emphasised that these data are from limited sources and are not necessarily representative of universal events. Very likely in practice the concern would be about frequencies of numbers of organisms on items which do not accord to a formal distribution and the way that these frequencies could influence sterility assurance. It is not difficult to imagine situations in the production of items destined for a sterilization treatment where irregular contamination of items would result. In fact, there are literature reports of "quite inhomogeneous" distributions of numbers of organisms at low mean values on two kinds of items (13). The influence on P of distribution of numbers of organisms is particularly important in relation to our earlier proposal (14, 15) regarding the use of sub-process data to monitor the overall microbiological quality of the production process. The basis of the proposal is the displacement of *ln P/D* curves upwards and/or to the right when conditions on items worsen microbiologically, i.e. when the average initial number of organisms/item increases or organisms of low response to radiation are present (Figures 1 and 5). For monitoring of this kind, we are interested in values of P (measured) at doses $<D_m$ that shift the above given pre-set limits.

Considerations such as the above have led us to examine possible means of assessing, by means of simulation models, the influence of the distribution of numbers and types of contaminants on items on the behaviour of the proportion of contaminated items with increasing dose. A simple example will serve to demonstrate the approach that we are presently adopting.

The basis of the approach is again the general model described by Equations [1] – [5]. For simplicity we consider items contaminated with organisms of one type, with each individual organism having an equal probability of inactivation by radiation. Assume that the contaminants are present initially at an average initial number of one viable organism/item on a population of four items. Two of the possible initial distributions of numbers of organisms on items are given in Figure 7 (Set 0); for present purposes the other possibilities are ignored. The two chosen, represent the extremes of distribution, one being symmetrical and the other exhibiting maximum asymmetry, and therefore best reveal the pertinent points. Imagine the application of an increment of radiation dose that inactivates 50 per cent of the population of organisms; the possible distributions resulting from this are given in Set 1. With a second increment of dose of equal size, the Set 2 distribution results. Clearly the behaviour of P on D, as shown by the curves at the bottom of Figure 7, differs in the two cases. The importance of this kind of differences in P to projected uses for measurements of P at dose $<D_m$ is self evident.

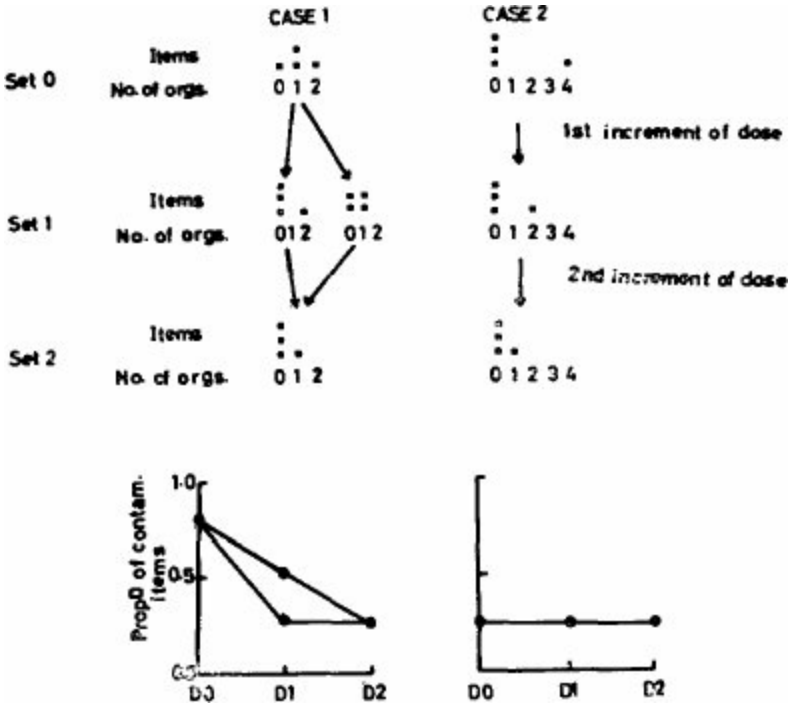

Figure 7. An illustration of how the initial distribution of numbers of organisms on items can influence the proportion of contaminated items *(P)* in a population of items.

The curves given in Figure 7 are analogous to curves derived from actual exercises involving the sub-process dose technique, although obviously, in practice, the initial conditions are open to greater variation and the populations of items are appreciably larger. An increase in population and in numbers and types of contaminants would result in a variety of permutations that are not readily handled in the simple manner depicted in Figure 7. Fortunately however, such variations in conditions lend themselves with relative ease to computing techniques. We are currently giving consideration to this problem.

Acknowledgement

This work was supported, in part, by research grants given by the U.K. Panel on Gamma and Electron Irradiation and by Irradiated Products Limited, Swindon, U.K.

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* The number of microorganisms of a particular type *(i)* surviving in an irradiated population is a function of radiation dose *(D)* according to

$$N(i) = N(o, i)\, \phi(D)$$

where $N(i)$ is the number of survivors of this particular type after dose (D), $N(o, i)$ is the number initially present $(D=0)$ and $\phi(D)$ is the dose/survival function for this type.

If $a(i)$ is the average number of organisms of type i/item at dose (D), then $N(i) = a(i).\, X$, where X is the number of items in the population and similarly $N(o, i) = a(o, i).\, X$, where $a(o, i)$ is the average initial number of organisms of type i at $D=0$.

In the evaluations, $\phi(D)$ was put equal to $1 - [1 - \exp(-kD)]^n$, the 'multi-hit' expression, thereby allowing the response of a given type of organism to be defined in a non-interpretive manner by substitution of appropriate values of the constants k and n.

Some Dose Rate Effects in Irradiated Micro-Organisms

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Abstract: *High dose rates of up to 10^{14} rads per second can be produced by high energy electron pulse generators. Dose rates of this order can alter the response of microorganisms to radiation relative to that obtained with conventional Co-60 γ- and X-rays at dose rates of order of 10^3 rads per second. These* direct *effects of dose rates are discussed with particular reference to effects on bacterial spores, since these organisms are often used as test systems in studies with radiation sterilization facilities. Factors which can influence the magnitude of the dose rate effects are: the duration of the electron pulse, the dose rate within the pulse and the chemical environment during irradiation.*

Apparent dose rate effects due to radiolytic depletion of oxygen are also discussed together with experiments where high dose rate is utilized to study the time scale of O_2-dependent radiation damage.

Introduction

Control of microbiological quality at all stages during the manufacture of medical and pharmaceutical products is necessary. This can be sometimes achieved by using biologically clean techniques with sterile starting materials during manufacture followed by appropriate treatment of the final product, in order to sterilize or reduce the level of contamination by a given species of micro-organism. High energy radiation can be used for this terminal treatment and it has been used with success in the treatment of pharmaceutical and other products where more conventional methods have proved inadequate (1).

For radiation sterilization, it is necessary to consider the response to radiation of both prokaryotic and eukaryotic organisms, since common contaminants are both bacterial and fungal in origin. Generally, all microorganisms are killed as an exponential function of absorbed dose although in some cellular systems there is a pre-exponential region, where at low doses the cells are more resistant to radiation. This resistant stage has been attributed to repair or to the accumulation of sub-lethal damage. Among the factors which may affect this pre-exponential stage or indeed alter the exponential response of organisms to radiation is the rate at which the cells are irradiated.

In this paper, we discuss the effect of dose rate on the radiation response of microorganisms with particular emphasis on the bacterial spore system. This is because the spore has not only an inherent

radiation resistance but it is used extensively as a test organism when determining the efficacy of radiation sterilizing facilities (2). In addition, apparent dose rate effects have been observed in systems which are initially in equilibrium with oxygen but are irradiated under conditions where the oxygen is consumed by radiolytic processes at a rate faster than that at which it is replenished from the surrounding environment. The implications of oxygen depletion, which can also occur in closed systems at low dose rates, will be discussed in relation to radiation sterilization. High dose rates have been used to study the time scale of radiation-induced O_2-dependent damage in various cellular species and results from such studies will be reviewed. This paper follows on from a previous paper in this conference series (3) and, for clarity, some of this earlier material is combined with more recent literature data.

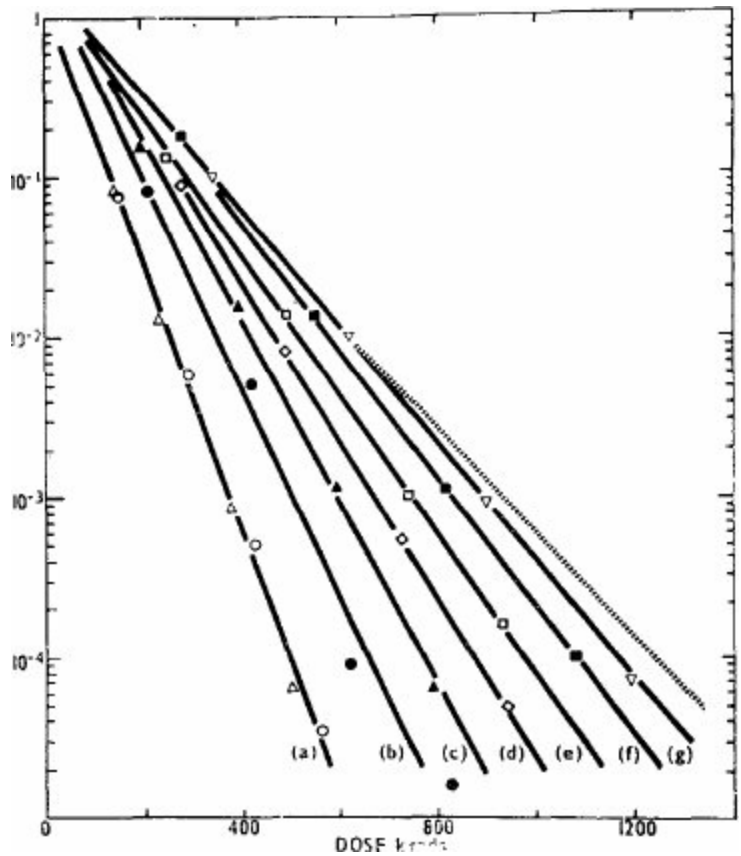

Figure *1.* Survival curves for *B. megaterium* spores irradiated in anoxia with 12 MeV electrons at dose rates ranging from 9870 rad/μs (b) to 51 rad/μs (g). Broken line, anoxic response to γ-rays. Reference (4).

Direct Dose Rate Effects in Cellular Systems

Irradiation under anoxic conditions

Purdie, Ebert and Tallentire (4) studied the effect of irradiation with pulsed 12 MeV electrons on *Bacillus megaterium* spores in aqueous suspension in an attempt to find effects that could be related directly to the reactions and lifetimes of transient radiation-induced chemical species in the spore. It was found that as the instantaneous dose rate was increased, i.e. the dose rate within the pulse at fixed pulse length and pulse frequency, a progressive increase in radiation sensitivity of the spore was observed (Figure 1). At the lowest dose rate of 51 rad/μs, the value of the inactivation constant *k,* the slope of the

survival curve, is 8.64×10^{-3} krad^{-1}, which is similar to that seen after γ-irradiation under anoxic conditions. As the dose rate increases, the value of *k* increases, giving a value of 14.3×10^{-3} krad^{-1} at 9870 rad/μs, the highest dose rate tested. In an attempt to find a relationship between dose rate and the increase in radiation sensitivity, these authors expressed the change in the slopes of the survival curves, i.e. the difference in values of *k,* as a function of the square root of the dose rate (Figure 2). This was to test a hypothesis that the increase in spore sensitivity at high dose rates is due to an increase in bimolecular reactions between radicals produced within the cell. The apparent linearity of Figure 2 suggests that radical-radical interactions, which are likely to become more important as the dose rate increases, lead to lethal events in the spore.

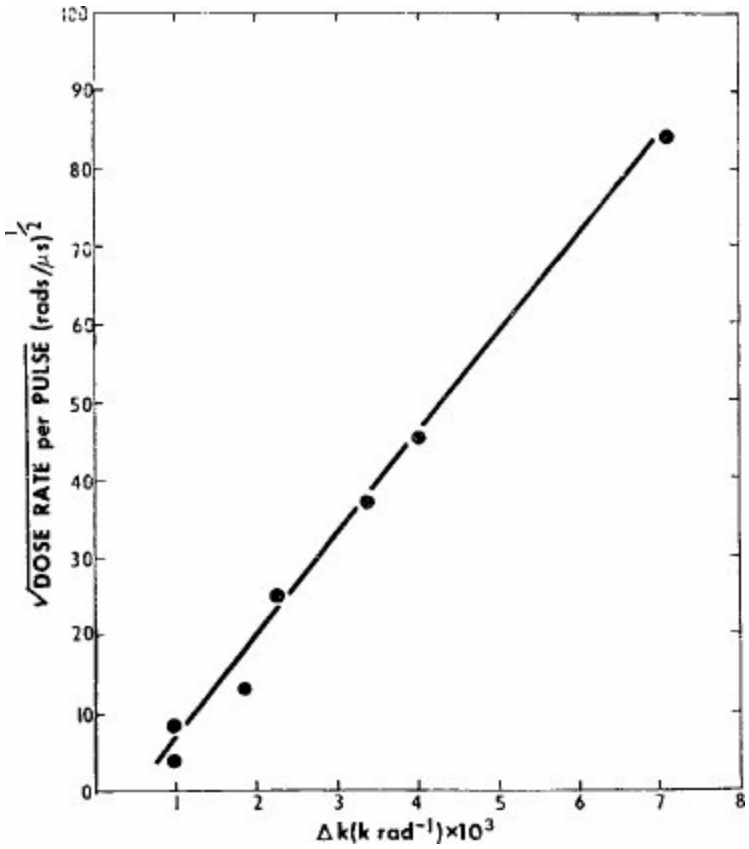

Figure *2.* The relationship between (dose rate)^{½} and Δ*k*, the increase in *k* over that value seen after γ-irradiation under N_2. Reference (4).

In order to investigate the involvement of free radical reactions, Purdie et al. (4) studied the influence of some free radical scavengers on the magnitude of the dose rate effect. The OH scavengers, potassium thiocyanate, t-butanol and sodium formate, were found to reduce the dose rate effect by up to 50%. Typical results for spores irradiated in the presence of 0.1M KCNS are shown in Figure 3. These results would indicate that since the scavengers act by removing OH, then half the dose rate effect must be due to processes involving OH radicals. This is in line with the results of Powers and Cross (5) who investigated the effect of N_2O on the radiation sensitivity of irradiated spores. N_2O reacts with hydrated electrons to produce an equivalent amount of OH radicals. Powers and Cross found that at low dose rates, the presence of N_2O increased the sensitivity of the spores by an amount equivalent to about half the increase observed in the dose rate effect. At high dose rates, i.e. 6934 rad/μs, above which no further increase in the dose rate effect is observed, the presence of N_2O did not affect the radiation sensitivity of the spores (4).

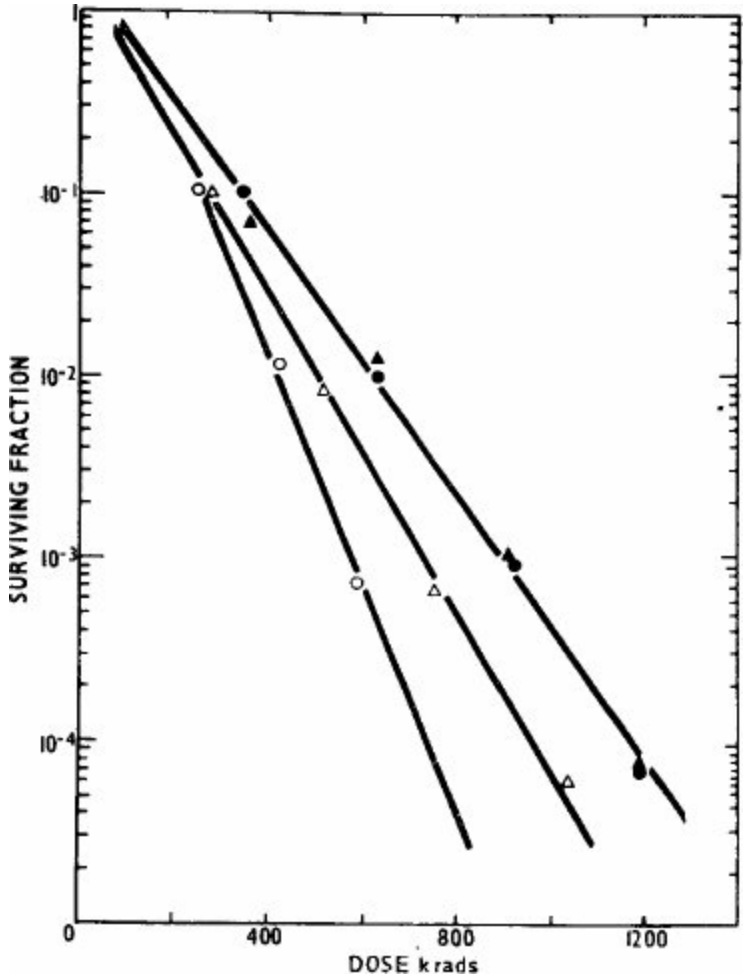

Figure *3.* Effect of 0.1M KCNS on the anoxic response of spores; ○, 6934 rad/μs; Δ, KCNS + 6934 rad/μs; ●, 51 rad/μs; ▲, KCNS + 51 rad/μs. Reference (4).

Table I. — Effect of electron pulse length on inactivation efficiency for irradiated *B. megaterium* spores. Reference (4).

Pulse length (μs)	Dose-rate (rad/μs) ($\times 10^{-3}$)	k (krad^{-1}) ($\times 10^{3}$)	s.e. of k ($\times 10^{3}$)	Extrapolation number (n)
5.00	6.9	14.71	0.26	4.03
2.00	9.1	12.40	0.29	2.13
1.00	10.8	10.77	0.24	1.79
0.50	11.2	10.46	0.17	1.90
0.10	11.8	9.07	0.15	1.73
0.05	11.7	8.98	0.10	1.64

Table I shows data from Purdie et al. (4) for anoxic *B. megaterium* spores irradiated with trains of electron pulses at a repetition frequency of 50 pulses/second. The pulse length was varied from 0.05 to 5μseconds. The instantaneous dose rate, i.e. the dose rate within the pulse decreased from 11.7×10^{3} rad/μsec. for the shortest pulse, to 6.9×10^{3} rad/μsec. for the longest pulse. However, in contrast, the

inactivation constants increased as the pulse length increased. This increase in inactivation efficiency with pulse length was attributed to build-up of transient radical species in the spore. When the concentration of these species is sufficiently high, radical-radical interactions occur, which it is suggested, lead to increased inactivation efficiency.

The results imply that the lifetime of these critical free radical species is considerably longer than the duration of the pulses. However, since no effect was observed when the pulse repetition frequency was changed from 50 to 5 pulses per second, the lifetimes must be much less than the shortest pulse separation time of 20 milliseconds.

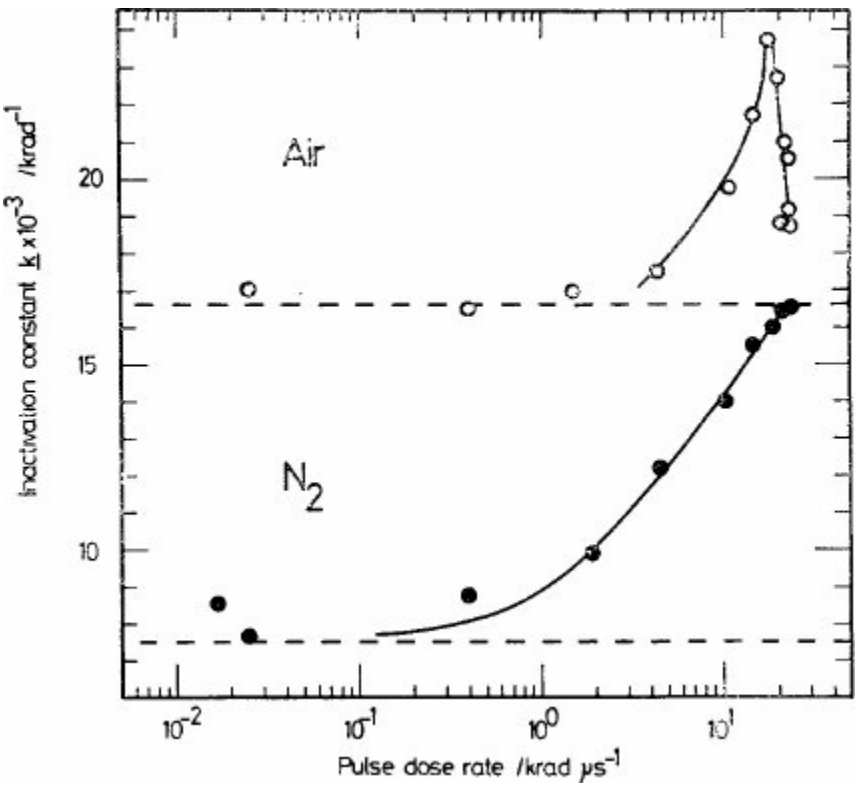

Figure *4.* The responses of *B. megaterium* spores irradiated in O_2 at various dose rates. For comparison, irradiation in N_2 at similar dose rates is shown as the dashed line. Reference (6).

Irradiation under initially oxygenated conditions

In an extension of the anoxic work reported above, Saleh carried out similar experiments with *B. megaterium* spores initially equilibrated with oxygen. Figure 4 shows that varying the instantaneous dose rate for spores in O_2 gives results almost the same as those seen when irradiation is in anoxia. For each experimental condition survival curves were linear over the dose ranges tested. Dose rates of up to 1000 rad/μs gave responses that were identical to these seen for continuous γ-irradiation. For dose rates above this value, the spores became more sensitive reaching a maximum response when the dose rates approach 2×10^4 rad/μs. Irradiation with higher dose rates result in a lessening of the response in oxygen. Thus, under these conditions, the oxygen enhancement ratio appears to fall.

Other experiments in air at high dose rates show a similar pattern to those seen in N_2. With the OH radical scavenger potassium thiocyanate present during irradiation in air, a reduction in response is observed (6).

The reduction in OER with *B. megaterium* spores described above is also seen with *B. pumilus* E601

spores (6). Similar effects were also obtained with *E. coli* K12, AB 2480, a uvr$^-$rec.A$^-$ mutant (7). With this organism it was found that increasing the dose rate up to 1000 rad/μs reduced the OER from 1.6 (for γ-rays) to about 1.0. This reduction in OER is not seen with wild type *E. coli* K12. These results illustrate that *direct* dose rate effects can occur in organisms other than bacterial spores.

In the results discussed so far, model systems using washed bacterial spores in aqueous suspension have generally been employed. In practice, for the sterilization of medical or pharmaceutical products, contaminants may be found in a wide variety of environments, ranging from dry or non-aqueous to aqueous media. Results from experiments, where *B. megaterium* spores have been irradiated while suspended in 50% ethylene glycol, a type of vehicle commonly used in the formulation of topical preparations, are relevant in this respect (8). Figure 5 shows that a dose rate effect is seen when radiation is delivered on the krad/min time scale, with responses varying from a k value of 16.2×10^{-3} krad^{-1} at a dose rate of 1.8 krad/min to $k = 10.7 \times 10^{-3}$ krad^{-1} at 7.2 krad/min. Under similar conditions, spores irradiated in an aqueous environment, over the same range of dose rates, give a constant response similar to that seen at 1.8 krad/min in Figure 5. These results show that variations in dose rate may arise as a result of the environment in which the cells are irradiated.

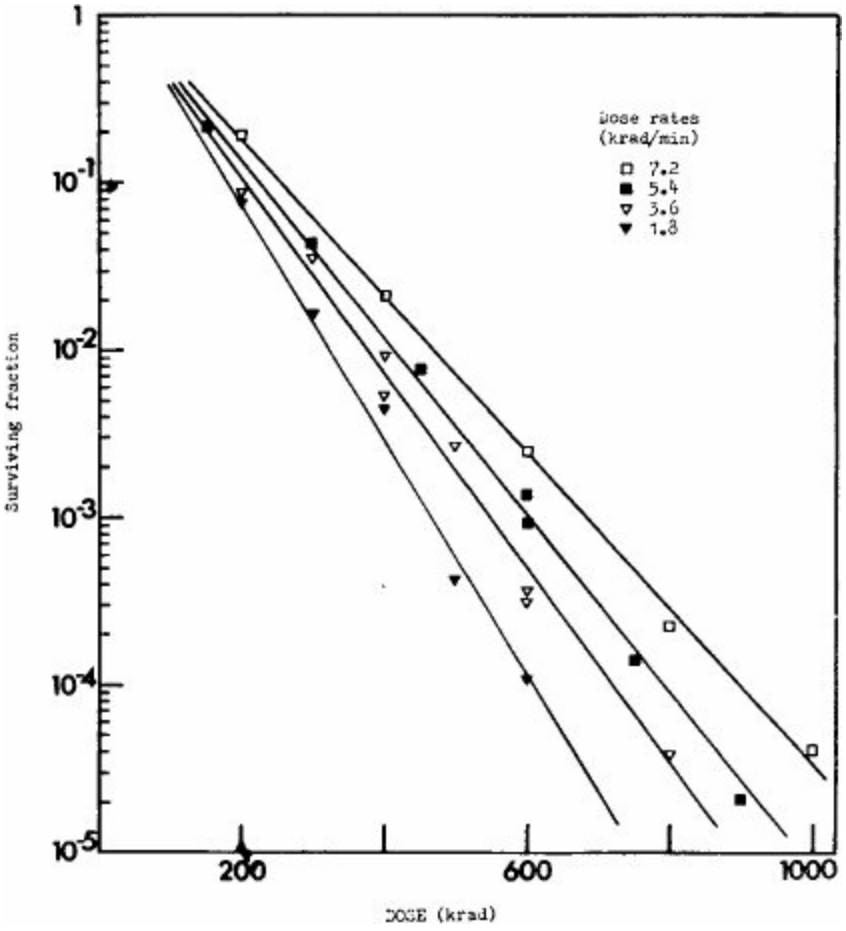

Figure 5. Survival curves for *B. megaterium* spores suspended in 50% ethylene glycol, irradiated in air at various dose rates. Reference (8).

So far the discussion has been restricted to dose rate effects on the exponential portion of cell survival curves. Many organisms show a resistant pre-exponential stage where the existence of a shoulder on single dose cell survival curves is thought to be due to the accumulation of sub-lethal damage (SLD). Cells can recover from this SLD after low LET irradiation, but a fractionated

experiment is required to demonstrate this recovery (9). However, if each fraction is delivered at ultra-high dose rates, such as 3×10^4 rad/μs, then it has been shown that recovery from SLD is not only reduced, but also delayed (10, 11).

Eukaryots and prokaryots, which have the capacity to accumulate and repair radiation damage, are also affected by very low dose rates. Doses delivered at the rate of tens of rads per hour will allow repair processes to occur while SLD is accumulating. Hall (12) has reviewed the effects of low dose rates on mammalian cells.

Dose Rate Effects Due to Oxygen Depletion

These were first recognized and characterized by Dewey and Boag (13) who compared the response of *Serratia marcescens* irradiated with X-rays and 1.8 MeV pulsed electrons (Figure 6). The lines labelled "X-ray" are the survival curves for *Serratia marcescens* irradiated in the absence of oxygen and in medium containing either 1% or 100% oxygen. The full OER is 3.1 under these conditions. The open circles on the upper line are the data for anoxic bacteria irradiated at a high dose rate with single 2/μs pulses of electrons and the closed circles the corresponding data for 1% oxygen. With the low dose rate X-rays, 1% oxygen is sufficient to produce 60-70% of the full oxygen effect. In contrast, bacteria which were pulse irradiated in the presence of 1% oxygen show the same radiation sensitivity as the hypoxic bacteria. The essential difference between the two modes of irradiation is that in the high dose rate case the O_2 is radiolytically consumed at a much faster rate than it is replenished from the surrounding environment. This is illustrated in more detail by the data of Epp, Weiss and Santomasso (14) who studied the sensitivity of *E. coli* B/r to a 30 ns pulse of electrons for a range of doses and oxygen concentrations. The curves for nitrogen and 100% oxygen follow simple exponential survival kinetics (Figure 7), but for lower concentrations of oxygen, the curves exhibit two distinct components. The 'break points' in each of the curves occur at the doses where radiolytic depletion of oxygen inside the cell becomes critical. In contrast to the experimental conditions used by Dewey and Boag (13), oxygen is present in the gaseous environment around the cells in the experiments of Epp and co-workers (14). Radiolytic consumption of oxygen *extracellularly* is much less efficient in the gas phase than it is in suspension. However, the rate of diffusion of oxygen into the bacteria is less than the rate of its consumption inside the cell and so the break point phenomenon is observed.

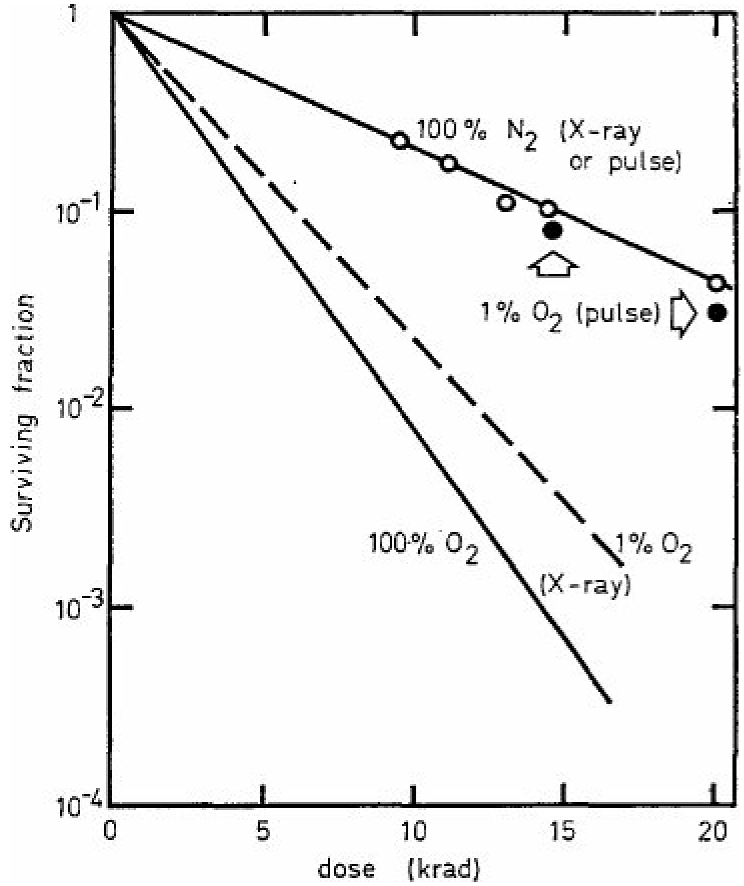

Figure *6.* Radiosensitivity of *Serratia marcescens* irradiated at low dose rates (X-ray) and at dose rates of about 10^4 rad/μs (pulsed electrons). Reference (13).

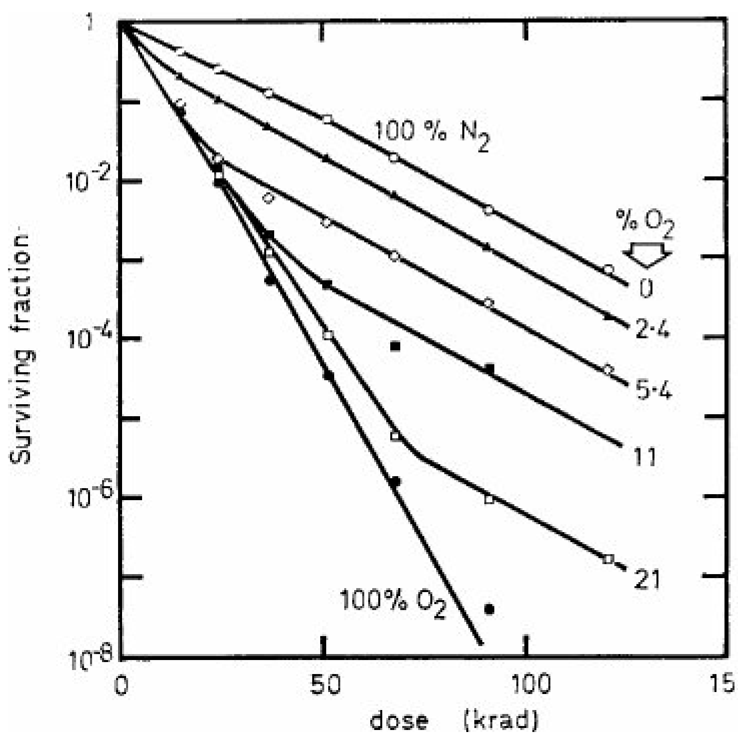

Figure *7.* Radiosensitivity of *E. coli* B/r irradiated with single 30ns pulses (dose rate about 2×10^6 rad/μs) at various O_2 concentrations. Reference (14).

If irradiations are carried out under conditions where replenishment of O_2 is impossible, i.e. in

closed systems, breaking survival curves of the type seen above will be observed irrespective of dose rate, indicating, that O_2-depletion is a *dose* effect rather than a *dose rate* effect. This is shown by some work of Weiss, Epp, Heslin, Ling and Santomasso (15), who irradiated with Co-60 γ-rays, *E. coli* B/r in suspensions initially equilibrated with various O_2 concentrations in sealed vessels (Figure 8). From these data, the authors calculated the doses required to remove all the O_2 from suspensions in a closed environment. They found that a suspension initially equilibrated with air requires in excess of 50 krad to remove all the O_2. Therefore, after irradiation with doses greater than 50 krad in closed systems, cells in suspension are, in effect, in an anoxic environment.

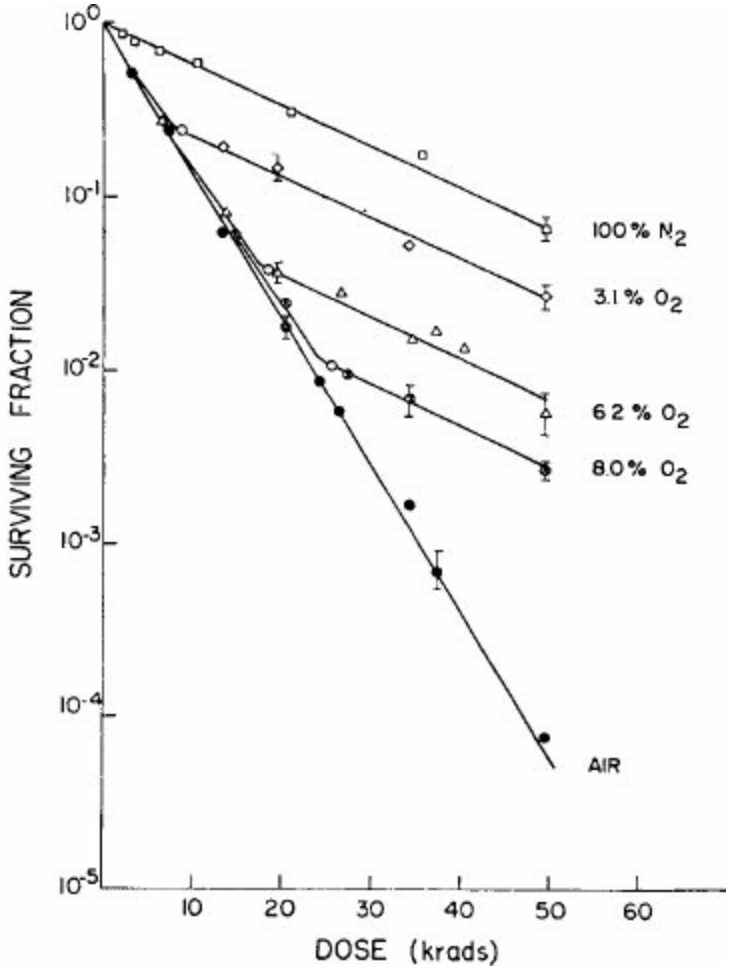

Figure *8.* Survival curves for *E. coli* B/r equilibrated with various concentrations of O_2 and irradiated in sealed vessels with Co-60 γ-rays. Reference (15).

The Lifetimes of Oxygen-Dependent Damage

Epp and his co-workers (16) have used the break point phenomena illustrated above to determine the lifetime of O_2-dependent damage and the time scale of O_2 diffusion into the bacterium *E. coli* B/r. In these experiments, bacteria held on a Millipore® filter in contact with a gas mixture containing a known amount of oxygen are exposed to *two* pulses of electrons. The pluses can be separated in time by amounts ranging from microseconds to seconds or longer. The first pulse is sufficiently large to consume by radiolytic action all the oxygen inside the cell. If the time required for oxygen to diffuse into the cell is longer than the time interval before the second pulse, the radiation response to the

second pulse will be that of anoxic bacteria. If, however, the time is shorter than the pulse separation time, the cells will become re-oxygenated and will be correspondingly more sensitive to the second pulse. Some of the data are reproduced in Figure 9 for oxygen concentrations in the gas phase of 4%. From experiments of this type it was concluded that, under these conditions, the upper limit for the lifetime of the oxygen-sensitive damage in the bacteria was 10^{-4} seconds.

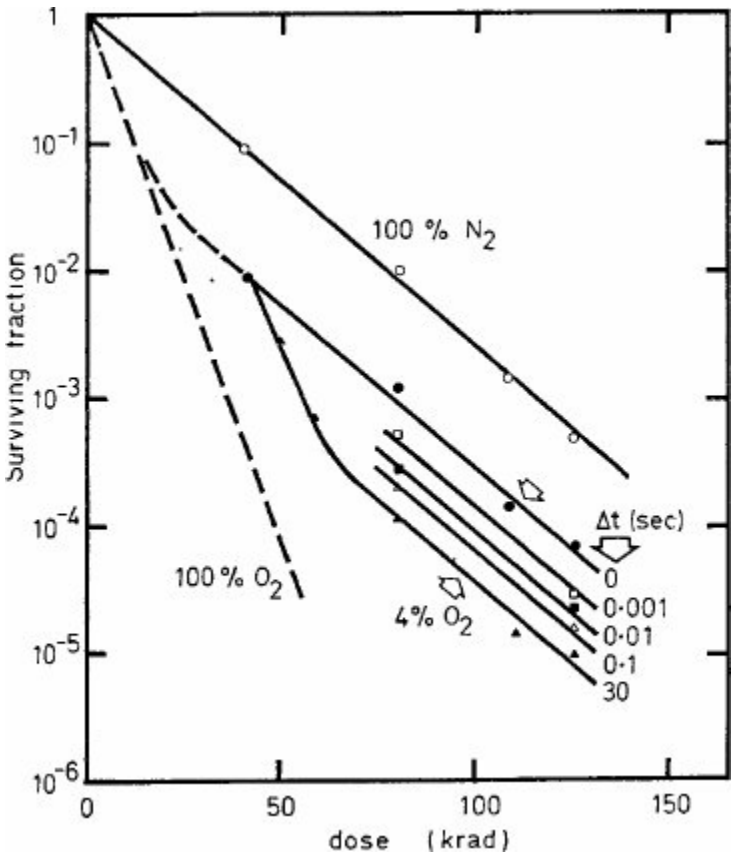

Figure *9.* Radiosensitivity of *E. coli* B/r irradiated with two 3 ns pulses (dose rate about 2×10^7 rad/μs) separated by a varying interval Δt. Reference (16).

An alternative method for looking at the time scale of the O_2 effect has been described (17). In these experiments the bacteria are mounted on Millipore® filters fitted inside a chamber which is flushed with humidified N_2. The cells are exposed to an explosion of O_2 released into the chamber through a fast acting solenoid-operated valve, either just before or just after irradiation of the bacteria with a single 2/ *μ*s pulse of electrons. Some of the data obtained by this technique are shown in Figure 10. The surviving fractions of bacteria irradiated at three dose levels are plotted as a function of time interval between irradiation and exposure of the cells to O_2. At each dose, when the bacteria are in contact with O_2 before irradiation even at the shortest resolvable time-interval of 100 *μ*s, the survival level is the same as that found for normal oxic irradiation. However, when the oxygen contact occurs after irradiation, the surviving fraction increases over the time range 0-2 milliseconds. When contact occurs later than 2 milliseconds the level of survival is that normally observed for anoxic irradiation. The results indicate that, under the conditions of this experiment, the damage which is sensitive to oxygen has a half-life of several hundred microseconds. This technique has been developed further to look at the time scale of damage in mammalian cells where the lifetime of O_2 sensitive species has been found to be about 10^{-3}s (18).

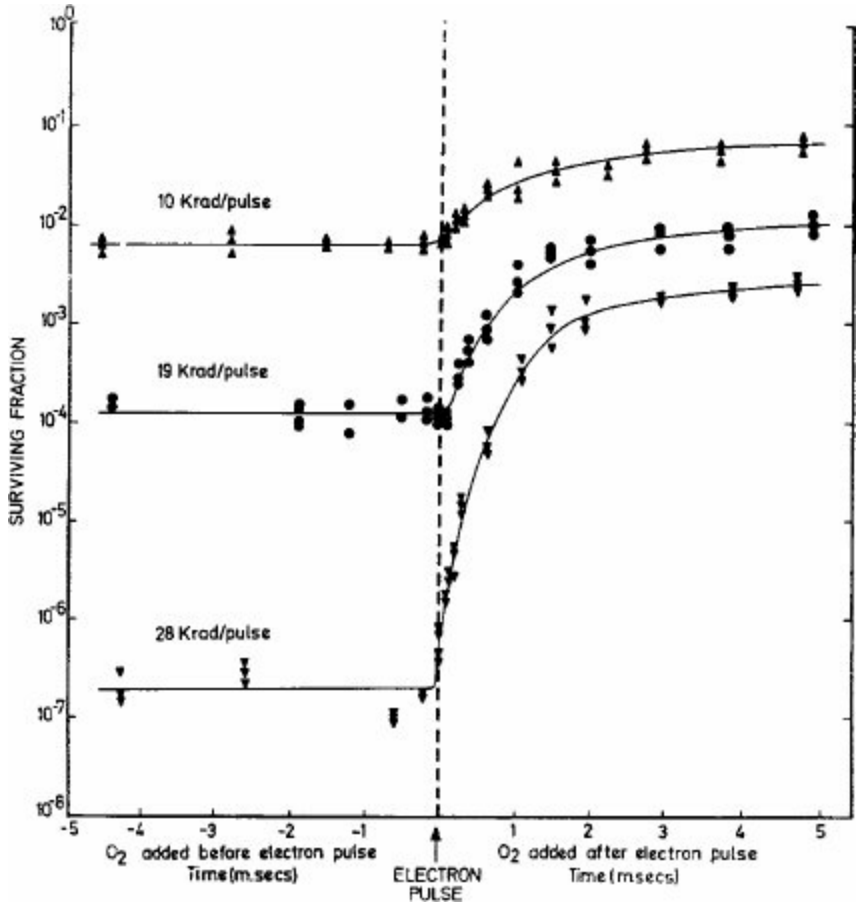

Figure *10.* Survival of *Serratia marcescens* irradiated with a single 2 *μ*s pulse (dose rate about 10^4 rad/μs) with O_2 added at varying intervals before or after the pulse. Reference (17).

The lifetimes of the O_2-sensitive species reported above for vegetative bacteria and mammalian cells differ by many orders of magnitude from those seen in fully hydrated *B. megaterium* spores (19). Here the potentially lethal damage persists for many minutes after irradiation and the long lifetime has been attributed to the low hydration status of the spore core. A liquid-mix stopped-flow technique was adopted for the work with spores. With it, spores in suspension are given a fixed dose of radiation with a train of 2-*μ*s pulses of electrons delivered at 650 Hz. After irradiation, which extended for about 1s, the spore suspension was mixed with oxygenated water. Figure 11 shows typical data for spores irradiated anoxically followed by post-irradiation addition of O_2. Kinetic analysis of the results in Figure 11 and other data enabled the authors to identify two independent rates of decay of the damage which is sensitive to oxygen, with respective half-lives of 9s and 120s (20).

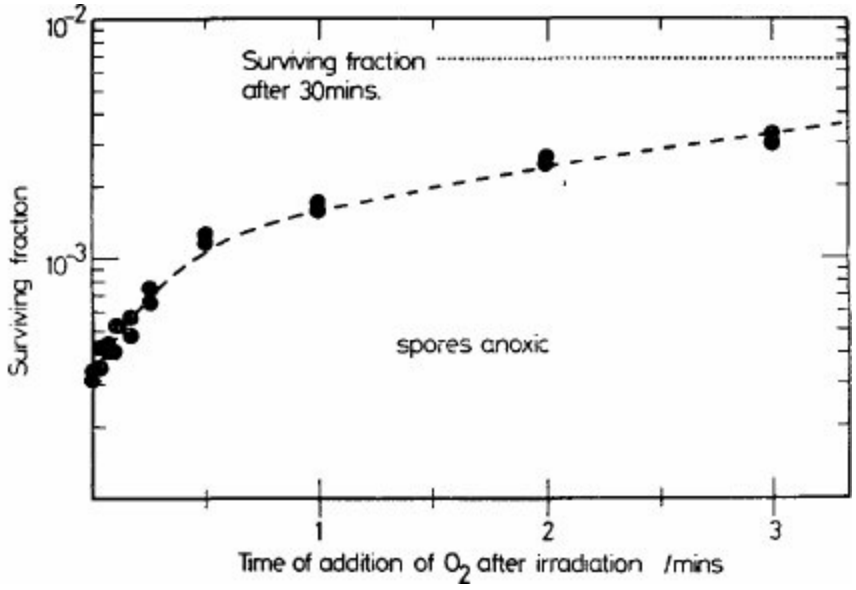

Figure *11.* Survival of *B. megaterium* spores irradiated in anoxia with pulsed electrons (dose rate about 10^4 rad/μs) with O_2 added at various times after irradiation. Reference (20).

Some Conclusions

Radiation sterilization is generally carried out with very large doses requiring the use of high-dose rate sources. If the dose rate is sufficiently high, oxygen depletion will occur early in the radiation procedure if the rate of diffusion of oxygen into the contaminating organisms is too slow to replace oxygen consumed by radiolytic action. Depletion will occur at all dose rates if the sterilization is carried out in a closed system with insufficient oxygen in the gas space.

It is likely that under the conditions normally used for sterilization with pulsed irradiation from a linear accelerator oxygen depletion will be a fairly common complication. Even with high-dose rate irradiation from continuous sources, depletion may still occur. This implies therefore that some lost efficiency of sterilization occurs because of anoxic radiation resistance.

The studies with spores and other microorganisms show, however, that if the dose rate is very high, an increase in anoxic radiation sensitivity can occur with pulsed irradiation due to the build-up of reactive free-radical species during the pulse. This phenomenon appears to become important at instantaneous dose-rates of the order of 10^4 rad/μs. This is somewhat higher than the dose rates which apply in commercial radiation sterilization procedures. It is debatable whether this particular dose rate effect contributes under such conditions.

Acknowledgement

The authors thank Drs. Harrop, Michael, Rupp, Tallentire and Watts for allowing them to quote their unpublished data.

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Water as a Modulator of Radiation Damage to Microorganisms

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Abstract: *The optimization of the sterilizing actions of ionizing radiation against bacterial spores is discussed in terms of the role of water in the radiation induced effects of these microorganisms. In the dry spore, which is the most sensitive state in O_2, three classes of damage are recognized, one O_2-independent and two O_2-dependent, and each responds differently to added water: anoxic sensitivity increases at highest water contents as water monolayer formation is complete, and both O_2-dependent effects are reduced, with the component due to a long-lived free radical interaction with O_2 being lost at saturation.*

The response to radiation of spores suspended in aqueous solutions is shown to be dependent on the redox potential and the concentration of added solutes. In this light, the effects of the radiation sensitizers, Ag^+, biacetyl, cobalt hexammine and O_2 are discussed and their relationship to damage induced by ·OH considered.

Introduction

For manipulation of the radiation sensitivity of living organisms one should attempt to know the roles of water in the initiation and development of the effects induced by radiation. Furthermore, those interested in maximizing the radiation sensitivity of organisms in the "dry" state that may be contaminants of materials that are desired sterile also might be interested in the extent to which water in small concentrations is important in determining radiation sensitivity. During recent years, several researchers have attended to this general problem, and a brief summary of the general state of knowledge concerning it is presented here.

Some Properties of Water Relevant to the Problem

General summary descriptions of the physical and chemical properties and the general behavior of water as well as its behavior in cells in terms of these properties can be cited here for the record. (1–6)

Extracting from these, we present here a brief summary of some of the characteristics of the water molecule in its two important phases that may relate to the biological evidence that vater does modify radiation sensitivity

Liquid Water — The water molecule itself consists of two hydrogen atoms covalently bound to a

single oxygen atom (Figure 1). The angle of 104.5°, together with the electron affinity of O_2, results in the setting up of an electronegative end on the oxygen opposite to that on the hydrogen ends of the molecule. This is the water dipole which gives liquid and solid water very particular characteristics, accounting for many of the empirically observed properties of water. Each molecule of water acts as a small dipole meaning that water molecules in bulk have special, ordered relationships among themselves. The orientation of plus to minus charges set up clusters of structured water (Figure 1). In ordinary water at ordinary temperatures, these clusters may consist of as many as 50 to 60 water molecules. These are transient structures, however. They break down easily, having an average lifetime (1/e time) in ordinary water of about 10^{-11} seconds (about 100 × the molecular vibration period). It is estimated that in a total volume of water approximately 30% mole fraction of the water molecules may be in this ordered or structured form. Because of their transient nature, the word "flicker" has been introduced as the colorful descriptive term.*

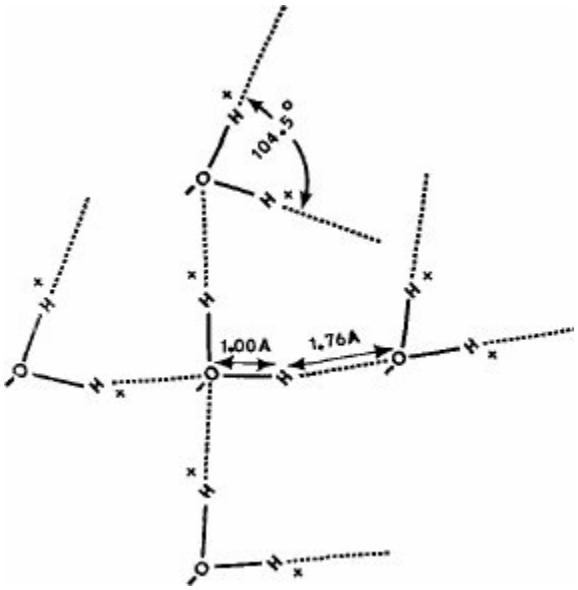

Figure *1.* The hydrogen bonds (dashed lines) between and among water molecules. The distances noted are from ice.

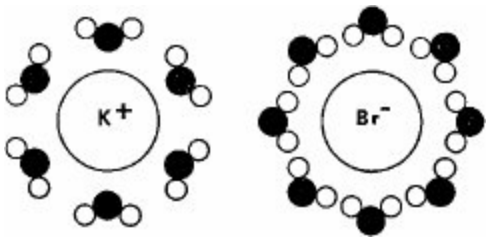

Figure *2.* The inner-most water shells associated with ionized KBr in aqueous solution.

The polar nature of water also determines its solvation properties. Water acting as the dipole actually can be thought of as the separating agent for ionic species such as potassium bromide, as in Figure 2. Polar groups on large molecules also "structure" surrounding water molecules, as in the diagram of the nucleic acid backbone (Figure 3). This is the water of "specific hydration" referred to again below. The biologically important class of compounds called amphiphiles with polar and non-polar ends have special relationships to water because of the polar nature of the water molecule.

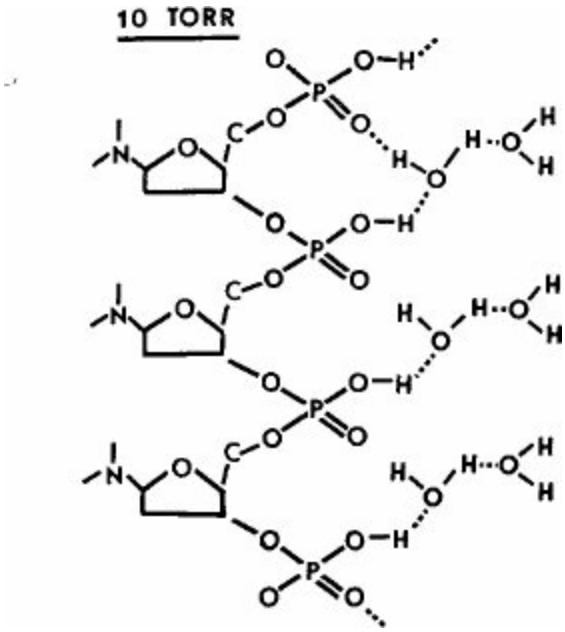

Figure *3.* Postulated relations between water and the sugar-phosphate chain of DNA. (5)

The ordering of water molecules around charged regions or particles can be extended even to the electron itself, which, when thermalized in polar liquids, has particular associations with the surrounding water molecules. This electron is the solvated electron and in water the hydrated electron has been demonstrated to be an important free radical species (Figure 4) produced in the radiolysis (break-down) process induced by high energy photons and charged particles in water and aqueous systems (8). The study of these hydrated electrons in chemical and biological processes affected by radiation is a thriving scientific industry. We shall refer to this activity later.

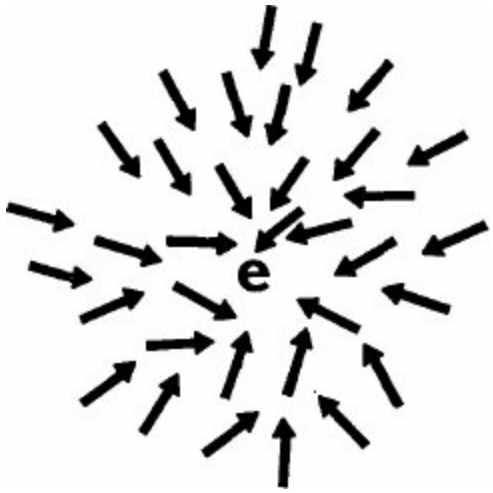

Figure *4.* A diagram of the hydrated electron, an electron of sufficiently low kinetic energy to allow ordering of water dipoles around it.

One other ordered structure, the hydrate clathrate which is a well recognized and described entity has not been investigated kinetically, as far as I am able to ascertain. Davidson (9) describes these clathrates and the parameters important in their formation and maintenance of stable configurations. Figure 5 demonstrates that the guest, in this case t-butylamine, induces orientation of water molecules in precise ways to form a highly ordered cage, stabilized by Van der Walls forces. This configuration is a much more stable and well ordered structure than the hydrated electron cage, in which electrical forces

cause looser orientation of the water molecules that are in more mobile states. Some authors postulate that the single water molecule itself could conceivably induce clathrate formation in a pure aqueous system. The conditions in the cell are such that it is allowable that clathrates are an important part of the structured water. However, there is little evidence for this, although the formation of clathrates has been invoked as a basis for anesthetic action, because of the ability of many of the anesthetic gases to induce stable clathrates in water systems.

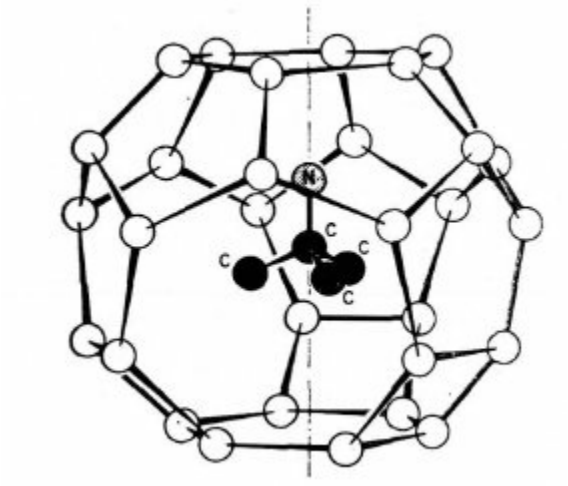

Figure *5.* A water clathrate, consisting of 30 water molecules about the "guest", in this instance *t*-butylamine. The number of surfaces (17) dictates the name heptakaidecahedron. (9)

Solid Water — The hydrogen bonding structures are much more stable in ice than in liquid water: the rotational period in ice is 10^{-5} seconds compared to 10^{-11} seconds in liquid water. An important innovation in ideas concerning the structure of ice is the proposition that the ice lattice has empty spaces, or shafts, which accounts for the decrease in the density of ice as it freezes (or, ice floats). This kind of structure is not necessarily that which obtains in relatively stable water molecules of biological macromolecules (see below). It is of interest that solid ice does exhibit electrical conductivity, although its rate is considerably less than seen in water. As a matter of fact, proton mobility is surprisingly high in ice, and we can wonder whether charge transfer of some sort might not be important in the water sheaths about large molecules in the living cell.

The Relations of Water and the Cell

How is water related to the structures in the living cell? The cell is itself a highly ordered structure on the ultramicroscopic level with ordered membranes that are polar and non-polar, of micelle-like structures that are polar and non-polar, of many kinds of molecules large and not large that are polar and non-polar, and many combinations of these. We can easily recognize now that there are many answers to the question. In times of less knowledge, early investigators of cell water introduced the single concept of "bound" water to account for the empirical observation that some water is easily removed from cells while other water is retained at ordinary temperatures and must be "boiled" out of the cell, indicating a tight relationship between a small portion of the water and the cell. The term, useful at first, was revealed a loose one that may not accurately describe any given cellular situation. The very fact that one can talk about 30° and 60° water means that we are dealing with a situation not definable except in terms of the empirical observation itself. Olmstead (3) uses the term "nebulous

concept" in describing it, and Berendsen (10) cites eleven different definitions of "bound" water each of which depends on the method that is used to recognize it.

But a terminalogy is needed for various cell-water relationships that are being described. For example, using nuclear magnetic resonance techniques that allow distinction between ordered and non-ordered water, Hazelwood et al. (11) recognize two phases of ordered water in skeletal muscle, a major and a minor that account for *most* of the water in the muscles; this means there are three phases. In our studies of the relation(s) between water and radiation sensitivity in the bacterial spore (12), we recognize at least three phases of water, two of which are "tight" and these may be ordered as in muscle. (This is discussed in detail below). There are compartments of water in cells recognizable by physical as well as functional tests.

But most of the studies have been made on macromolecules in solution rather than intact cells. Berendsen's (10) general summary of the relation of water to biomacromolecules is that there are two kinds of binding relationships. The first is *specific hydration* in which water is irrotationally bound with at least two — and sometimes three or four — hydrogen bonds to particular appropriate sites on the macromolecule. The water molecules can also hydrogen bond with each other. The residence times are long — 10^{-6} sec to a few seconds, and the total amount of water is small — for proteins in solution 0.2 – 0.5 moles water/100 g of macromolecule, or about 5% (w/w) specific hydration. There is additionally a larger component that is different from both bulk water and the water of specific hydration. It can amount to 3 moles water/100 g macromolecule (50% w/w). It does not freeze even at very low temperatures; yet the rotational time (the measure of rigid structure) is only slightly lower than that of non-associated water. Exchanges between this layer and bulk water are rapid.

Thus most of the associated water is liquid — and not ice-like — and the differences between the two liquid waters are not great. But Berendsen remarks that marked thermodynamic change can be brought about by forces that cause only minor differences in dynamic behavior, and large functional differences could exist in the two liquid waters — associated (water of *nonspecific hydration)* and bulk. His summary ends with "Critical and clear-cut experiments, rather than solid-state theories, are needed to evaluate such a role of water in biological systems."

There follow now descriptions of experiments in our spore system that are addressed to the roles (we use the plural) of these waters in modifying radiation sensitivity.

Water as a Modifier of Radiation Sensitivity in Cells

There are two general methods useable for study of this complex phenomenon. In the first, one uses cells that are dried and then rewetted to controlled degrees and irradiated in several physical and chemical conditions. This requires considerable demand on the cell, for it must retain biological integrity after severe, un-physiological treatments: extreme desiccation, very high and low temperatures, exposure to toxic substances in high concentration, and combinations of these. The second is like unto the first, except that the cell is investigated in suspension. The rigor of the treatments and the demands on the cell are like those made in the first e.g., suspension of cells in 100% alcohol and hexane with retention of viability has been required in some of the experiments.

Years ago the bacterial spore was chosen by us as the cell that could be considered experimentally almost as another chemical, and, with proper consideration of its basic biology, we still could ask of it proof of biological integrity: it had to germinate and produce a colony of cells. It is the most

appropriate cell-type for investigation of the many questions of water function. It is appropriate that the bacterial spore is the biological object of most interest to the conveners and participants of this conference. For these two reasons, the following considerations are confined to the bacterial spore.

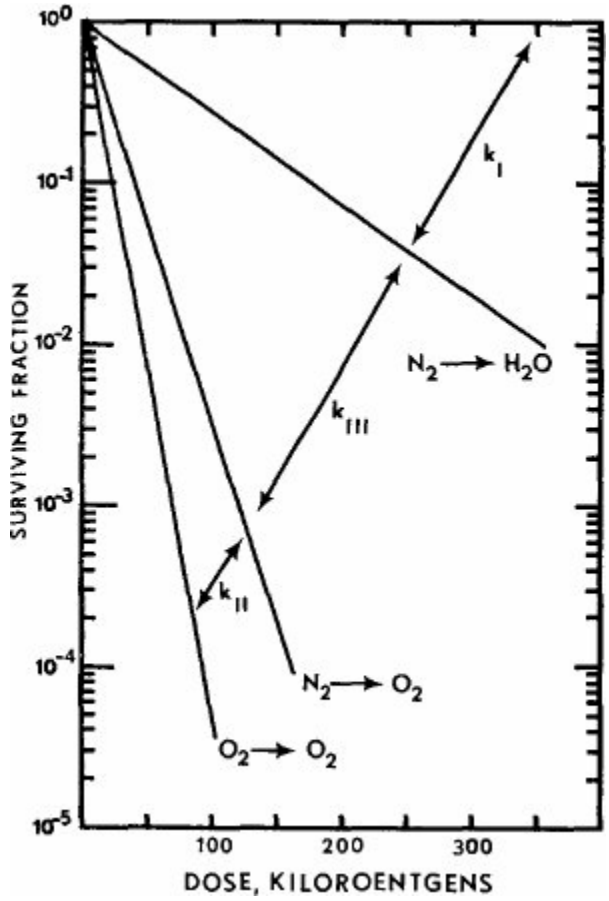

Figure *6.* Survival of dried bacterial spores irradiated in three experimental conditions: $N_2 \rightarrow H_2O$, irradiated in N_2 and exposed to H_2O prior to exposure to O_2 and the growth medium; $N_2 \rightarrow O_2$, irradiated in N_2 and then exposed to O_2 and incubated; $O_2 \rightarrow O_2$, irradiated in O_2. I, II and III are described in the text.

The "Dry" Spore — It was established early (13) that drier spores are inactivated by X-rays according to first-order kinetics

$$N/N_o = e^{-kD}$$

and that *k* is a convenient measure of radiation sensitivity (D is radiation dose). These first order kinetics are exhibited under various experimental conditions as shown in Figure 6, indicating that the physio-chemical reasons why the response curve is moved successively from least sensitivity ($N_2 \rightarrow H_2O$, meaning that the cells are exposed to X-rays in nitrogen and after irradiation are exposed to liquid water prior to germination), to medium sensitivity ($N_2 \rightarrow O_2$), are different from and independent of the reasons why the response curve moves from medium sensitivity ($N_2 \rightarrow O_2$) to highest sensitivity ($O_2 \rightarrow O_2$), and that these two sets of reasons are in turn independent of the physico-chemical reasons why the spore exhibits the base line sensitivity ($N_2 \rightarrow H_2O$). We can identify three rate constants as shown on the diagram, and the total probability of survival when irradiated in O_2 is

$$N/N_0 = e^{-(kI + kII + kIII)\,D}$$

which is the product of the three separate and independent probabilities that the spores will survive the three separate and independent sets of physico-chemical reasons for inactivation following irradiation.

The three components revealed here are: Class I — that described by the rate constant k_I that is entirely O_2 independent; Class III — that described by k_{III} that is developed only if O_2 is presented to the cell following irradiation after irradiation in N_2 (it has been shown to be the consequence of O_2 addition to a radiation-induced free radical that is relatively stable in the dry spore); and Class II — that described by k_{II} that is seen only when spores are irradiated in O_2. There are two different O_2 effects and one O_2-independent effect demonstrated by this figure.

The general experimental plan must be to characterize the overall radiation response in terms of its particular components, (I, II, and III) and their particular behaviors as the experimental variables are applied to the system. With respect to water we examine the conduct of each component as water content is varied (14).

The striking characteristic demonstrated in Figure 7 is that water is largely protective against radiation damage in the spore. The overall inactivation constant in the dry spore (100×10^{-3} kr^{-1}) is reduced to approximately 1/3 of that (35×10^{-3} kr^{-1}) in the fully wetted spore in oxygen. Analysis of the details of this change reveals that two oxygen dependent components of damage are most affected in this reduction of sensitivity, both k_{II} and k_{III} being reduced rapidly, k_{III} apparently disappearing at the fully wetted conditions. As we will demonstrate below this is perhaps an operational disappearance, for there is evidence that this component and perhaps another is retained in the wet conditions and can be recognized with proper experimental procedure. The component k_I is relatively stable during the wetting process at low water levels, but increases a little at high water contents. The details of the behavior of k_I at high water contents (Figure 8) are that there are actually two additional water dependent processes seen at high water content. One of these is called "hydrogen sulphide dependent" and the other "hydrogen sulphide independent" meaning that H_2S can remove one but not the other. These increases in radiation sensitivity as water content increases are small relative to the large decreases that take place in the oxygen dependent components. But these studies show at least four kinds of water dependent damage: one associated with k_{II}, and one associated with k_{III}, both decreasing as water content increases; one that we call the water dependent hydrogen sulphide dependent part of k_I; and one the water dependent hydrogen sulphide independent part of k_I, both of these increasing as water content increases. Aside from the overall general picture of the large decrease as water content increases (or the large protective capacity of water against radiation damage in this dry system), the lesson is clear that one must seek the different properties of the different components of damage. In other words, there is no single description of the interaction of water, the cell, radiation, and the effects brought about by radiation.

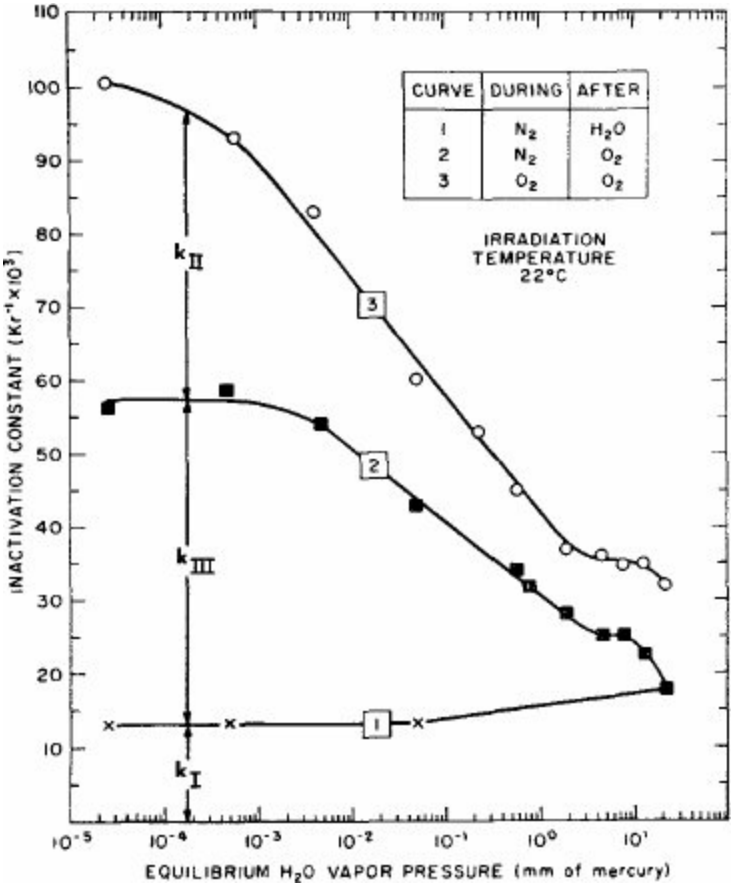

Figure *7.* Radiation sensitivity of the spore influenced by water content. The abscissa is the vapor pressure of H_2O to which the spores are equilibrated prior to and during irradiation. At the temperature of these experiments, 22 torr is 100% relative humidity that in turn is equal to aqueous suspensions. (14)

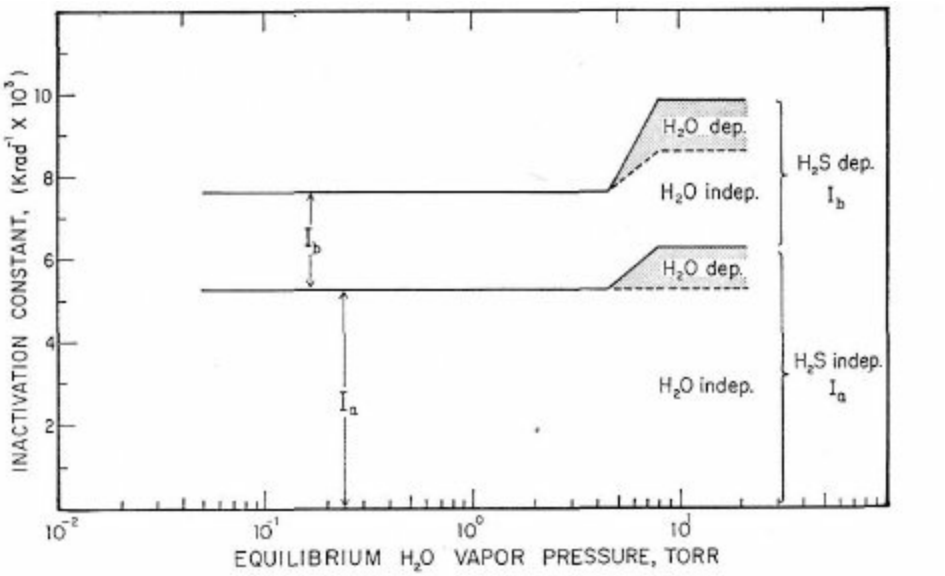

Figure *8.* The behavior of kI, the measure of the anoxic component of damage, at high water contents in the spore. (12)

Another approach used is the introduction of deuterium water as a substitute for H_2O, that is, change the kind of water as well as the amount. There are two kinds of experiments. Grow the cells that

produce the spores in high concentrations of D_2O and compare these spores with those grown in H_2O; and, then, using these two kinds of cells, add H_2O or D_2O to the dry cell and measure changes in radiation sensitivity.

The general experience is that H_2O grown spores are more sensitive than D_2O grown spores in a variety of experimental situations, or, sensitivity is reduced in the D_2O spores. While this cannot be said to be due to D_2O water in the spores, we must always allow for the fact that there is water associated in these spores that cannot be removed at ordinary temperatures.

Added D_2O causes the same reductions as added H_2O in the two O_2 effects. But when D_2O is the added water in the high regions of water content, kI responds as follows: always the spores receiving D_2O are more sensitive than spores receiving H_2O (Figure 9) (15). This action is different and opposite to the effect of D_2O when incorporated into the spore.

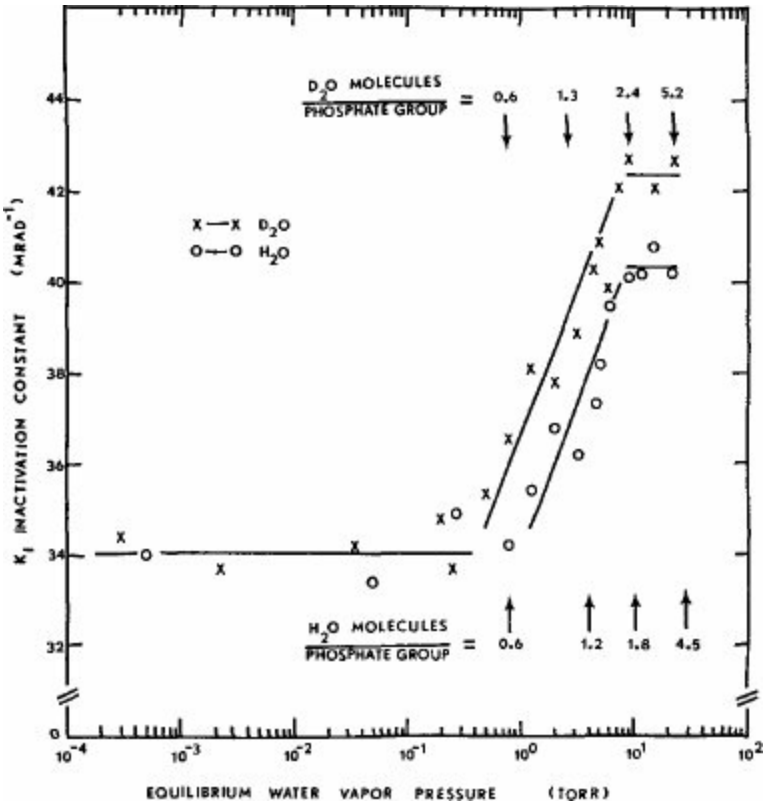

Figure *9.* When D_2O is the added water in the region of high water content, the increase in sensitivity (Class I) is greater than when H_2O is the added water. (16)

A series of experiments with spores exposed to different vapor pressures of water permitted investigation of the relationships between amount of water in the spore and radiation sensitivity (15, 16, 17). The adsorption isotherms (16) are of the typical type II, with constants that agree with those obtained by other authors. They show the regular hysteresis effect. In the paper cited, the reason for considering the values as monolayer values are given. The difference in sorbed H_2O and sorbed D_2O on a weight basis is in the ratio of their molecular weights, meaning that both H_2O and D_2O can compete equally for the same sites. The monolayer value indicates a total adsorbing area of 246 m^2/g spore compared to 6.4 m^2/g spore outside surface area available, meaning that we are dealing with internal surfaces. Extending these to the biological studies (15) results in Figure 9, in which the

behavior of added D_2O and added H_2O in terms of increasing radiation sensitivity is interpreted as related to the number of water molecules associated per phosphate group on a postulated DNA molecule. We see that for both H_2O and D_2O, Class I begins to increase in value when added water is at about one half water molecule per phosphate group. The maximum increase of k_I is reached at approximately 2.4 D_2O molecules per phosphate group, and approximately 1.8 H_2O molecules per phosphate group. These two limits bracket the value obtained (16) at which the monolayer is complete with the two kinds of water, giving us strong evidence that this increase in k_I is truly the consequence of the completion of a water sheath about the DNA molecule. We should note that of the total radiation sensitivity involved in Class I, this change represents 25%. We should also note that increasing the water content beyond these levels to saturation has no effect on radiation sensitivity. These values should also be compared with the more than 20 water molecules per nucleotide for *in vitro* DNA. This may be the consequence of restrictions placed on spore DNA by its special structure, as well as the confines dictated by the spore coat.

Our analysis of the changes in radiation sensitivity induced by added D_2O and H_2O in Class I, the size of the layers, and the greater yield of ·OD over ·OH, suggests that the representation of Kimler (5) (Figure 3) should be approximately correct and should indicate the layers from which hydroxyl radical is involved in sensitization in this class of radiation damage. Note that this is at most a two water molecule shell.

We should note that addition of water at levels lower than these accounts for the marked decrease in the size of the two oxygen dependent classes of damage (Figure 7). No mechanism for the Class II decrease is recognized; and the Class III decrease is probably a reflection of the decreased lifetimes of O_2-dependent free radicals as water level increases. It does not, apparently, have much to do with the hydroxyl radical effects observed after the addition of certain sensitizers such as NO_3^- and Ag^+ as we shall discuss below.

The Spore in Aqueous Suspension — The second large category of research in this area is the study of spores suspended in water to which is added various solutes that react with species produced in water by high energy photons. The basis for these studies is the large body of evidence that has been accumulated since the advent of the pulse radiolysis techniques with high energy electron beams.

The following general reactions occur.

The products

$$H_2O \rightarrow \cdot H\ (G = 0.5),\ \cdot OH\ (G = 2.5),\ e^-_{aq}\ (G = 2.5)$$

are primary, and

$$\rightarrow H_2\ (G = 0.5),\ H_2O_2\ (G = 0.7)$$

are secondary molecular products.

The G values refer to the number of molecules formed for each 100eV of ionizing radiant energy absorbed. Even in pure water, these primary species have short lifetimes, with half-lives about 5-20μs, and with added solutes that react with them these times decrease markedly. Indeed, it is by this general technique that reaction rates of these species with a very large number of chemical compounds have been measured; and these data can be used for designing experiments intended to determine specific absolute and relative concentrations of the species under investigation.

Inquiry into the role of these in radiation biology (particularly here the radiation biology of the

spore), was made by this general technique: addition of substances known from radiation chemistry either to increase or decrease the lifetime of particular radiolytic species.

In these experiments, there are several important experimental variables that maximize the sensitivity of spores; two of these are discussed briefly here to demonstrate methods in rationalizing radiation biological response in terms of radiation chemistry of water: the redox potential of the added solute; and its concentration.

A large number of experiments (18) indicates a relationship between redox potential of a variety of chemical substances and their ability to sensitize bacterial spores and certain other cells to X-rays (Figure 10). Independently, this relationship for another general class of sensitizers (and over a more limited range of $E^{o\prime}$) has been described for metabolizing cells (19).

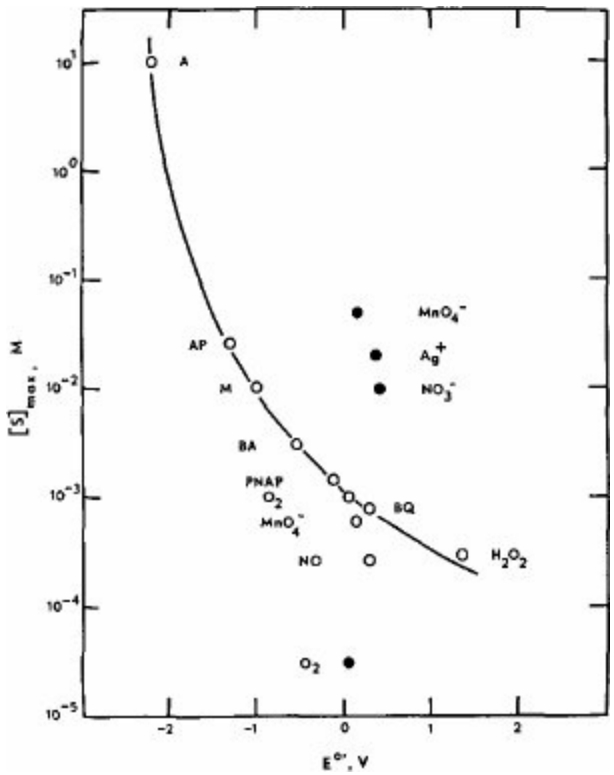

Figure *10.* Relation between redox potential of a sensitizer and its concentration at which it exhibits maximum effect on spores. (18)

We deal here with the ability of the compound to accept electrons, that is, to become reduced. It is difficult from these observations to say that it is the redox process itself that is the operating cause of sensitization. Much of our experience says that it is not the absence *per se* of the electron in the solution that is the direct cause of the increase in sensitivity, but rather the chemical consequences of its removal or sequestration. For it is most likely that an increase in hydroxyl radical concentration can be the more proximal cause for increases in radiation sensitivity. In this instance, we can think of the electrons ordinarily being removed theby the reaction

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

the hydroxyl ions being without biological effect. Efficient competition by an added solute for e^-_{aq} would result in at least local increase in [·OH].

This general conclusion is based on the action of hydroxyl radical scavengers, such as alcohols, in interfering with sensitizer action. Tertiary butanol is one of the favored hydroxyl radical scavengers

suggested by radiation chemistry and was used routinely in our experiments. Earlier experiments showed that other alcohols such as methanol and ethanol also interfere with the sensitizing abilities of a number of different kinds of radiation sensitizers.

The situation here allows that the hydroxyl radical is directly involved in the sensitizing series of events. The alcohol, *t*-butanol, that is without effect on the absence of the sensitizer, does reverse the action of a number of sensitizers, and their effectiveness is in the order of their reaction rates with ·OH, the faster the reaction with ·OH, the lower the effective concentration of the alcohol. This conclusion for this action of sensitizers via ·OH is experimentally sound for the bacterial spore. Note that this conclusion does not include speculation concerning the chemical action of ·OH in bringing about sensitizing action.

Concentration as a variable is of special importance. In our spore studies, we demonstrated early that for biacetyl there is a particular concentration at which the activity peaks, and on either side of this the sensitizing action disappears (20) (Figure 11). Other sensitizers, such as nitrate, increase in effectiveness as concentration increases up to a plateau that extends over a large range of high concentration. Still others, such as Ag^+, show increases in radiation sensitivity as concentration increases up to a peak, and then the effect divides: there is then a decrease in radiation sensitivity as the concentration increases for one part of the radiation effect, that is the low dose component (the "sensitive" component), and a plateau effect for the radiation effect in the high dose component (the "resistant" component). Here we see a dependence of the action of the sensitizer on the total dose being given. In O_2 there is a marked peaked over -O_2 effect.

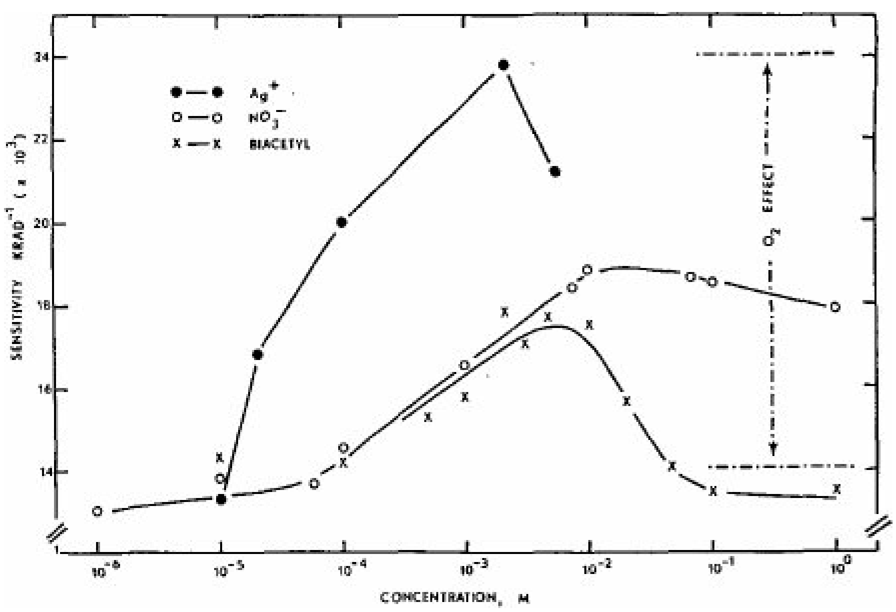

Figure *11.* Three general relations between increases in concentration of additive and sensitivity of bacterial spores. (20)

In all of these complicated reactions, the common thread is that the hydroxyl radical is involved. For some of these, it can be argued that the decrease at high dose is actually the reaction of the sensitizer with the hydroxyl radical and with consequent decrease in radiation sensitivity. This together with the alcohol scavenger experiments form the basis for the electron sequestration model described previously (20).

An interesting question relating to ·OH production by these sensitizers is what water

compartment(s) is (are) involved. Even though there is evidence that the water of specific hydration is the source of ·OH for the increase in Class I anoxic damage (see above), this does not explain the action of added sensitizer. Richmond et al. (21) point out that sensitizers, biacetyl and Ag^+, exert their action only when the spores are in suspension, and are not effective when the spore is equilibrated to water vapor, even 100% R.H. Cobalt hexammine, however, does sensitize spores equilibrated to 100% R.H.; but the degree of sensitization is only half that seen in aqueous suspension. This suggests that although the ·OH may be operating in all three instances, the production of ·OH is not due simply to the presence of the sensitizer but depends upon some unknown relationship between the sensitizer and water structure and/or position.

Though not ready yet for exploitation, one other approach to water function in radiation processes deserves mention. Kimler (5) has changed radiation sensitivity of bacterial spores by irradiating in argon, krypton and xenon, three gases that structure water by forming clathrates in pure water. These increase radiation sensitivity, and in the order of their clathrate forming ability. He interprets these effects as the consequence of better energy transfer in the structured water. This kind of experimental attack could lead to fundamental insights into the role of water in energy transfer processes in cells.

The O_2 Effect — This is a special case that deserves special mention because of its general importance in radiation biology (we must point out that in many *in vitro* systems oxygen can be demonstrated to be a protector under certain circumstances and that extrapolations from the *in vitro* experiment to the cell should be done with extreme caution and with explicit regard to the uncertainties involved). We had suggested as early as 1963 (14) that there are probably two sensitizing actions of oxygen in cells in aqueous suspension, even though we were able to operationally demonstrate but one. Since then Tallentire and Jacobs (22) have shown that the response of bacterial spores in aqueous suspension exposed to gamma rays is dependent upon the concentration of oxygen in that suspension and that the response indicates a two-phase dependence suggesting two actions of oxygen. More recently, in mammalian cells (or in bacterial) on the basis of fast mixing experiments (23), two actions of oxygen are being invoked to explain the results. In our laboratory (24) a study of the concentration dependence of oxygen on the radiation response of the spore (in this instance for 50 KVP X-rays) supported qualitatively the results of Tallentire and Jacobs (Figure 12). An added feature in these experiments, however, is a study of the effects of alcohols as hydroxyl radical scavengers, in this case both *t*-butanol and *t*-amyl alcohol, (Figure 12). The hydroxyl radical shows different reaction rates with these alcohols, but these two when normalized for this difference are identical in reducing part of the oxygen effect in the region of low oxygen concentrations. There is indeed a hydroxyl radical component in the oxygen effect, but not at very low concentrations of oxygen nor at very high concentrations of oxygen (approximately 10 – 20 micromoles oxygen in water). Here we see that at least three oxygen effects can be observed in aqueous suspensions, and one of them is associated with H_2O, the source of the ·OH radical.

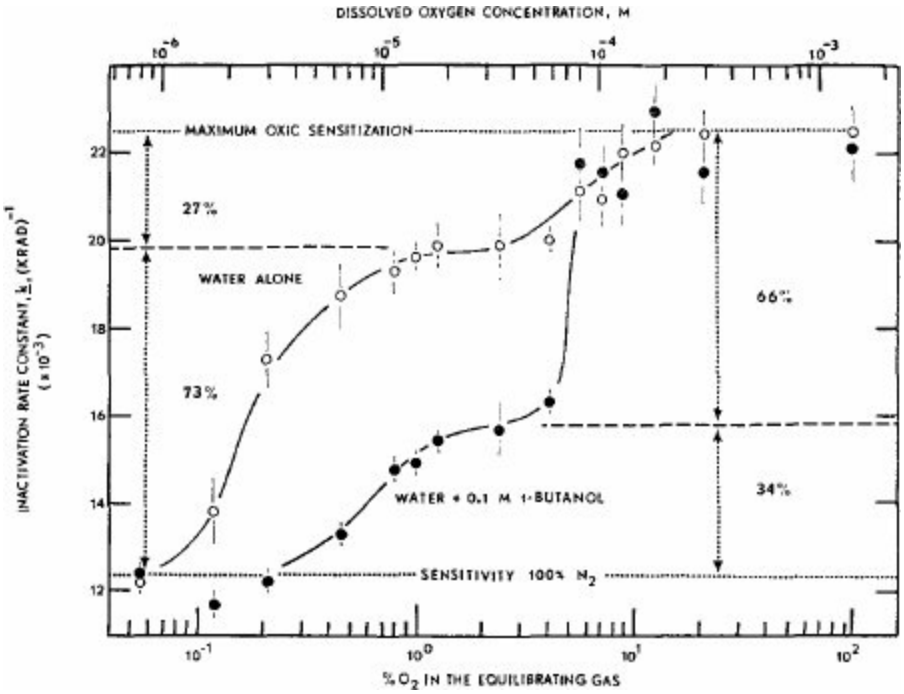

Figure *12.* Sensitivity of bacterial spores as affected by varying concentrations of O_2 in solution and the presence of an ·OH scavenger. (24)

Summary

In a general summary of the studies on the relations among kinds of water, amounts of water, and radiation sensitivity of the bacterial spore we include the following:

a) if O_2 is involved, the bacterial spore is at its most sensitive in its driest state;
b) the sensitivity is due largely to two oxygen effects, one of which is a reaction of radiation induced free radicals with oxygen and the other an as yet undescribed interaction among oxygen, the spore, and radiation;
c) the free radical component of damage appears to disappear at the very highest water content;
d) the anoxic effect is mostly invariate with water content except at the very highest water contents;
e) when D_2O is used as the suspending water during the biological formation of the spore, the cell produces a spore which is less radiation sensitive (oxic and anoxic) than those grown in H_2O;
f) when, however, D_2O is added water rather than the incorporated water, spores receiving D_2O show increases in radiation sensitivity in the anoxic component at the highest water levels greater than those receiving added H_2O;
g) this increase in anoxic sensitivity occurs as water monolayer formation (the water of specific hydration) becomes complete;
h) it is addition of this water of specific hydration that apparently causes reductions in the two O_2-dependent classes of damage;
i) addition of the water of non-specific hydration, i.e., water external to specifically absorbed water, causes a final drop in the two O_2 classes and no change in the O_2-independent part;
j) some added chemicals appear to sensitize spores to radiation in proportion to their ability to take

electrons (redox);
k) two added radiation sensitizers, (biacetyl and Ag^+) that act in water suspension by increasing [·OH] do not operate when the spore, though fully wetted, is not in aqueous suspension;
l) but another sensitizer, cobalt hexammine (II), does sensitize in suspension as well as at 100% R.H. water equilibrium i.e., *not* in aqueous suspension);
m) in aqueous suspension in O_2 there can be demonstrated a hydroxyl radical component of damage, but only at intermediate concentrations of O_2;
n) hydroxyl radicals are shown *not* to be important in N_2 in the absence of added solute;
o) but the action of many substances that act to increase anoxic sensitivity can be shown to be due to increases in [·OH].

Practical Considerations

As it can be influenced by water, optimization of the sterilizing actions of ionizing radiations against bacterial spores requires that:

a) radiation of dry materials must be accomplished in the driest state (equilibrated to less than 10^{-4} torr water vapor pressure);
b) O_2 must be present to a level of at least 10% of the ambient gas phase in this dry state;
c) these two conditions increase sensitivity of bacterial spores by a factor of five over that seen in the wet or moist anoxic spore;
d) if the material is in aqueous suspension, chemicals with large positive redox potentials can be added to increase spore sensitivity at the lowest concentration of additive;
e) since hydroxyl radicals are important in these sensitizing actions, ·OH scavengers should be absent to prevent interference with sensitization;
f) the concentration of the added sensitizer is of critical importance, and should be tested empirically over a wide range to recognize the concentration of highest efficiency.

Acknowledgment

The Laboratory of Radiation Biology is supported by PHS Grant GM-13557 and ERDA Contract E-(40-1)-3408.

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*Actually the theoretical problems presented by liquid water are of such magnitude that it is impossible to describe accurately the structure of water in the terms used above except in an approximate way. The reader is referred to Rao (7).

Experiences with Radiation Sterilization in Czechoslovakia

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Efforts to solve problems connected with requests on sterility of a number of medical supplies resulted in experiments making use of information already available in the field of radiation sterilization. After several years of examinations with the Van de Graaff electron accelerator (400 W, 2 Mev, 200 μA, TUR Dresden GDR), the biggest radiation plant in Czechoslovakia has been established in the Centre for Research and Application of Ionizing Radiation, which is a branch of the State Textile Research Institute in Veverská Bitýška near Brno. The radiation plant, which has been serving for research, examining production and industrial sterilization practices, is equipped with the radiation source of Co-60 type J-6000 produced by AECL Canada. Initial activity of the Co-60 source corresponded to 2.22×10^{15} disintegrations/sec (60 kCi). Encouraging experiences and results, especially with some medical supplies, resulted in increasing demands for sterilization from manufacturers. This has resulted in further loading of the source to the present-day activity of 8.33×10^{20} disintegrations/sec. (255 kCi). In the near future another extension of the sterilization service is to be taken. Construction of new manipulation and storage premises, with a modern fully automated technique based on computer programs, will be used in connection with these plans.

The radiation plant offering sterilization service to manufacturers, hospitals and research institutes is aimed primarily at the sterilization of medical materials. Ionizing radiation is used for sterilization of dressings, surgical gloves, transfusion sets, IUDs and other disposable medical products. Some antibiotics in the solid state and freeze-dried epidermal grafts are sterilized in smaller amounts.

The sterilization dose for disposable medical supplies has been determined at a minimum of 25 kJ/kg if the pre-sterilization microbial counts do not exceed an average of 10^3 and a maximum of 5×10^3 microorganisms on one product. The pre-sterilization contamination is tested for every kind of sterilized goods once every three months. Such a testing gives information which enables us to evaluate hygienic conditions on products prepared for sterilization, even though a sudden accidental increase in counts may not be noted. A disadvantage to this approach is the fact that the resistance of microorganisms is not examined (1, 2).

In the last Pharmacopoea Bohemoslovenica III (3) the minimum sterilization dose of 25 kJ/kg is recommended. The dose, however, is not considered constant. There are examples of products which are sterilized by higher doses such as 50 kJ/kg. Among these products animal diets or polyurethane split sheets can be mentioned. The dose is determined by microbial contamination and conditions during irradiation. That is why lower doses are not excluded and might be used in exceptional cases, although they have not yet been applied in routine sterilization.

The control of the sterilization process is an important point in running the radiation plant. The dosimetry control of our plant has been carried out on commissioning and on further loading of the Co-60 source, by means of ceric sulphate. For routine monitoring Red Perspex® is used. Packages are further over labelled with Megarad indicators.

According to Pharmacopoea Bohemoslovenica III (3) microbiological control should be based on direct testing of products taken at random from the sterilized batch. This kind of control is done only rarely in the case of radiation sterilization, as many specialists in Czechoslovakia are prone to believe that this way is inadequate to prove the sterility of the batch in question. For this reason, biological indicators containing spores of *Bacillus sphaericus* C_1A also are checked after the radiation treatment.

Despite the considerable amount of early efforts in the establishment of criteria for microbiological control and safety aspects of radiation sterilization practices, the situation remains controversial and as yet has not been satisfactorily solved. The required standards of such criteria by the various national public health authorities differ widely (4, 5). Three principal approaches to sterility control have been suggested by the critics of conventional methods based on random sampling and testing of equipment from a sterilized batch:

— biological indicators are to be used as a basis for a sterilization standard,

— assessment of the total counts of microbes on products are to be established prior to the sterilization, and

— the efficiency of radiation sterilization is established using the sub-sterilization irradiation doses.

The model developed by Tallentire et al. (6) describes the dependence of the proportion of contaminated items in a population of items on radiation dose. The practical application of the method, which is based on establishing the efficiency of sub-sterilization doses, seems to be a very acceptable way for routine control of radiation sterilization as it exploits the original method of direct testing of random samples and at the same time allows the evaluation of the resistance of the bacteria present on products that are to be sterilized. For the model, assumptions were made, the distribution of microorganisms on items was that of Poisson and radiation inactivation corresponded to "multiple-hit" kinetics.

We were interested in the application of such a model in a practical situation. The work has been carried out on samples of hydrophilic gauze swabs (5.6 cm) taken from the production line on the last day of every week at the end of working hours for a period of one year.

Examined samples were divided into groups, each of which consisted of 10 pieces. In the first group, the pre-sterilization counts of microorganisms were assessed. Seven other groups were exposed to increasing doses of gamma irradiation in the range from 1 to 15 kJ/kg and the fraction of samples yielding positive growth of bacteria was evaluated after cultivation.

The observations of pre-sterilization counts on studied items in the period from October 1975 to September 1976 are shown in Figure 1. It has been proved on the basis of Barttlet's test that the variability in the average contamination number is statistically significant. The highly significant difference among individual months was the consequence of a great inhomogeneity in the material studied. The highest average number of microorganisms found on a sample began in autumn and at the beginning of winter reached in January 6,428.5, thereafter decreasing to the minimum 492.5 found in March. The maximum average was noted in August (8,083.3 colonies on one product). Seasonal alterations may be caused not only by changes of climatic conditions and epidemics among workers of

the factory, but above all the most changeable factor seems to be the raw material itself, i.e. gauze from which the swabs were produced.

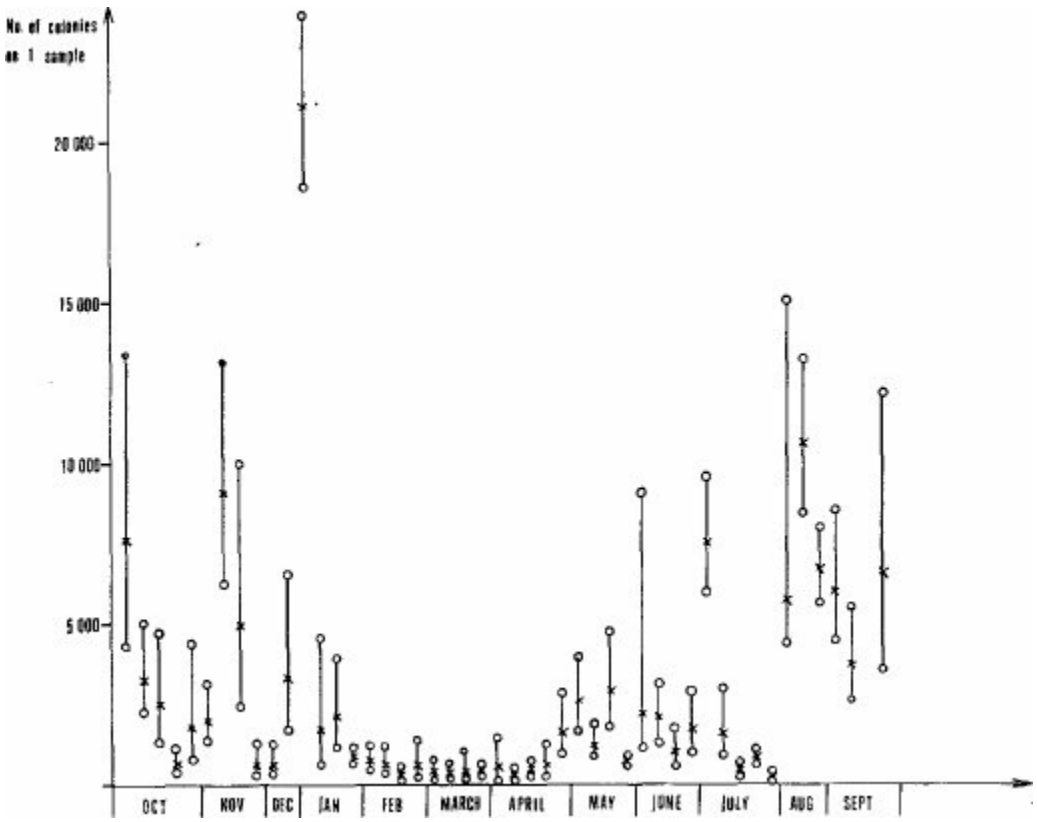

Figure *1.* Pre-sterilization counts on hydrophilic gauze swabs over the period from October 1975 to September 1976.

In order to prove the hypothesis that the microorganisms on items prior to irradiation are distributed according to the Poissonian distribution (6), sample means and sample variances have been compared. It follows from differences between these two characteristics that in most cases the distribution of microorganisms on items is not a Poissonian one. Based on evaluation of histograms it would be possible to approximate the number of microorganisms on samples according to the log-normal probability distribution. The practical difference in comparison to the Poissonian distribution would appear to take into account the higher upper limits for the probable appearance of samples with sporadic high contamination.

The inactivation effect of increasing sub-sterilization doses on samples of hydrophilic gauze is summarized in Figure 2. It is evident that the higher the dose used, the fewer the samples found to yield positive cultivations after 14 days of incubation. If the ratio of positive results to the whole number of samples tested are plotted against the given dose, a sigmoidal curve is obtained.

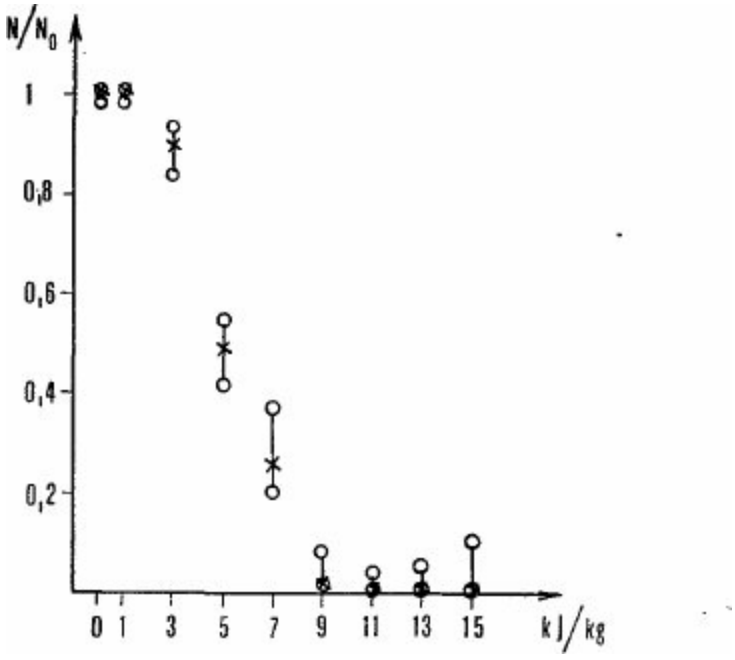

Figure *2.* Inactivation effect of increased sub-sterilization doses on the microorganisms on hydrophilic gauze swabs.

It was also proved that when higher pre-sterilization counts were assessed, a higher dose was needed to reach inactivation of all samples, as shown by the methods employed. In the limits of the method used, however, no surviving microorganisms were found after irradiation by 13 kJ/kg even by such a high average count as 8,000 cells/one test-piece or the maximum 21,480.

Strains isolated after irradiation by sub-sterilization doses have been identified and evaluated as to their resistance to ionizing radiation. After finishing this study a definite conclusion of the whole experiment will be made. Already, the preliminary results indicate the sterility can be guaranteed by a dose of 25 kJ/kg, even under given unfavorable conditions.

To close this discussion contribution, it is possible to state that the method of radiation sterilization has been successfully established in Czechoslovakia primarily for the treatment of disposable medical products. Proposed studies are aimed at working out adequate control methods and looking for new ways to apply ionizing radiation for sterilization of sensitive pharmaceutical or cosmetic products and biological tissues.

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Panel
Questions and Answers

To: T. ALPER — England, by: V. G. JENSEN — Denmark

Q. Do your statements and explanations related to the shoulders of survival curves have any consequence in practice for "fractionated" radiation sterilization, that is 2.5 plus 1.0 megarad?

A. It is an important aspect of what I call the "Alpine" effect, that is to say, the effect of fractionating dose that the cells should be metabolizing between doses of radiation. I think this has been quite effectively shown. Of course, an inherent aspect of this particular dose fractionation effect is that the survival curve has a shoulder to it. If you have an exponential curve you will get no fractionation effect. The two effects, that is to say, a shoulder to the survival curve, or if you like, an initial region of comparatively no sensitivity and the cell's ability to recover that property are intimately connected. For example, if you use the very same organism and use it as you can in certain cases, use it in conditions in which the extrapolation under the survival curve is two, then if you fractionate the radiation, you will get twice the survival after the fractionation interval, as you would have done if you had no fractionation interval. Or if the extrapolation number was six, then you would get six times the survival, but this is not a very general experience. So you have the two conditions necessary for the fractionation effect. One is that the survival curve does have a shoulder to it, and two, that the cells are able to metabolize in the interval between doses. Now, whether or not those two conditions are satisfied in conditions of radiation sterilization, I would not know because it is not a field about which I am familiar. I would imagine that perhaps in radiation of pharmaceuticals, you could have appropriate condition for fractionation effects. The size of the initial dose does not matter. You can give an enormous dose and still get this effect, which still is called recovery.

To: W. S. MILLER — U.S.A., by: T. OLEJNIK — U.S.A.

Q. In comparing plastic syringes and metal needles, in what materials are the doses calculated?

A. Doses were calculated for each device at the least accessible points, but on the outside of the devices. The tests on needles and syringes were conducted at different times under perhaps different test conditions. A direct comparison of results with needles and with syringes was not intended. Rather it was the purpose of the two separate models to show the response of the devices to sterility test, after being exposed to greater dosage to show the relative dosage effects, as a function of the contamination levels shown for each device. So there was not a direct comparison intended.

To: E. A. CHRISTENSEN — Denmark, by: A. CHARLESBY — England.

Q. We are interested in the practical use of sterilized products and this involves a number of steps, radiation treatment being only one. To obtain an overall picture we must therefore compare the risks involved at each step. Could Dr. Christensen give us any figures on the possibility of recontamination during handling of the product, during use, in order to put the whole process into perspective?

A. The risk for microbial contamination during the handling of medical devices in treatment under normal conditions has, as far as I know, not been demonstrated by examinations or experiments. However, the magnitude of the probability can perhaps be evaluated by a comparison to the frequency of microbial contamination during the handling of such items in sterility tests. In the latter case we have controlled examinations and reliable data.

You have heard Professor Tallentire's estimate of this frequency today — about 1:1000. My estimate should be between 1:100 and 1:5000, depending on the kind of product tested and the technique used for the examination. The physicians and the nurses cannot perform the aseptic technique better than the trained technicians in the control departments.

The gap between the norm $1:10^6$ and the estimated frequency of microbial contamination during the routine use of sterilized medical devices is — also in my opinion — larger than needed for a number of different medical products. However, the norm $1:10^6$ is the official norm for all sterilization methods in the Nordic countries. This norm is based on experience and practice at the hospitals in these countries and I am sure that the norm is justified for several medical products. With only one norm for sterilized medical products at hand I have to advocate procedures and control methods giving a reasonable balance between the safety demanded when the heat sterilization methods are used and the safety obtained with the substitutes, the gas and the radiation methods.

To: G. E. ADAMS — England, by: A. CHARLESBY — England

Q. The effect of oxygen in influencing radiation sensitivity in bacterial spores, for example, is obviously very complex. Oxygen can react by capturing electrons, whereby increasing the ionization component. It can react differently with geminate and non-geminate electrons, and of course, it can undergo much slower reactions with radicals. Since most of the basic radiation induced reactions are, at first, of a simple chemical nature, would it not be advisable to carry out parallel experiments with simple macromolecules, where many effects such as radiation protection and temperature have an analogous character?

A. I am not really quite sure what your question is. Are you asking, "Can you study the complexities of the oxygen effect in a cellular system by carrying out parallel model experiment in a chemical system?" I am not quite sure what you mean.

Comment by

A. CHARLESBY — England

Well, it seems to me that we are dealing with a material which is really complex and starting a very complex radiation effect. Therefore, we should find out what these effects are in a chemically simple system and then translate this information, where possible, into more complex systems.

A. Well you raise several possible mechanisms of the oxygen effect. You talk about relatively slow reactions with free radicals; you talk about electrons scavenging. Now if you go to a model system, you can demonstrate each and every one of those processes occurring under some particular set of conditions. For instance, in dilute solution, the oxygen effect, be it sensitizing or more generally protecting, as Dr. Alper said this morning, is indeed reaction with free radicals. If you take a solid state system, particularly with molecules with semi-conductive properties like DNA, there is plenty of biophysical evidence now showing that the electrons do indeed migrate great distances and interact, or rather compete with the geminate recombinations, so you will see what you are looking for in the appropriate set of conditions. But the variability in the model situations which you can choose is just as great as the variability which is naturally there in the cellular system. You have to characterize the oxygen effect in the cellular system, then you might investigate each one of these particular hypotheses in the appropriate chemical system.

Comment by

E. L. POWERS — U.S.A.

I am sorry that I have to disagree that the study of a macromolecular model system can give information which is directly usable in the cellular system. We know of many instances in which, as a matter of fact, Dr. Alper mentioned several of these this morning, in which oxygen is protective in the macromolecular system. This is a very rare phenomenon in cellular system. Oxygen is a sensitizer. It is obvious that oxygen is acting differently in the cells than it does in the macromolecular system or people are looking at the wrong end point in the macromolecular system. One does not know until he does it on the cell. The second point is that I disagree that the bacterial spore should be considered a system more complex or more difficult to understand than an irradiated polymer molecule. I think that the results we have presented in the literature of the last 15 years and the study of radiation biology of the spore is a highly organized, highly reproducible set of numbers which is explainable partially in terms of known radiation chemistry.

Comment by

T. ALPER — England

I would like to add to what Dr. Powers said, or perhaps amplify something he just hinted at. As he said, maybe we have been looking at the wrong macromolecular system, when we have been looking for a model of what provides the sensitization of oxygen in the cell. I would suggest that if radiobiologists and radiation chemists started turning their attention to model membrane systems, it would be a very rapid transition to finding what is responsible for oxygen sensitization in the cell.

To: T. ALPER — England, by: K. H. CHADWICK — The Netherlands.

Q. The question arises from our ideas that DNA double strand breaks are the critical radiation induced lesion. These ideas predict that there should be a direct correlation between mutations per survisor and cell survival. Recent publications (Thacker and Cox in Nature — 1976) have demonstrated this correlation. If mutation is a DNA damage and is directly correlated mathematically with cell

survival, how can membrane damage be reconciled with mutation?

A. My answer to that question is at least two parts. First of all, I would not agree with the premise that there is direct correlation between mutations per survivor and cell survival. The scoring of mutations is notoriously difficult. No satisfactory method has yet been established. If you score mutations per survivor and you look at modifying agents, are you looking at all the effects of a modifying agent on survival or are you looking at the effect of the modifying agent on mutation induction? In some modest experiments we did with induction of mutations in bacteria on the correlation between mutation induction and survival, we found that to be sure of quantitative work on mutation induction, we had to use a part of the survival curve where there was effectively 100% survivors. When we used that dose range, we would score mutations and be sure that the effects we observed were on mutation induction and not on cell survival. So, I do not agree with the premise of this excellent correlation between mutation induction and cell survival, because once you get to rather low levels of survival, obviously that is your denominator, and it must affect the results. Now, if mutation is DNA damage, and there is an "if" there, I would certainly expect that some mutations can be attributed to energy deposited directly in the DNA. I should be extremely surprised if energy deposited in the membrane to which the DNA is attached could not ultimately, after certain cellular processes, result also in a mutation. I would turn the whole thing around and I would say that seeing DNA damage outside the cell does not demonstrate an oxygen effect, whereas things, such as mutation induction in the cell, do demonstrate an oxygen effect. This to me is evidence of the involvement of the membrane; that is to say, a primary deposition event in the membrane as being involved in mutation induction. There is in fact a whole book based on mutations as a cellular process. There is a whole sequence of biochemical events after the initial one, and I may just add that it is certainly held by some very eminent workers in the field of carcinogenesis and mutation induction that certain mutation events due to chemical mutagens primarily act through their effect on the nuclear membrane.

Comment by

G. E. ADAMS — England

I would like to substantiate what Dr. Alper said. The idea that membrane damage can be important in a cellular system does not necessarily mean that it should be taken separately from DNA damage. The idea of damage occurring in a DNA membrane complex of some kind is consistent both with DNA damage and membrane damage. The fact that DNA in some form is involved, the circumstantial evidence in radiobiology is very strong indeed. The effect of ploidy, the correlation between chromosome volume and cellular radiataion sensitivity, all point to DNA in some way. But the fact that radiation sensitivity changes through the cell cycle in the mammalian cell and the effect of oxygen does not, would indicate that something other than just a pure DNA is involved.

Comment by

E. L. POWERS — U.S.A.

I am not disputing Dr. Alper's membrane proposition at all. She looks at it this way and she has

very persuasive evidence that membranes are associated with this damage and it is appropriate that she think of the DNA membrane complex as the target. That is very, very good and that is arguable about the bacterial cell. I am not sure that it is arguable for the eucaryotic cell. I do not know if there is clear evidence that the eucaryotic chromosome is associated with membranes. Perhaps you can help me.

T. ALPER — England
Comment by

This morning I showed one diagram and one photograph that I think pointed very clearly to the very close and intimate association of DNA with the nuclear membrane. In the eucaryotic cell there is good evidence that the initiation of DNA synthesis in the late synthetic phase of the cell cycle is right at the nuclear membrane. If you are looking for cell biological evidence, I think that this does exist.

To: W. S. MILLER — U.S.A., by: J. L. WHITBY — Canada

Q. What is your reaction to Dr. Tallentire's paper dealing with extrapolating from fraction sterile figures obtained from low irradiation doses?

A. I will answer this in a general way. In proposing a new approach to a technical process we must be willing to have the proposal scrutinized objectively by academic, regulatory and industrial scientists. Further, we seek their contributions. In the case of variable radiation sterilization doses, I would welcome, not only mathematical models for use with sub-process dose data to establish doses, but also ideas on models for better estimation of bioburden distributions and limits, bioburden sampling protocols, etc. I am convinced that the use of lower radiation doses can result in satisfactory assurance of sterility, if adequate bioburden data is obtained and if manufacturing processes are under control. However, European scientists and regulatory officials must also be in agreement before devices can be sterilized for their markets.

To: E. A. CHRISTENSEN — Denmark, by: J. MASEFIELD — U.S.A.

Q. When quantifying the bioburden, what is used as the basis for the count? The entire product and its package or a part of the product by weight or volume?
If it is the entire product, are we to presume that under the Scandinavian approach it would be out of the question to use radiation to sterilize such articles as hospital packs, and surgeons' gowns, because of the initial high count of many thousands of organisms?

A. The Nordic recommendations for radiation sterilization are based on the recommended Code of Practice for Radiation Sterilization of Medical Products published by IAEA in 1967. (Please notice 1967, not the 1974 version). In this recommendation, the presterilization bacterial contamination (the bioburden in the terminology preferred by our colleagues from North America) is measured by the initial count, defined as "the number of colony forming microorganisms that can be isolated from the item itself and from the inside of the packaging" (3.2 Note 1). The philosophy behind the use of the count per item instead of per gram or per square centimeter was that the risk for harmful effects to the patients is related to exposure during a treatment. A single dose of a drug, a single infusion set, a single syringe are relevant units from this point of view, and the gram and square

centimeter are less relevant. It is, however, not forbidden to use so-called common sense, and a reasonable "translation" of the term *item* can, of course, be found also for textiles and other products not directly covered by the original recommendations. I doubt that a single case can be referred to where the health authorities in a Nordic country have given an unreasonable interpretation of the definition of *initial count* used in the IAEA recommendation when this definition was not directly applicable.

To: E. L. POWERS — U.S.A., by: A. CHARLESBY — England

Q. The effect of water concentration can readily be seen when simple macromolecules (polymers) are irradiated in water. The effect observed (network formation) increases as the concentration is reduced, that is the molecules are further apart. This is because there are more OH radicals available to react with each macromolecule. However, at very low concentration, this situation no longer applies, because molecules react internally. I would like to draw your attention to the effect of temperature, which affects the observed changes, or example in irradiated food.

A. I am sorry I cannot quote any information that we have on the effect of temperature in our system to modify radiation chemistry. We have just recently tooled up for accurate control of temperature in the range from 5 to 50 degrees. Is that the range?

Comment by

A. CHARLESBY — England

Ten to minus 30 degrees.

A. No we will not be able to do that. So I have no information to contribute.

Comment by:

A. CHARLESBY — England

I would like to carry the point a bit further with Prof. Powers, if I may. It does seem to me that the many directions he has been talking about are very convincing. I am looking forward to seeing his paper in print and that will give me more time to study it. But ultimately what we are studying in this case is just what are the technicalities of radiation under various conditions. We are not saying, "What is the radiation doing other than killing?" That is about the end of the story, but it seems there is really where the story starts. What is the mechanism by which a cell is, in fact, killed? Not just what are the ingredients which caused it to die, but what is happening to it while it is being killed? This is the kind of thing which is much more complex and perhaps even more interesting from an academic point of view. There, I think, some model systems are extremely valuable. I came into this field when I was a physicist and I still am. Without asking some biologist about the temperature effect of radiation, it is known that bacteria show significant changes in temperature sensitivity. This involves some rather elaborate theory of biological effect, variable temperature and so on. When we did the same experiment with some very simple polymers, we found exactly the same temperature dependence. In other words, in that case temperature sensitivity of bacteria has nothing to do with biology; it is simply a radiation chemical

effect. That is just one example; there are many others. Radiation sensitization and radiation protection by additives work just as well in simple macromolecules as they do in biological systems. So there again, it is a simple chemical effect, if you like, almost like a physical effect and not a biological effect. Some other things are coming up now. For example, we find the radiation sensitivity of macromolecules depends not only on their structure or even their concentration, but depends on their morphology. So if we assume that cells in subdividing change their morphology, as they presumably do, they will have a different radiation sensitivity, a different face to their structure just due to the fact that they have a different morphology. This might produce a departure from the exponential relationship. If you have the same cell under two different conditions during its various transitions, you get different radiation sensitivities. If you get vastly different apparent responses though, we are in fact getting one damage in one particular species. This is the kind of thing I am thinking of.

The question of food, which is the one I raised, was another use of sterilization which is becoming, I hope, quite important. The problem is that you are trying to kill the bacteria without spoiling the food. Your trouble is, if you irradiate at room temperature, you kill the bacteria with a dose of, shall we say, 2.5 megarads, but the food becomes almost uneatable. At least steak tastes like goat meat. If you like goat meat that is fine, but most people do not. So the problem is how to kill the bacteria without affecting the food. The answer seems to be that if you irradiate below room temperature, typically minus 30 degrees, you destroy the bacteria and do not affect the food. This causes problems because we know the temperature sensitivity of a bacterium is very much the same as most simple macromolecules, why then should protein be so utterly different? Obviously, if you destroy the bacteria and do not destroy the food it must be that there is an enormous temperature variation in the case of the food, but not in the case of bacteria. To explain this is rather awkward. But I think the answer might be that when you irradiate at low temperatures you are irradiating not in water, but in ice. If you are irradiating in ice, all the effects that you are producing are rather different than in water. Therefore, the subsequent effect on food due to the free radicals is utterly different.

Comment by:

E. L. POWERS — U.S.A.

I should like to subscribe to Dr. Charlesby's theory that we should look to simple systems for explanations and I think we have attempted to do that over the years. In 1958, we published a paper in Experentia in which we described the effects in the very low temperature regions on the radiation sensitivity of bacterial spores starting with liquid helium temperatures. We saw an invariance in radiation sensitivity up to 125 degrees absolute. From 125 degrees absolute to room temperature there was a temperature dependance with a 1 kilocalorie apparent activation energy. Further, we demonstrated that it made a difference whether the spore was an H_2O grown spore or a D_2O grown spore. The temperature dependence of a D_2O grown spore began not at 125 degrees absolute, but 150 degrees absolute. We looked into the plastic literature and I think that it was Dr. Chapiro who had done a number of experiments like this.

His curves in plastic are similar to ours, or if you like, ours are similar to his. And we looked, Dr. Charlesby, in vain for any explanation of these effects which we could apply to our bacterial spores. In other words, we are waiting for the simple system to tell us something, to explain our results. We have

the same results exactly. The spore is acting like a piece of plastic.

To: E. L. POWERS — England, by: J. KOMENDER — Poland

Q. What would be the effects on microorganisms irradiated at approximately minus 70 degrees centigrade to minus 190?

A. As I said we have temperature independence up to 125 degrees absolute, and then the increase in sensitivity of only 1 kilocalorie dependence, which is a very small activation energy. This suggests that we are dealing with a physical process and this is not a chemical process at all.

Anonymous Comment:

I agree that we are dealing with a physical property rather than something associated with diffusion. But the other point is when you freeze a cell down to that temperature and you irradiate it you might modify a great deal of the free radical lifetimes because they are frozen. But you do not assess the viability of a cell at minus 70 degrees, but assess it at 37 degrees. Of course, all those free radicals are liberated and behave as if irradiated at that particular temperature.

Another thing, Dr. Charlesby, the people at Natick, Massachusetts in the Army Laboratories have been using low temperature radiation rates for the preservation of taste, as you know, for a number of years.

To: E. A. CHRISTENSEN — Denmark, by: G. KOPPENSTEINER — Germany

Q. How do you produce a *B. pumilus* indicator with a D_{10} of 0.3 Mrad? This D_{10} seems relatively high.

A. A pumilus indicator with a D_{10}-value of 0.3 megarad can be prepared from spores of *B. pumilus,* strain E601, when the spores are not damaged by washing procedures and are dried from a suspension with small amounts of organic compounds, e.g. proteins. The D_{10}-value 0.3 for this strain was, as far as I remember, first published by Dr. C. Artandi (Artandi, First Int. Symp. UK Gamma and Electron Radiation, Taylor & Francis Ltd., London, 1964, p. 89), and standard preparations with this strain and a D_{10} value of about 0.3 megarad can still be obtained from my department.

To: E. A. CHRISTENSEN — Denmark, by: K. KERELUK — U.S.A.

Q. (1) What is the D_{10} or D_{Mrad} value for your spore sand biological indicator?

(2) In ethylene oxide sterilization, how does the spore sand biological indicator "determine" relative humidity or when are the spores "wetted?"

A. (1) I understand the question as referring to the radiation resistance of the biological indicator delivered from my department for control of dry heat, ethylene oxide and formaldehyde sterilization.

The radiation resistance of this biological indicator is not measured routinely. Only the resistance against dry heat and ethylene oxide of each batch is measured with regular intervals until the batch is outdated. However, if the radiation resistance of the test strain is unchanged — and most likely

it is — our data from experiments more than 10 years ago should still be correct. At that time the D_{10}-value of the test strain in a sand preparation was about 0.25 megarad.

(2) Our biological indicators for ethylene oxide sterilization are designed to have about the same resistance against the combined effect of ethylene oxide and water vapour as samples of dust from uncontrolled indoor environments in Denmark. We can of course not examine the characteristics for the biological indicators under all possible combinations of the parameters determining the biological efficiency of an ethylene oxide sterilization procedure. We have to choose a few sets of parameters for our calibration of a new batch. The indicators "determine" the relative humidity as *sufficient* when the indicators are inactivated and as *insufficient* when growth can be demonstrated. The indicators can not measure *when* the spores are "wetted." The indicators demonstrate only sufficient or insufficient biological efficiency of the sterilization procedure, regardless of the details in the sterilization program used.

To: E. A. CHRISTENSEN — Denmark, by: T. ALPER — England

Q. (1) I am puzzled by the conclusion you drew from irradiating *colonies* grown from human-associated microorganisms. Does this represent a realistic situation in radiation sterilization? That is, do contaminants tend to have grown into colonies?

(2) Can "biological dosimeters" possibly work when survival curves depend as strongly as they do on, for example, oxygen concentration, method of growth of the culture before irradiation, method of growth afterwards?

A. (1) The use of colonies of bacteria as objects for isolation of microorganisms with unusual high resistance to radiation is a consequence of the method used to collect the organisms from the air in clean areas. We obtain the samples as colonies on agar surfaces because we use the slit-sampler technique. The resistance of the microorganisms is not evaluated on the basis of the resistance in the mixed population only. The resistance of the organisms in pure cultures is determined as well.

The conclusions related to the choice of dose are based on the frequency of occurrence of microorganisms with the demonstrated resistance, not to the artificial system used for the isolation of the resistant organisms. If one of the organisms from the air in the clean area develops to a colony of resistant cells we draw the conclusion that at least one of the particles picked up from the air was carrier for at least one cell with unusual high resistance. Consequently, the number of resistant strains collected is a minimum measure for the number of dust particles carrying resistant organisms in the air of clean rooms. In our critical analysis of the data obtained we have not overlooked the obvious risk for laboratory contamination in a department dealing with a number of very resistant laboratory strains. However, we have so far no isolates indicating that kind of failure.

(2) I have to apologize for not being specific enough when I presented the proposal for control on the microbiological efficiency of the doses measured in radiation plants used for sterilization.

It is a condition for a routine use of these methods that standardized preparations and dose-response curves are available from a reference plant. Information about the culture medium,

growth temperature, counting technique, etc., shall be available also. The reference to our use of the method in cooperation with IAEA is given in my paper.

To: T. ALPER — England, by: I. GALATZEANU — Rumania.

Q. As I understand from your excellent presentation, the behaviour of the cell membranes during and after irradiation is not well known. Do you think that the free radicals formed inside of the cell will occur on exchange with external media, through the membrane? Do we have in this case a similar exchange, like Donnan membranes? Can you imagine such a mechanism?

A. I do not think I have a good answer to this question. I think we must remember that the questioner has bacteria in mind and we must remember that there is the cell membrane and outside of that is the cell wall. Now whether or not we should expect an exchange of either external radicals through the cell wall or other molecules which could subsequently affect the radiation response is just a matter of speculation. We know, of course, that certain molecules can get through the cell wall and interact and if my speculation has anything to it, then they could very well be acting through interaction with membranes. But I really have no satisfactory answer.

General Discussion

Comment by:
Co-Chairman, J. L. WHITBY — Canada

It is obvious that there is some degree of conflict of interest because there has been a great many high-powered physical questions and there is obviously a small in-group which has some very interesting material and there are, of course, more practical papers not being subjected to quite the same degree of questioning at the moment. I had hoped for instance, when Dr. Charlesby asked Dr. Christensen a question, he might have been referring to recontamination at the time of use. In fact, people do not open packages properly. I always spend lots of time trying to tell medical students that you must learn how to use that sterile article, if you are to get any of the benefits. There are many other factors I think which we ought to consider. Are our results comparable to some other investigators? Are we using the same methods? What about recovery of organisms after irradiation? What is the best method to use? I had a discussion with Dr. Kallings relating to Dr. Khrushchev's paper. He pointed out that there are several different experimental problems involved in the ecological studies on the sensitivity of microorganisms to ionizing radiation. One obvious problem is to design a culturing system that allows fastidious and slow growing radiation resistant microorganisms to be demonstrated. If the culturing system is not optimal to pick up the microorganisms, then the results will give false impressions that radiation resistant microorganisms do not occur. And a second closely related problem is how to check that the medium used for plating for survival curves will actually allow the partly damaged irradiated cell to multiply. This has very important practical implications in determining the results from the survival curves and he noted that this was abundantly clear in Dr. Alper's presentation this morning. When working with well-known culture, such as *Streptococcus faecium,* which is used as an indicator, one has good possibilities of control because one can control the count satisfactorily. But when one is working with unknown organisms, it is very difficult to ascertain whether they will grow or not. If one overlooks this aspect, one will conclude that the organisms are of low resistance. Results of such an ecological study will be very much dependent on some critical experimental conditions. Without details of the technique and controls used it is impossible to evaluate results. This is a plea that we may be given the methods employed in this very large scale Russian survey. I would welcome contributions on the basic microorganisms, on the best methods of culturing, how long we should incubate cultures, and which media we should use. If we do not use the same methods, our results cannot be integrated. I would like a contribution or two on culture methods.

Comment by:

E. A. CHRISTENSEN — Denmark

Indeed it is a complicated question and the number of resistant organisms will always be at minimum levels. In our results you see no anaerobes. It was a matter of choice based upon the type of products. We have been interested in the number of anaerobic organisms which occur at low levels and

therefore we have used our efforts only to pick up the organisms most easily found. There you see one example of the restrictions of the method. We have several examples where organisms can grow on blood agar and not on the other substrates. We have the other situation where you only can pick up organisms on very limited and narrow substrates. This means that you may be able to demonstrate twice as many resistant organisms if you use a broad spectrum of substrates. But the fact of twice as many in this relation means nothing.

General Question by:

T. OLEJNIK — U. S. A.

Q. Not being a microbiologist, I will ask a rather naive question. Since in the final analysis the nutrient medium that will be sustaining surviving microorganisms is going to be composed of *Homo sapiens,* would it not seem logical to try to culture organisms in a nutrient medium which most mimics the human nutrient medium in which they are expected to survive?

Anonymous Comment:

Based on tradition, microbiologists have designed their culture media to pick up organisms harmful or pathogenic for human beings.

Comment by

T. ALPER — England

I was just wondering whether there is such a thing as a bacteriological medium which approximates to a mammal or a human being.

Anonymous Answer:

A. When hydrocele was common, we used to use hydrocele fluid as a source of human proteinaceous material. I think it is a very good medium for growing fastidious organisms. But we do not use it much nowadays. I think we all adopt the same principle in that we use what we find comfortable, until we find a better medium. If we find a better medium, we then discard all our previous media. I think most Americans culture in Trypticase Soy Broth, but I think there are better media, more universal recovery media. Personally, I think time is more important than the composition of the medium.

Comment by:

T. ALPER — England

I really do not think it is important because I believe you do allow a very large safety factor, particularly when you are dealing, as we heard, with materials that are pretty clean to start with. But I think it should not be assumed that a good medium, that is to say a rich medium full of nutrients, is necessarily one in which you get the highest survival. You could, if you like, at least in the sort of work I

have done, almost cast your microorganisms into two groups. Those which show a better survival rate in a highly nutrient medium and those which show a much poorer survival in a highly nutrient medium. I doubt if your needs could be met by one universal medium.

Comment by:

F. J. LEY — England

I think our confidence about whether organisms will survive these high doses of radiation comes from accumulated experience. It is true now that laboratory animal diets have been treated with ionizing radiation for quite a number of years. I know that most of it is treated at 2.5 megarads and it has been used for the keeping of germ-free animals very successfully. The initial number of organisms involved are in the order of 10^6 per gram. I should estimate there is probably a thousand tons a year of this diet processed. The other experience, of course, is with germ-free animals where 4 or 5 megarads is being used very successfully without causing positives in the medium, where the culture medium is the laboratory animal.

SECOND SESSION

Co-chairmen

K. Ostrowski

R. W. Campbell

The Immunogenicity of Radiation Sterilized Biostatic Grafts

K. Ostrowski

Department of Histology and Embryology, Institute of Biostructure, Medical School, Warsaw, Poland.

Abstract: *The use of radiation sterilization for tissue banking evokes changes in the tissues which are from the clinical point of view usually negative, such as a decrease of the mechanical values or decrease in some biological properties of the graft e.g. the ability of induction of osteogenesis. Some alterations, however, are positive, for instance the decrease in immunogenicity of the irradiated tissue. This paper reviews the changes in the immunogenicity of irradiated grafts, stressing the differences in the immunological situations evoked in the three kinds of clinically grafted tissues a) dense fibrous connective tissue (tendons, fasciae or dura mater), b) peripheral nerves and c) bone tissue.*

The thesis of this paper, which is the introduction to the session, is that the damage involved in the interaction of the sterilizing dose with the irradiated tissues is not fully balanced by such advantages as the sterility of the grafts and decreased immunogenicity. Changes in the preservation procedure should be planned to optimize the existing situation.

Introduction

It is my pleasure to open this session and to begin the discussion concerning some changes (mainly damage, in only few cases — improvements) caused by the interaction of the huge doses of ionizing radiation used for sterilization with biological tissues. I am not competent to discuss the problems concerning the irradiation of drugs. Probably the problems which we encounter in the activity of the tissue banks, based on the radiation-sterilization technique are similar, if not the same as in the drug industry.

Before I discuss the problems on which we are working in our Laboratory let me formulate some remarks on the fundamental recommendations for the application of ionizing radiation in the range of megarads in the practice of radiation sterilization of tissue grafts.

I hardly dare discuss microbiological problems in the presence of experts taking part in this meeting, but I would like to summarize the situation as it looks from the point of view of the tissue bank:

The Code of Practice for Sterilization of Medical Products published by IAEA(1) and other scientific publications(2, 3) encourage elasticity in the doses of ionizing radiation used to kill the microorganisms contaminating the sterilized product. The idea is to adjust the sterilizing dose to the initial contamination according to the nomograms describing the logarithmic process of sterilization in the form of the dose-survival curve. The recommendation seems ideal, but unfortunately, three

questions are still open:

1. There is no way of sampling the tissues from the cadaver so as to be reasonably sure that the samples represent the initial contamination in all tissues taken for grafting.

2. Diminution of sterilizing dose in cases when initial contamination is low, implies the danger of producing radiation-resistant microbial strains (3).

3. Although some scattered information on the sensitivity of viruses to radiation sterilization has been published (4, 5) no routine method exists for evaluating viral contamination and the effect of the sterilization procedure in this respect.

The paradox in the situation existing nowadays in tissue banking is that one big center has been using for years lyophilized tissues after checking their initial contamination without any sterilization at all — and with excellent results(6), while others(7–10) are using huge doses of radiation in the range of Mrads to observe the regulations for sterilization of biological products, thus, inflicting damage which probably in many cases could be avoided. There is nothing intermediate between these two extreme situations.

In the following paper by Dr. A. Dziedzic-Gocławska you will hear a discussion on the effects of radiation sterilization on the physical and chemical properties of different tissues. Most of the research in this field was done on bone tissues. You will see that, apart from an obvious decrease in the mechanical properties and biological ones of the irradiated bone, the changes are not too big and in some situations, reversible after rehydration.

Dr. J. Komender will discuss later the clinical results with some radiation sterilized grafts. He will show that an overall 85 per cent success after clinical application of radiation sterilized and radiation damaged bone grafts is achieved and might be acceptable. After all, the proof of the pudding is in its eating.

I will concentrate now on the specific immunological situations connected with nonviable, biostatic tissue grafts, irradiated for sterilization purposes. This problem is not included in the other authors papers, but it is of some importance.

The immunological problems in the transplantation of biostatic grafts are different from those encountered in transplantation of viable tissues or organs. The specific situation in this field is twofold:

Firstly, the aim of applying a biostatic graft is different. It is to serve temporarily as a biological prosthesis and finally to be resorbed and substituted in the creeping substitution process by the host's own tissues. The second part of this statement might not be true in some special cases as, e.g. in grafting of preserved cartilage in reconstructive surgery.

Secondly, in most cases the immunogenicity of the grafted tissue is much lower than in the case of transplantation of living skin or kidney. The main source of histocompatibility antigens — the cell surface — is nonexistent or denaturated. On the other hand, the main protein in most of the preserved biostatic grafts is collagen. The immunogenicity of this protein is relatively low.

Radiation sterilization, in the aspect of induced changes in immunogenicity, seems to be useful causing as an end effect a decrease of the immunogenicity of the irradiated tissues.

This statement based on experimental data which I am going to discuss might be generalized in the following way: *the future processes of graft preservation, including radiation sterilization, should be optimized so that the unavoidable damage connected with the interaction of ionizing radiation with the preserved tissue would not overbalance the advantage of sterility.*

The main constituent of devitalized biostatic grafts such as bone, skin, tendons, meninges, fasciae

and even cartilage, is collagen. This is not true for the preserved peripheral nerves where lipid components of myelin are present, although collagenous epi-, peri- and endo-neurium are constituents of the grafted nerves.

Collagen is a biopolymer which has been very well described as to its biochemical structure. The immunogenicity of this polymer is low, but very well defined.

In fact immunological techniques were used together with biochemical and biophysical ones to elucidate the structure of this protein. In most connective tissues there are two genetically distinct alfa-chains termed alfa 1 (I) and alfa 2. These chains occur in ratio 2:1 and form the familiar type I collagen. A different collagen was found in cartilage. It contains three identical chains termed alfa 1 (II) which are different both in sequence and amino acid composition from the chains forming collagen I. Different antigenic determinants have been located in collagen. Some of them were found to be species specific. The other ones were specific to the collagen chains themselves, notwithstanding from which source they were derived. The antigenic determinants involve a small number of amino acids. Some of them show immunogenic activity dependent on one single amino acid as, e.g. tyrosine residue at the position 1033 of alfa 1 chain. Synthetic polypeptides helped in elucidation of the antigenicity of collagen. Cross-reactivity of antisera prepared with the use of collagen from different species as the antigen visualized the species specific differences. An important similarity from the point of view of tissue banking practice was found between calf and homologous human collagen peptides, as shown by the complete cross-reactivity of specific sera.

By using collagen derived polypeptides and comparing them with the immunogenic effects of some synthetic polypeptides, amino acid sequences responsible for antigenic activity of collagen were revealed. It was found that native collagen and also synthetic polypeptide $(Pro, Gly, Pro)_n$ evoke thymus independent immunological reactions. Heat denaturated collagen evokes a thymus dependent reaction analogous in this respect to the immunological activity of the synthetic $(Pro, Gly)_n$ polypeptide. Denatured collagen can, therefore, potentially evoke cell dependent reactions of delayed hypersensitivity type. (Review and literature in(11).)

No data exist on the immunogenicity of cohagen denaturated by ionizing radiation. If we assume that immunological data based on experiments where tendons, fasciae or dura mater were used to illustrate mainly the immunogenicity of collagen, one can draw some conclusions as to the effects of ionizing radiation on the immunogenicity of collagen. Unpublished data and incomplete results of experiments performed in our Institute by Dr. A. Komender(12) on dense fibrous connective tissue are available. Xenogenic fasciae were used and different steps of the preservation procedure were applied to show the differences in the host's reaction. The modified lymph node reaction technique (13) was utilized for evaluation of the immunological situation. This modification allows for precise quantitation of the intensity of the reaction. It was found that even in the xenogenic situation the blastic reaction was not high. Denaturation of fasciae by ethanol treatment, lyophilization and irradiation caused an intensification of the reaction as shown in Figure 1. This is not the proper place to discuss the blastic reaction of the regional lymph node. Not all of this reaction is necessarily of immunological origin. Nevertheless, the experiment shows that the immunogenicity of denaturated xenogenic collagen contained in the dense fibrous tissue is certain, although not very pronounced.

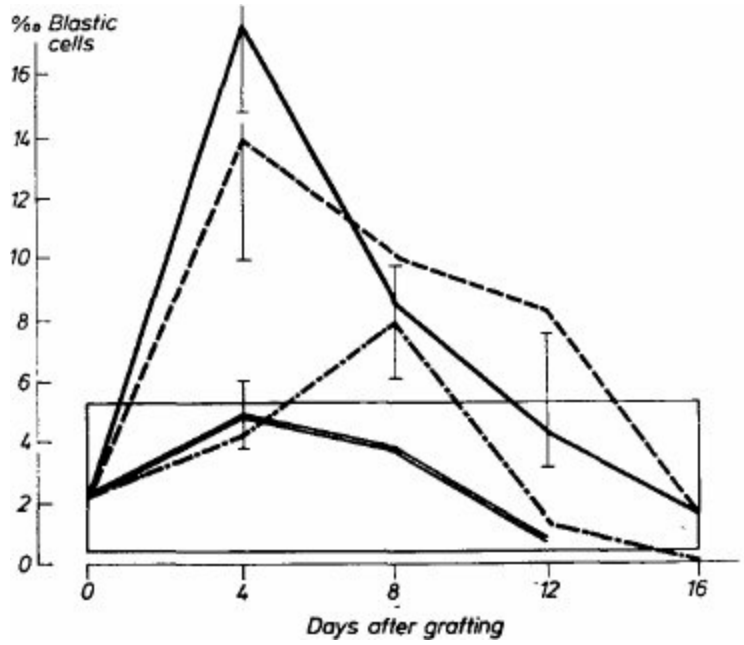

Figure *1.* Blastic reaction evoked in C57B1 10 × Sc/Sn mice in the regional lymph node after grafting of xenogenic (bovine) fasciae treated in different ways:
——— 70% ethanol denaturation, lyophilization, 3.5 Mrads from Cobalt-60 source.
- - - - - 70% ethanol, lyophilization.
—•—•— lyophilization.
═══ mock operations.
rectangle = contains value of all contralateral controls.

By histological evaluation of the infiltration reaction, which is not a very specific way of evaluating immunogenicity, Rosomoff and Malinin(14) found that the dura mater even in a xenogenic situation shows low "antigenicity" after freeze-drying.

The situation is different in the field of research on the immunogenicity of peripheral nerves sterilized by ionizing radiation. More precise data exist in this field. Marmor (15) provided proof, based on histological estimation of the cellular infiltration in the vicinity of the graft, that radiation sterilization decreases the intensity of this reaction as compared with the reaction caused by untreated nerves.

Data more adequate from the immunological point of view were provided by Campbell and Wright (16), who compared the immunogenicity of irradiated and fresh untreated peripheral nerves, adopting allergic autoaggressive peripheral nerve inflammation as proof of immunogenicity. Homogenates of peripheral nerves together with Freund's adjuvant were injected into the foot pads of guinea pigs. No reaction was found after injecting the homogenate of irradiated nerves, in contrast to the allergic inflammation of peripheral nerves when fresh nerve homogenate was used for immunization.

Pollard et al (17) working on fresh and freeze-dried allografts in rats showed that the situation is not as simple as it seemed in the older literature. The time factor is important in the sensitization by preserved nerve grafts. When a longer time of regeneration was needed for the substitution of long fragments of inserted grafts, an immunological reaction occurred in the host. Immunosuppression by immuran aided regeneration through peripheral nerve allografts.

This observation is supported by the yet unpublished work by Kassakowski et al.(18) from our Institute. They used the highly specific migration inhibition test to evaluate the sensitization of inbred mice by grafts of peripheral nerves taken from a strain differing in the H-2 locus. The results are shown in Figure 2.

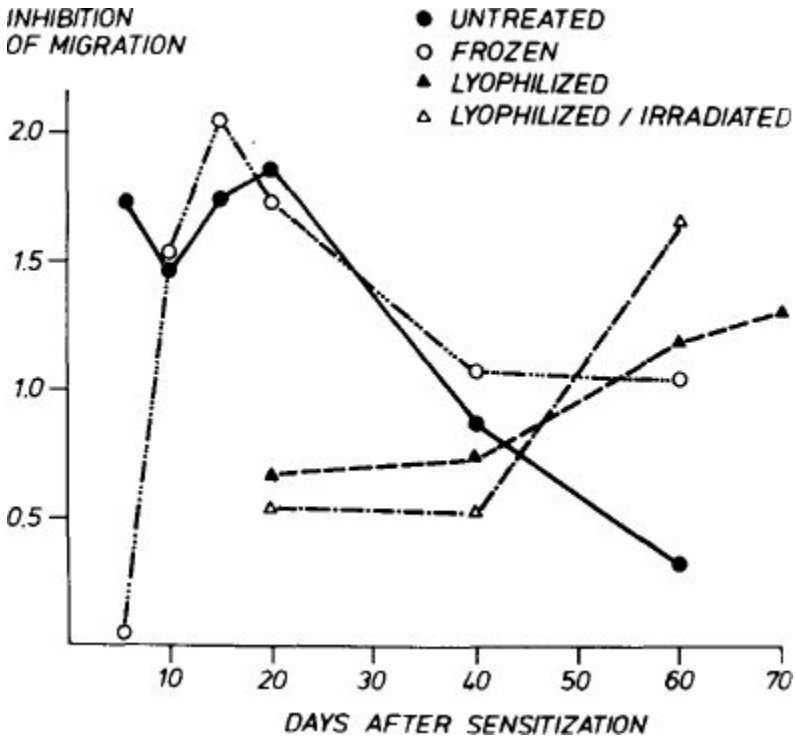

Figure *2.* Sensitization of B10.D2 inbred mice after grafting of peripheral nerves of B10.LP mice — evaluated by migration inhibition test. Inhibition is measured by the relation of radiuses of experimental and control areas of migration of macrophages on inverted scale, where zero means no inhibition.

It seems that lyophilized as well as lyophilized and irradiated peripheral nerves sensitize the host as much as fresh untreated ones. The difference, which was probably overlooked by other authors is, that sensitization in these cases is delayed and occurs not earlier than 60 days after grafting as compared with the period of 15 – 20 days in which the peak of sensitization was reached when frozen or fresh nerves were used. These data are yet unpublished and the work is in progress.

I left the discussion concerning bone tissue for the end because of some complications involved. Bone is the major product of tissue banks. Most data concerning the immunogenicity of preserved grafts concern bone. The confusing thing is that some authors do not always realize, or rather are not inclined to admit, that it is very difficult indeed to obtain results concerning bone tissue free of bone marrow, even in experimental conditions. This factor is — or rather was — of importance because nowadays we realize that immunogenicity of bone tissue is rather low, but if contaminated with bone marrow, it might be high because the haemopoietic tissue is highly immunogenic. This is why some literature data are confusing and difficult to compare.

One can find in the literature data on immunogenicity of bone and on trials to find "bone-specific" antigens (19, 20). As the situation looks now no conclusive proof of the existence of such tissue specific bone-antigens has been found. The fundamental papers in this field by Burwell(21) show that bone tissue purified from its content of bone marrow shows only weak antigenicity. This finding was based on the regional lymph node reaction. Bonfiglio and Jeter(22), Chalmers(23), Brooks et al. (24) checked

the immunogenicity of bone allografts after various preservation procedures. All the cited authors found a decrease in the immunogenic activity after freeze-drying and after irradiation. Cellular infiltration and mean survival time of skin grafts in sensitized hosts were used as parameters.

Kossowska(25) carried out a systematic study on the influence of the particular steps of the preservation procedure on the immunogenicity of bone. As shown in Figure 3 radiation sterilization lowers the immunogenicity of bone grafts as evaluated by the regional lymph node blastic reaction. Freezing and lyophilization itself provokes similar effects. Zaleski et al. (26) found that if bone is not contaminated by bone marrow, the same is true even in the xenogenic system. These results are in line with the data provided by Friedlaender(27) who used the Cr-51 release assay to study the humoral and cell mediated response in rabbits sensitized by bone allografts. Frozen lyophilized bone grafts never sensitized the experimental animals. Two review papers communicated at the IAEA Advisory Group Meeting in Athens in 1976 (28, 29) show conclusively that the antigenicity of allogenic bone measured by the degree of humoral cytotoxicity produced after grafting is highly reduced by the process of freezing and by lyophilization. Irradiation of lyophilized grafts (2.5 Mrads) does not change the situation.

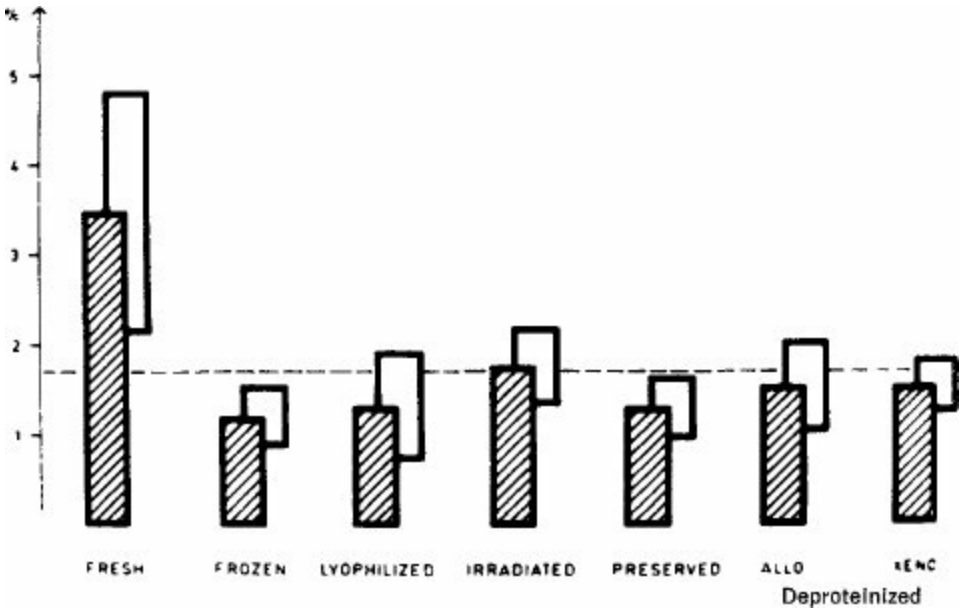

Figure *3.* The changes in the immunogenicity of allogenic bone by single steps of preservation procedure, as evaluated by regional lymph node blastic reaction.

Kamiński et al. (30) used the migration inhibition test to evaluate the sensitization of bone graft recipients. These experiments performed on mice (Figure 4) show a low immunogenicity of preserved bone.

I would like to close this discussion by the statement that the immunological problems involved in transplantation of preserved biostatic grafts are less serious than those encountered in the field of transplantation of living tissues. The risk of sensitization of patients is much lower and the immunogenicity can be reduced by modification of the preservation procedure.

The best example to illustrate this general statement would be the situation in skin transplantation. Those centers where living, deep-frozen skin is preserved and used are obliged to organize and use the tissue-typing procedure to match the preserved living skin with the donor (31). On the other hand when devitalized, lyophilized skin is used, even in the xenogenic situation, the problem is almost

nonexistent. Of course lyophilized skin is used only for skin dressing and is never allowed to stay long enough to be penetrated by the blood vessels of the healing wound. Literature data exist showing that multiple changes of xenogenic skin in the same host never induced sensitization (29, 32). Devitalized, lyophilized skin used for wound dressing is closer related as far as its immunogenicity is concerned to the membranes formed from reconstituted collagen than to the living skin transplant.

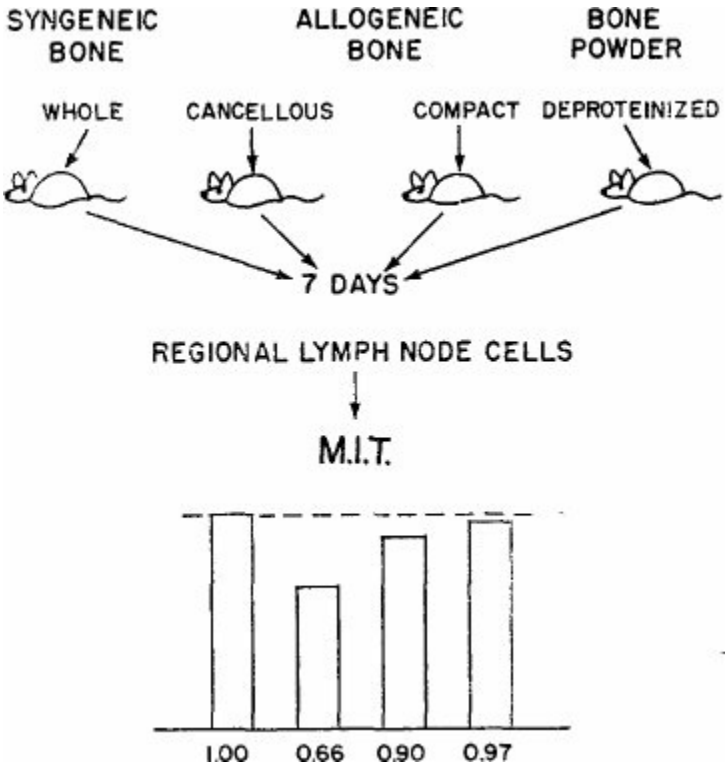

Figure *4.* Differences in immunogenicity of allogenic cancellous, compact and deproteinized bone, as evaluated by migration inhibition test (MIT). CBA mice were used as donors and Balb/e as recipients.

In my final remarks of this introduction I would like to stress that whatever the changes induced by preservation in the tissues, the end result as evaluated by the clinicians is all that counts. The next two papers will discuss problems which are much more serious than the immunological ones involved in biostatic graft transplantation. I hope that after this session you will have a general picture of the situation in the field of tissue banking which will allow for a constructive discussion.

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Effect of Radiation Sterilization on Biostatic Tissue Grafts and Their Constituents

Anna Dziedzic-Gocławska

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Abstract: *The use of ionizing radiation for sterilization of grafts induces many physical and chemical changes, which influence the biological properties of irradiated tissues. Some of these alterations are positive, e.g. decrease of immunogenicity, but most of them are undesirable, e.g. changes of mechanical parameters, decrease of osteoinductive capacity of radiation sterilized bone grafts.*

This paper reviews data on the effects of ionizing radiation on the main intercellular constituents of biostatic grafts: collagen, elastin and ground substance. Subsequently, experimental data concerning the effects of radiation sterilization on bone, heart valves, skin, dura mater, fascia and tendon grafts are discussed.

Biostatic, nonviable grafts such as bone, cartilage, peripheral nerves, dura mater, heart valves and skin serve as a kind of biological prosthesis, and in most cases undergo subsequent resorption and substitution by the host's own tissues.

The biological properties of these grafts, their immunogenicity, resorbability, ability to induce regeneration processes, e.g. osteoinductive ability of bone grafts, and, in some cases the mechanical properties of grafted tissues, are of great importance from the clinical point of view.

It should be stressed that the requirements are different for various types of grafts, depending on the role which they should fulfill in the host. For instance, in the case of peripheral nerve grafting the quick degeneration and resorption of myelin would be desirable to facilitate the ingrowing of new axons. On the other hand, some cartilage implants used in reconstructive surgery should be unresorbed as long as possible. The mechanical properties are very important in the case of vessels, heart valves or bone grafts, but they are not so significant when nonviable skin is used as a dressing material in the treatment of burns.

When choosing the method of preparation and sterilization, the particular requirements as regards grafted tissue should be taken into consideration in order to optimize the preservation procedure.

Since tissues prepared for grafting are usually taken from cadavers, they should be sterilized. Any kind of sterilization affects the biological properties of tissue grafts. There is only one tissue bank which organizes the sectioning of cadavers and the whole preservation procedure under sterile conditions which allows one to omit sterilization of the grafts (1).

Ionizing radiation has been widely used for over 25 years in tissue banking practice (2–4). This method of sterilization is very convenient and offers many advantages. It allows one to sterilize the material in closed vials and in this way to avoid additional contamination during packing. Gamma radiation particularly has a high penetration ability, thus allowing sterilization of even hard tissues such

as bone. With the sterilizing doses applied, the temperature rise is not very high, which is important when heat-sensitive biological materials are sterilized.

On the other hand, it should be kept in mind that high doses of ionizing radiation (in the range of megarads) used for sterilization purposes evoke many physical and chemical changes, which influence the biological properties of irradiated tissues. Some of these changes are profitable, e.g. decrease of tissue immunogenicity, discussed in the paper by Dr. Ostrowski, or increase of tissue resorbability. However, most of them are undesirable, e.g. decrease of osteoinductive properties, or changes in mechanical parameters of bone grafts. The general discussion concerning the direct and indirect effects, as well as the nature and time scale of physical, chemical and biological events resulting from the absorption of ionizing radiation, has been outlined elsewhere (5, 6).

Effect of ionizing radiation on intercellular constituents of connective tissues

Before we start to discuss the effects of radiation sterilization on the mechanical values and biological properties of particular biostatic grafts, some physical and chemical changes induced by ionizing radiation in the main extracellular components of connective tissues should be presented. Connective tissues in general are characterized by a low density of cells and abundance of extracellular material.

The common intercellular component of biostatic grafts such as bone, cartilage, heart valves, dura mater, tendons, fascia is an amorphous ground substance in which collagen and/or elastin fibres are embedded. In the case of hard tissues such as bone, enamel, dentine or aging cartilage the organic matrix is impregnated with calcium salts which in general are a mixture of amorphous calcium phosphates and crystalline hydroxyapatites.

Radiation-induced changes in collagen

Collagen is the major component of most biostatic grafts. A knowledge of radiation-induced physical and chemical changes in collagen is very important from the practical point of view because these changes may affect the mechanical parameters and biological properties of radiation sterilized grafts. Since such grafts undergo progressive resorption and substitution by the host's own tissues, it is important to know how the sterilization procedure affects the resorbability of collagen. One of the parameters which might allow one to predict the rate of resorption of collagen *in vivo* is its solubility *in vitro.*

In recent years a number of investigations have been made on the effects of ionizing radiation on collagen. Native, cross-linked or denaturated collagen obtained from different sources was irradiated with a wide range of doses, in dry or wet state with X-rays, high energy electrons and gamma radiation.

Various methodological approaches have been applied to study radiation-induced damage in collagen. The appearance, yield, stability and origin of collagen free radicals have been investigated by the electron spin resonance (ESR) technique. Other studies concern the determination of the tensile strength of collagen, shrinkage temperature, its solubility, amino acid analysis and reactivity with some enzymes. Histological and electron-microscopic observations of irradiated collagen were also performed.

Using ESR spectrometry several authors (7–17) studied the appearance, yield, stability and origin of free radicals in irradiated collagen. It is very often difficult to compare the results obtained, because the investigations have been made under different experimental conditions: the samples were irradiated in an air atmosphere or degassed at room temperature, or deep-frozen, and different energy and doses of ionizing radiation were used.

It has been reported that the ESR spectrum of irradiated collagen gives a complex pattern formed by two- and four-component radicals with relative intensity approximately proportional to its glycine-to-alanine ratio (10).

Fisher et al. (16) analysed the low temperature ESR spectrum of irradiated tendon collagen and found that it was similar to that obtained by Termine and coworkers (12) from irradiated 29-day-old rat femora. They found that most of the damage in irradiated collagen is concentrated randomly along the main peptide chains and that relatively few radicals are formed in the side chains of the amino acid residues. It has been observed that the yield of collagen radicals is dose-dependent, which was checked up to 0.6 Mrad, but the overall radical yield at room temperature was found to be low (G value of 0.3). The authors suggest that this may indicate a general resistance of collagen to radiation damage.

Stachowicz et al. (13, 14) analysed the ESR spectra of tendon collagen and organic bone matrix irradiated and measured at room temperature *in vacuo* and in the presence of air. A strong symmetric doublet line with weak outer components of the quartet was observed when collagenous material was irradiated and measured *in vacuo* at room temperature. The radical yield from organic bone matrix was much higher (G value of about 5.0) than those from radiation-induced defects in bone mineral (G value of about 0.1-0.2) when samples were irradiated at room temperature. Radiation-induced damage in bone mineral will be discussed later. The kinetic studies showed that the collagen radical is quite stable in degassed samples at room temperature, but decays rapidly after heating to 150°C, probably owing to interchain radical recombination. After admission of air oxygen to the nonheated samples, diffusion controlled decay of collagen radicals takes place owing to formation of peroxy radicals which are probably the intermediates. The peroxy radicals might react to form stable diamagnetic molecules (14) (see Figure 3).

It has been shown that the radical yield in irradiated collagenous materials varies widely and it is strongly dependent on the method of preparation of the samples and the experimental conditions under which the irradiation and ESR measurements were done, e.g. porosity of samples, temperature at which irradiation and measurements were done, presence or absence of air oxygen (14, 17).

Irradiation of collagen has been shown to lead to extensive changes in its physical properties (5, 18–20) and relatively small changes in the chemical composition (21). It has been suggested that these changes are primarily due to disorganization of the secondary structures (5, 20).

The radiation-induced changes differ markedly depending on whether collagen was irradiated in the dry or wet state.

Bailey and Tromans (20) investigated the effects of electron radiation on the ultrastructure of collagen fibrils using an electron microscope and a negative staining technique. They found that high doses are required to produce any changes in fibril structure. When fibrils were irradiated in the presence of water, no radiation-induced effects were observed up to doses of 10 Mrads, but above this dose a gradual destruction of the fibril band pattern occurred until at 40 Mrads the cross-banding completely disappeared. In contrast to irradiation in the wet state, irradiation with a dose of 4 Mrads of dried fibrils caused fragmentation of the filamentous units as well as extensive longitudinal band

splitting. The overall pattern of the fibrils disappeared and the fragments were completely disorganized. These observations were confirmed by Grant et al. (22) who found that the structural changes in irradiated collagen are dose dependent and that they are more pronounced when collagen was irradiated in the dry state.

Bailey et al. (18) studied the effect of irradiation with 2 MeV electrons with doses up to 40 Mrads on the shrinkage temperature of rat tail tendon collagen. It has been observed that the hydro-thermal shrinkage temperature decreased progressively with dose.

Bowes and Moss (21) investigated the effect of gamma radiation with doses ranging from 5 Mrads up to 50 Mrads on the shrinkage temperature of collagen irradiated at different moisture contents (5-80%). The shrinkage temperature of dry collagen could not be measured as it began to disintegrate almost at once. On irradiation with 18% moisture the shrinkage temperature was decreased nearly 20°C by a dose of 5 Mrads, and after a dose of 50 Mrads the collagen disintegrated and dissolved at 25°C. Collagen irradiated at 80% moisture did not show shrinkage even in boiling water.

The radiation-induced reduction in shrinkage temperature of collagen fibres is believed to be due to disorganization of the triple helix (5).

The tensile strength of irradiated collagen is dose-dependent, but the decrease of this parameter is more pronounced when collagen is irradiated in the dry state (19, 23, 24). Braams (23, 24) observed that, to reduce the tensile strength of dry tendon to about 1/3 of its original value, a dose of 18 Mrads was needed, while the reduction of tensile strength of hydrated collagen by the same value required about 46 Mrads. This observation was confirmed by Bailey and coworkers (19), who found that the relative tensile strength of native collagen irradiated in saline decreased logarithmically with dose (5 Mrads reducing the value by 50%), and that this effect was about three times as great, when the tendon collagen was irradiated at 10% moisture content. On the other hand, the authors showed that the relative strength of tendons, irradiated in saline and subsequently thermally contracted, increased with increasing dose to about ten times after 10 Mrads, while the irradiation of dry tendon, after rehydration and thermal contraction, caused a logarithmic decrease in strength (Figure 1).

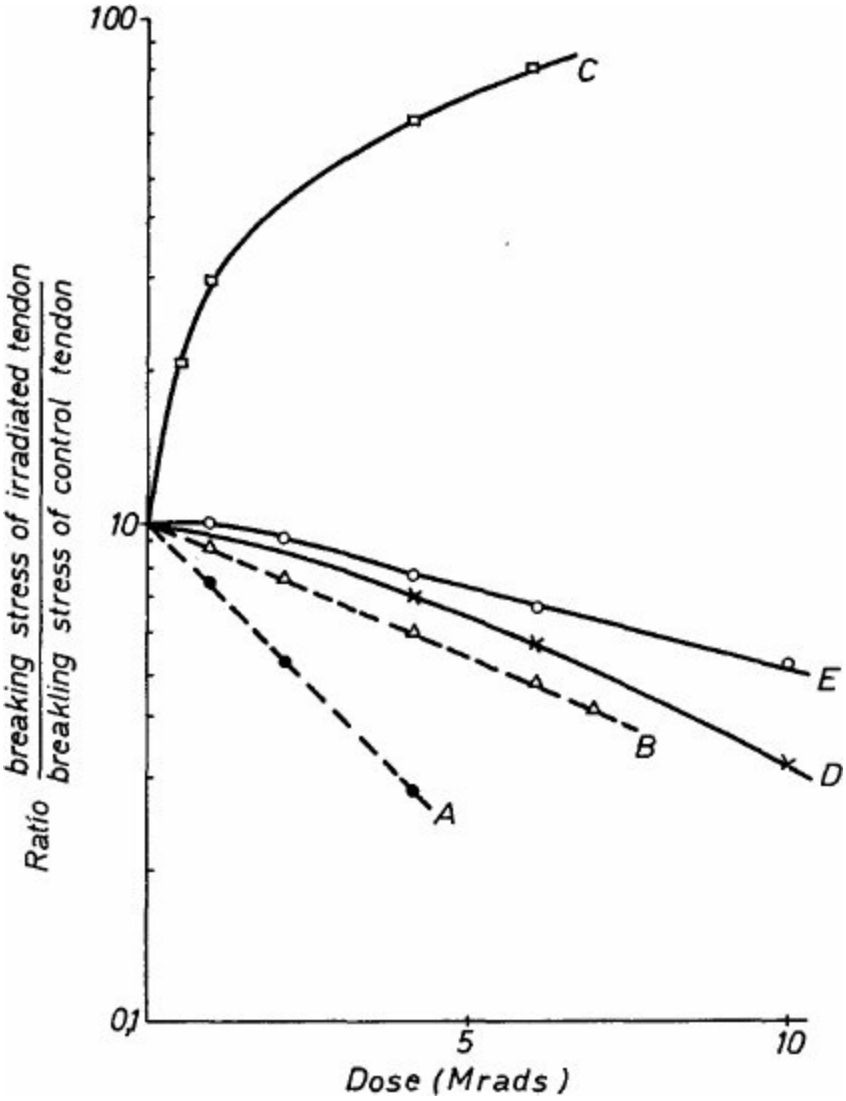

Figure *1*. Relative tensile strengths of irradiated rat-tail tendons measured native and after thermal contraction. A — irradiated dry (10% moisture), measured native; B — irradiated in saline, 0°C, measured native; C — irradiated in saline, 0°C, measured after thermal contraction; D — irradiated dry, measured after thermal contraction; E — irradiated in saline after thermal contraction (Bailey et al. 1964).

It has been shown that irradiation of native collagen in a wide range of doses (1-100 Mrads) caused a dose-dependent decrease of the solubility if the collagen was irradiated in the wet state and an increase of its solubility after irradiation in the dry state (19, 20, 22).

A detailed examination of the effects of irradiation (2-20 Mrads) performed at various temperatures on the solubility of collagen at different moisture contents was undertaken by Bailey et al. (19). A progressive increase in solubility was observed when rat tail tendon collagen was irradiated in the absence of water or at 10% moisture content. The solubility of wet, irradiated rat tail tendon collagen decreased with dose from the initial value of 90% to 80% after a dose of 5 Mrads and to 20% after a dose of 15 Mrads. The collagens from kangaroo and chicken tendon and from steer hide were initially less soluble than that of rat tail tendon, and the effect of irradiation in saline was much less marked. On the other hand, it has been observed that irradiation performed at low temperature (–20°C and –78°C) increased the solubility of wet rat tail tendon collagen and that this effect was more pronounced in the more insoluble collagen from kangaroo tendon.

Grant et al. (22) studied the effects of 14 MeV electron radiation with doses from 25 to 100 Mrads on the solubility of native and cross-linked rat tail tendon collagen irradiated in the wet and the dry state. They confirmed the observations of Bailey and coworkers (19) that the solubility of collagen irradiated in the dry state increases, while irradiation of collagen in the wet state causes a decrease of its solubility. Collagen cross-linked by the glutaraldehyde treatment was altogether insoluble after irradiation, whether it was irradiated in the wet or the dry state.

Sliwowski and Dziedzic-Gocławska (25) studied the effect of gamma radiation on the solubility of dry collagen derived membranes prepared for wound dressing. It has been found that the solubility increased with dose of radiation (Figure 2). The dose of 3.5 Mrads (the dose routinely used for sterilization in our tissue bank) caused about a 50% increase in solubility. This finding is in agreement with data concerning native collagen (19, 20, 22) and with the observations of Buring (26) on lyophilized organic bone matrix irradiated with up to 10 Mrads. It has been shown also that it is possible to lower the solubility of radiation sterilized collagen membranes by subsequent heating of the specimen at 110°C for 5 hours (Figure 2-B).

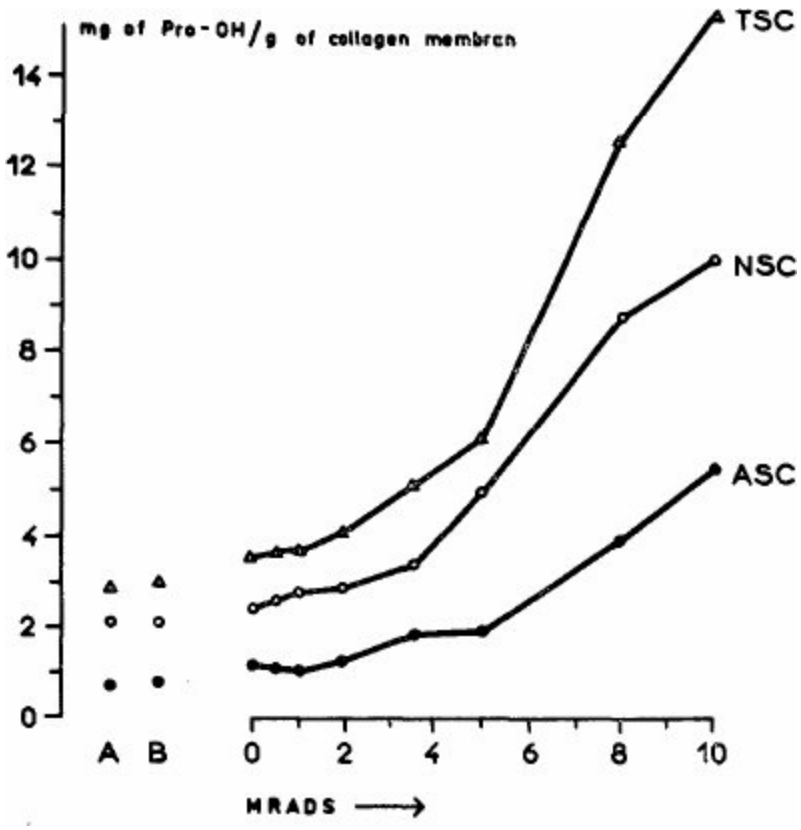

Figure *2.* Relationship between the solubility of collagen membranes and the dose of gamma radiation. NSC — neutral soluble collagen, ASC — acid soluble collagen, TSC — total soluble collagen; A — heated at 110°C samples, B — membranes irradiated with 3.5 Mrads and then heated at 110°C for 5 hrs. (Sliwowski and Dziedzic-Gocławska, 1976).

The considerable decrease in solubility and the increase in the tensile strength of thermally contracted collagen irradiated in the presence of water and the increase of solubility accompanied by a decrease in tensile strength of irradiated dry collagen were consistent with the hypothesis that irradiation of collagen in dry state results in scission of polypeptide chains and that in the presence of water the process of cross-linking predominates (5, 19, 22). The presence of cross-links was confirmed by stress-strain analysis (19). It has been postulated that the formation of covalent, intermolecular cross-

links probably results from random reaction of free radicals arising from scission of the main polypeptide chains (22).

The irradiation with high doses (25-100 Mrads) altered the reactivity of collagen with collagenase and elastase. Wet, but not dry, irradiated collagen became resistant to collagenase. Both wet and dry specimens were digested with elastase (22).

In general, not very pronounced differences in amino acid composition were noted between native collagen and that irradiated with high doses (25-100 Mrads) (21, 22).

It has been postulated that the observed changes in physical properties of irradiated collagen are primarily due to disorganization of its secondary structure (20).

It should be pointed out that very high doses (often many times higher than those used for sterilization) were applied to evoke marked changes in the physical and chemical properties of collagen, and that these changes were more pronounced when the collagen was irradiated in the dry state. Some of these properties can be altered by chemical or physical treatment of collagen before or after irradiation, e.g. it is possible to lower the solubility of irradiated dry collagen by pretreatment with glutaraldehyde (22) or by the heating of the specimen after irradiation (25), which is important from the point of view of tissue preservation practice.

Effect of irradiation on elastin

Elastin has been identified both histologically and chemically as a prominent component of the intercellular matrix of elastic cartilage, the walls of large vessels and some ligaments. It is also widespread in many tissues and organs such as skin, soft connective tissue, heart, heart valves and bone. Elastin fibrils examined by electron microscopy in conventionally stained sections, appear to comprise an amorphous core composed of elastin and a surrounding layer of microfibrils, approximately 100 Å in diameter, consisting of polar protein associated with hexose. In the electron microscope, negatively stained elastin fibrils were resolved into primary filaments arranged in a lateral array of about 50 Å periodicity. Anisotrophy was also indicated by optical polarization analysis and in low-angle diffraction patterns (27). Elastin possesses remarkable physical properties, such as ability to sustain large deformation without rupturing and to revert spontaneously to its original conditions when tension is released. In contrast to collagen, elastin shows considerable stability towards alkalis, acids and thermal treatment.

Data concerning the effect of ionizing radiation on elastin fibres are scarce.

Jelinek (28, 29) studied the effect of radiation on elastin *in vitro* and *in vivo.* He reported that *in vitro* after a dose up to 5 kilorads morphological changes such as fragmentation, disarray and swelling of the fibres occurred. Susceptibility of the fibres to radiation at doses as low as 3 kilorads was observed after irradiation *in vivo.*

Van Herpen [cited by Bailey (5)] observed an increase in rigidity of the elastic fibres after irradiation, and concluded that this indicated an excess of cross-linking. In contrast to the effect of ionizing radiation on dry collagen, where the process of chain scission predominates, irradiation of elastin fibres in the dry state produced extensive cross-linking. Van Herpen postulated that in elastin, having a much looser structure than collagen, the chains are normally too far apart to allow extensive cross-linking. On the other hand, in the dry state, the probability of two reactive groups meeting is greatly increased. In collagen the packing may be so tight that the probability of cross-linking is

maximal and the removal of water decreases the mobility of the molecules sufficiently to inhibit cross-linking. A progressive decrease in rigidity was observed at doses higher than 20 Mrads due to the process of chain scission, which predominates over cross-linkiing.

Effect of ionizing radiation on the ground substance

The ground substance is amorphous interfibrillar material located in the extracellular compartment. The character and function of connective tissue depend on the structural relationship between the ground substance and the fibrous components. The main components of the ground substance are glycosaminoglycans which may be divided into two groups: hyaluronic acid and sulphated mucopolysaccharides. Hyaluronic acid has a regular chemical structure and it is covalently bound to a very small amount, if any, of protein. In contrast, the sulphated mucopolysaccharides have a much less regular structure, lower molecular weight and they are covalently bound to the protein core. They form the side chains of proteoglycans. Hyaluronic acid aggregates proteoglycans by interacting with their protein cores. Recently it has been postulated that an interaction occurs between collagen fibres and proteoglycans. The protein core of proteoglycan is covalently bound to its sulphated mucopolysaccharide chains which, in turn, bind electrostatically to the collagen fibrils (30). The ground substance, beside being important in the laying down of collagen fibres, is believed to be involved in the permeability of biological membranes.

Several authors (31–43) studied the effects of ionizing radiation on mucopolysaccharides.

Ragan et al. (31) and Brinkman et al. (32) used synovial fluids as a model for the study of the effect of irradiation on mucopolysaccharides. After a dose of 5 kilorads a rapid decrease in viscosity, down to 30% of the original value, was observed (31), due to degradation of mucopolysaccharides.

Investigations concerning the effect of irradiation on hyaluronic acid were performed by several authors (33–39). It has been found that, after irradiation, the viscosity of hyaluronic acid also decreases and that it is more susceptible to hyaluronidase (33, 34). The sensitivity of hyaluronic acid to ionizing radiation has generally been attributed to glycosidic bond cleavage. Balazs et al. (35) reported that irradiation caused depolymerization of the high molecular weight polysaccharides to low-molecular dialyzable compounds, and that the destruction of hexosamine and hexuronic acid moieties was observed. Lamberts and Alexander (36) studied the postirradiation effects on hyaluronic acid. They attributed the fall in viscosity to the reaction with hydroxyl radicals, which caused main-chain scission. Addition of thiosulphate immediately after irradiation prevented a further decrease in viscosity. The delayed diminution is viscosity was observed when hyaluronic acid was irradiated in the presence of 0.1 M NaCl. This was explained by the reaction of chloride ions with the radiolysis products of water to form another entity capable of causing main-chain scission, but at a relatively slower rate. Later, Balazs and coworkers (37) concluded that radiation sensitivity of hyaluronic acid was due to facile reaction with both major products of water radiolysis, e^-_{aq} and OH. Hyaluronic acid was found to be more reactive to e^-_{aq} than other carbohydrates. The observed protective effect of oxygen was accounted for by the reaction $e^-_{aq} + O_2 \rightarrow O_2^-$, assuming that O_2^- as little affinity towards hyaluronic acid.

Other studies on hyaluronic acid have been performed with the use of the vitreous body of the eye (38) or artificial mixtures of collagen gels and various concentrations of hyaluronic acid (39). Hyaluronic acid stabilizes the gel against the release of liquid, but following irradiation the stabilizing effect decreases with increasing dose. It has been postulated that hyaluronic acid was more degraded

than the collagen (39).

Jooyandeh et al. (40) studied the effects of gamma radiation on heparin and keratin sulphate. They found that, during irradiation, acid and reducing products were formed, mainly as a result of attack by OH radicals. Depolymerization, giving lower dextrins, also occurred without loss of sulphate groups. The protective effect of oxygen was accounted for by the scavenging reaction $e_{aq}^- + O_2 \rightarrow O_2^-$.

Ranu et al. (41) studied the mechanical properties of skin irradiated with doses from 1000 to 3000 rads. From the observation that the elastic properties of skin associated with collagen fibre alignment are little affected by these doses of radiation, the authors conclude that the ground substance is radiation resistant.

Recently Phillips et al. (42) investigated the effects of gamma radiation with doses ranging from 1.6 to 100 Mrads on glycosaminoglycans of human cartilage. They also tried to find a method of protection of the tissue from radiation damage. The chemical changes following irradiation have been monitored by the use of "critical electrolyte concentration" (CEC) measurements utilizing alcian blue/magnesium chloride and toluidine blue/sodium chloride systems.

In this method the glycosaminoglycans of untreated and irradiated cartilage were examined by metachromatic staining with the use of alcian blue or toluidine blue solutions containing different molar concentrations of $MgCl_2$ or NaCl. The decrease in the "critical electrolyte concentration" with dose of radiation was related to the decrease in hexose and hexosamine contents of the cartilage as a result of degradation of glycosaminoglycans. After a dose of about 4.8 Mrads a very slight decrease in the hexosamine content (about 1%) and almost no changes in the hydroxyproline content were observed. The former is a measure of glycosaminoglycan content in cartilage, and the latter is related to the amino acid of collagen.

It has been concluded that glycosaminoglycans are more susceptible to radiation damage than collagen, although in chemical terms both do not appear to be grossly degraded by radiation. It has been found that the glycosaminoglycans of cartilage can be successfully protected from radiation damage by a suitable cationic detergent, cetylpyridinium chloride. The energy transfer method used for radiation protection in experiments with heparin and other glycosaminoglycans (43) was now applied to protect cartilage glycosaminoglycans. It should be stressed, however, that the agents used for protection of tissue from radiation damage may also protect microorganisms, decreasing their radiation sensitivity, which may affect the sterility of radiation sterilized grafts.

The ground substance is involved in the permeability of biological membranes. The effect of ionizing radiation on this parameter will be discussed later.

Effect of radiation sterilization on biostatic tissue grafts

The effect of ionizing radiation on tissues as a whole is not necessarily the sum of the known effects on their constituents. The analysis of radiation-induced changes in tissues is far more complicated for many reasons. Some new phenomena, nonexistent in isolated compounds appear, e.g. transfer of free radical electron spin energy between bone mineral and bone protein (12). The available methods of evaluation of radiation damage in irradiated tissues are different from those used for analysis of radiation-induced changes in isolated, chemically defined tissue constituents.

The effect of radiation sterilization on tissue graft immunogenicity is discussed in the paper by Dr. Ostrowski. The clinical results with the use of irradiated implants are analyzed by Dr. J. Komender and

Dr. A. Komender (49).

In this subchapter some experimental studies performed *in vitro* and *in vivo* and concerning the evaluation of mechanical, physical and biological properties of particular radiation sterilized grafts are presented.

Bone grafts

Bone allografts constitute the majority of the grafts prepared by tissue banks and used in clinical practice. The most commonly used methods of preservation of bone tissue are deep freezing and lyophilization. Since these methods do not ensure the sterility of tissues, which are usually taken from cadavers, in many banks the grafts are subsequently sterilized by ionizing radiation with doses ranging from 2.0 to 3.5 Mrads (13, 44–49). Such high doses of ionizing radiation affect the tissues and many experiments were performed to evaluate the biological properties of radiation sterilized grafts. Some of the experimental data concerning the appearance of radiation-induced paramagnetic entities in bone tissue are presented and the effect of ionizing radiation on mechanical values, remodelling and osteoinductive properties of bone grafts are discussed.

Electron spin resonance studies of radiation sterilized bone grafts. Ionizing radiation induces in biological material, among other intermediates, the formation of free radicals, which are often very stable. Free radicals are highly reactive chemical entities and their potential interference with the metabolic processes of the recipient of radiation sterilized grafts should be taken into consideration. There have been suggestions that free radicals might be potential mutagenic agents, but this hypothetical effect has never been proved or disproved. The potential harmful effect was studied in research on irradiated food, where free radicals have been found (50).

As concerns the potential danger of free radicals introduced with radiation sterilized grafts into patients, the control consists in monitoring the irradiated tissues by electron spin resonance (ESR) spectrometry. This technique allows one to detect paramagnetic entities, including free radicals, and to establish their yield and stability. In some cases qualitative analysis of paramagnetic species is possible (51).

Very often it is difficult to compare the results obtained because irradiation and ESR measurements of bone and its constituents have been performed under various experimental conditions: at low or room temperatures, *in vacuo* or in the presence of air oxygen, irradiation was performed *in vitro* or *in vivo,* various sources of ionizing radiation and different doses were applied.

The first data on ESR signals in irradiated bone were published by Gordy and coworkers in 1955 (7). Since then several attempts have been made to establish the stability, yield and origin of the ESR signals appearing in irradiated bone and its constituents (8–16, 52–59). One part of the investigations concerns biological material, another based on simpler chemical models provides basic data concerning radiation-induced changes. Some of the data concerning radiation-induced free radicals in organic bone matrix collagen have been discussed previously in the section concerning the effect of irradiation on collagen.

Slager and coworkers (8, 9) found an ESR signal in radiation sterilized bone grafts. This signal persisted for 12 weeks when irradiated grafts were kept *in vitro* at room temperature or at 37°C. The authors claim that 35 days after grafting into animals the signal in the grafted fragment of bone disappeared.

Cole and Silver (52) analyzed ESR spectra of a tooth after X-ray irradiation. They found three different kinds of paramagnetic species, one of which was identified as a hydrogen atom.

Swartz (11) found a dose-dependent relationship of the ESR signal intensity in bone irradiated *in vitro* and *in vivo*. After analysis of the overall decay of radiation-induced ESR signals of deproteinized and decalcified bone, the author concluded that the exact nature of the resonance had not been elucidated in this experiment.

Termine et al. (12) analyzed low temperature spectra of irradiated bone and compared them with those obtained after irradiation of artificial mixtures or organic bone matrix and synthetic calcium phosphates. The complexity of the low temperature ESR spectra did not allow definitive conclusions. The authors postulate that a transfer of free radical electron spin energy may take place between bone mineral and bone protein and that there is an intimate interaction, perhaps through chemical bonding, between the organic and mineral phases of skeletal tissue.

Hauben (15) examined the influence of various parameters such as Ca:P ratio, degree of crystallinity and impurities on the yield and stability of radiation-induced paramagnetic species in synthetic apatites and collagen. The investigations were performed at room temperature and at –180°C. He found that the yield of paramagnetic species derived from apatites depends on the density of bone.

Fisher et al. (16) using the ESR technique studied radiation damage in collagen and calcium phosphates similar in composition and structure to the principal inorganic components of bone and tooth. The low temperature ESR spectra of gamma irradiated calcium phosphates were interpreted in terms of PO_4^{-3} O^- and H radicals.

Stachowicz and coworkers (13, 14) examined the ESR spectra of bone tissue and its constituents irradiated and measured at room temperature *in vacuo* or in the presence of air oxygen. This allowed the analysis of simplified ESR spectra, because the shortlived, quickly recombined radicals did not interfere. It was concluded that in bone tissue irradiated and measured under the described conditions two main ESR signals derived from its mineral and organic constitutents can be detected. The results obtained with a variety of bone samples, teeth, purified collagen and synthetic hydroxyapatite allowed assigning the long-lived stable ESR singlet (Figure 3) to radiation-induced defects in bone hydroxyapatite (G value of about 0.1-0.2). The relation between spin concentration of stable paramagnetic centers and absorbed dose of gamma radiation has been examined within the range of doses from 0.1 to 12.0 Mrads. The linear relationship was observed up to 1.5 Mrads. At higher doses the relation loses its linear characteristic (56, 57). Since these paramagnetic centers were found to be stable for years in samples kept *in vitro,* non-reactive at various temperatures and, when kept in aqueous media *in vitro,* there seems to be no danger of their interference with metabolic processes of the recipient of radiation sterilized bone grafts. Another component of the ESR spectra is a strong symmetric doublet derived most likely from collagen radicals. These radicals are quite stable in degassed samples (G value of about 5.0), but after admission of air oxygen, diffusion-controlled decay takes place (Figure 3). The prolongation of storage of bone grafts after irradiation causes complete decay of collagen radicals.

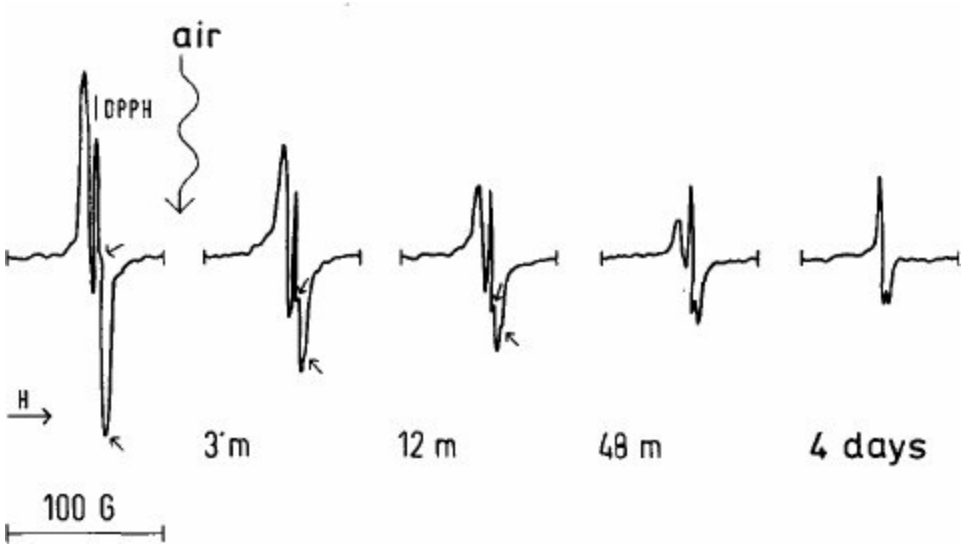

Figure *3.* The first derivative ESR spectra of degassed compact bone powder irradiated in a Co-60 source with a dose of 3.5 Mrads, recorded immediately after irradiation (first signal) and 3, 12, 48 minutes and 4 days after admission of air into sample tube. Samples were irradiated and measured at room temperature. After complete decay of collagen-derived symmetric doublet an asymmetric stable ESR singlet (last signal) arising from defective hydroxyapatite crystals is seen (Stachowicz et al. 1970).

In experiments performed on hydroxyapatite synthesized *in vitro* it has been proved that the stable asymmetric ESR singlet is derived from radiation-induced defects in the crystalline lattice of hydroxyapatite. The ESR spectra of irradiated amorphous precursors of hydroxyapatite are more complex and differ from this characteristic for hydroxyapatite. They disappear completely within a few days of storage at room temperature. The spin concentration of radiation-induced stable paramagnetic centers depends on the average crystal size hydroxyapatite and on its content in the measured sample (58).

One of the important new developments in the study of biological calcification processes was the discovery that the mineral part of bone is not homogeneous, but consists of two fractions: amorphous calcium phosphates and crystalline hydroxyapatite. The proportion of both fractions, i.e. crystallinity of mineral changes in the course of development, aging and in pathological processes (60).

Since the described stable radiation-induced changes in bone mineral are derived only from the defective lattice of hydroxyapatite crystals, the relation of spins to the total amount of ash is a measure of crystallinity of mineral (61, 62). This approach was used for evaluation of the crystallinity of minerals of physiologically (Figure 4) and pathologically calcifying tissues (61–63). The high sensitivity and accuracy of this method allow one to use it on a microscale. Single osteons isolated from 100 μm thick undecalcified sections (61, 64), as well as subcellular fractions. e.g. isolated Ca-loaded mitochondria (65), were measured.

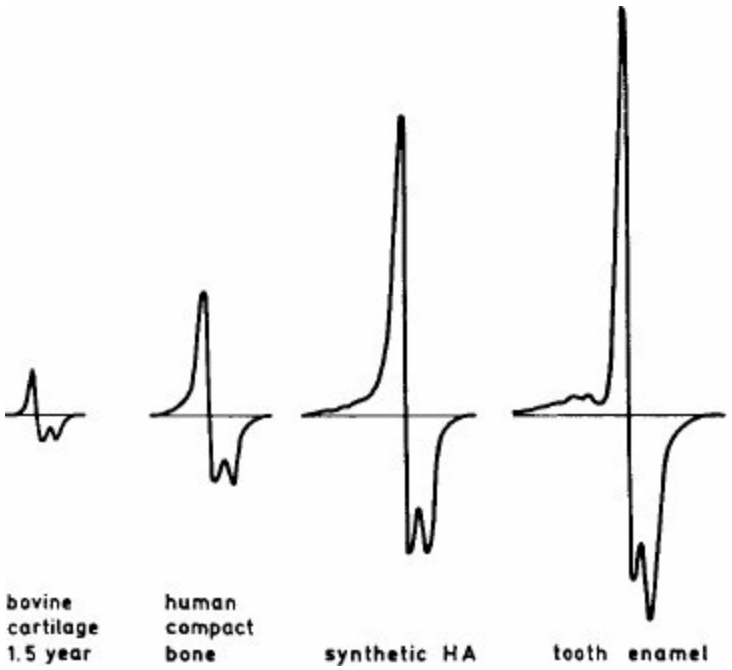

Figure *4.* Comparison of the intensities of the first derivative ESR signals arising from radiation-induced paramagnetic centers in hydroxyapatite for various kinds of mineralized tissues and for synthetic hydroxyapatite, after normalization as regards weight of samples and sensitivity of measurements (Ostrowski et al., 1974).

Slager et al. (9) were the first to describe the disappearance of the radiation-induced ESR signal after grafting of irradiated bone in an allogeneic system in dogs. The nature of the observed ESR signal was not discussed in the paper. It is now known (13, 14, 58) that this signal is derived from radiation-induced defects in the crystalline lattice of bone hydroxyapatite. The high stability of these paramagnetic centers enables their use as a new type of label of radiation sterilized bone grafts for quantitative description of the process of graft resorption and creeping substitution (56, 66).

The idea to use the ESR active paramagnetic species produced during irradiation in skeletal tissues for dosimetry of absorbed dose of ionizing radiation was formulated in the papers by Swartz (11) and Houben (15). Although the nature of ESR signals was not clearly defined, Brady et al. (67), by applying low temperature ESR measurements and averaging of signal intensities, were able to measure the doses in the order of a hundred rads. A more precise approach to such dosimetry was possible when the nature of the complex ESR spectrum in bone and stability of its main paramagnetic components were defined (13, 14, 58). After identification of stable radiation-induced defects in hydroxyapatite and plotting of the dose-dependence curve, a bone powder dosimeter was proposed for control of the radiation sterilization process (57). The ESR spectrometry also can be used for low dose dosimetry in cases of therapeutic and/or accidental exposure (68, 69).

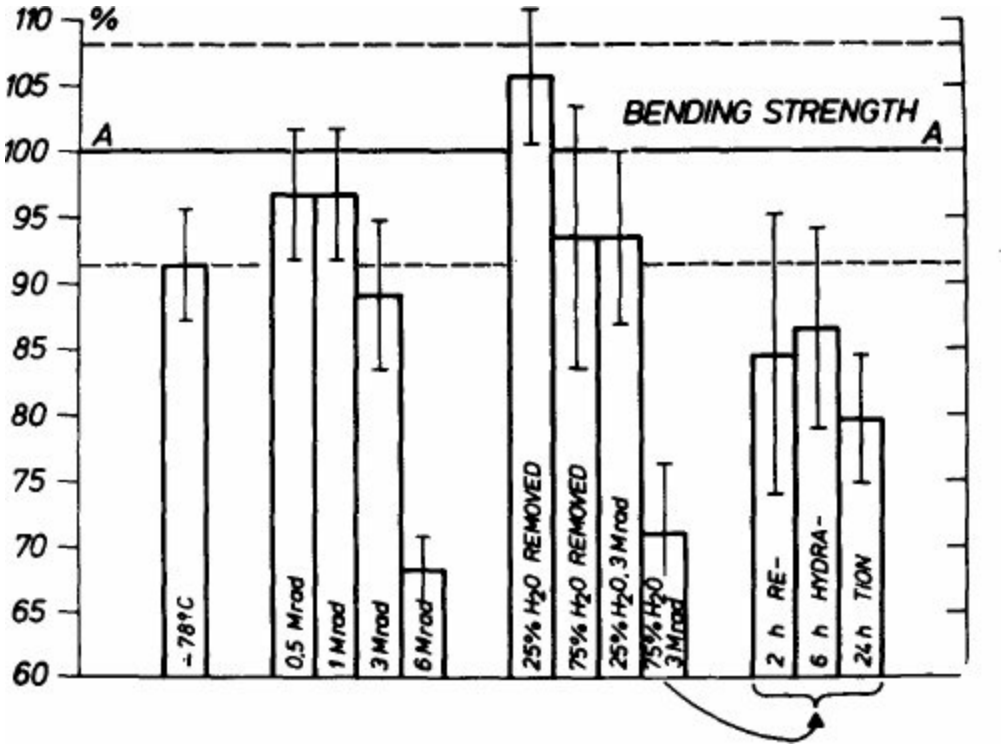

Figure 5. Relative bending strength values (mean ± SE) for bone samples treated in different manners (Komender, 1976).

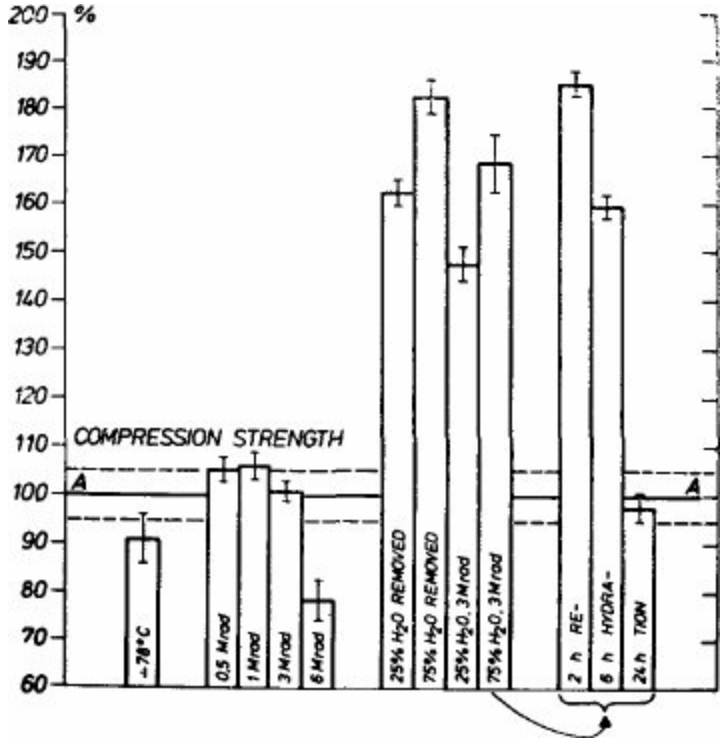

Figure 6. Relative compression strength values (mean ± SE) for bone samples treated in different manners (Komender, 1976).

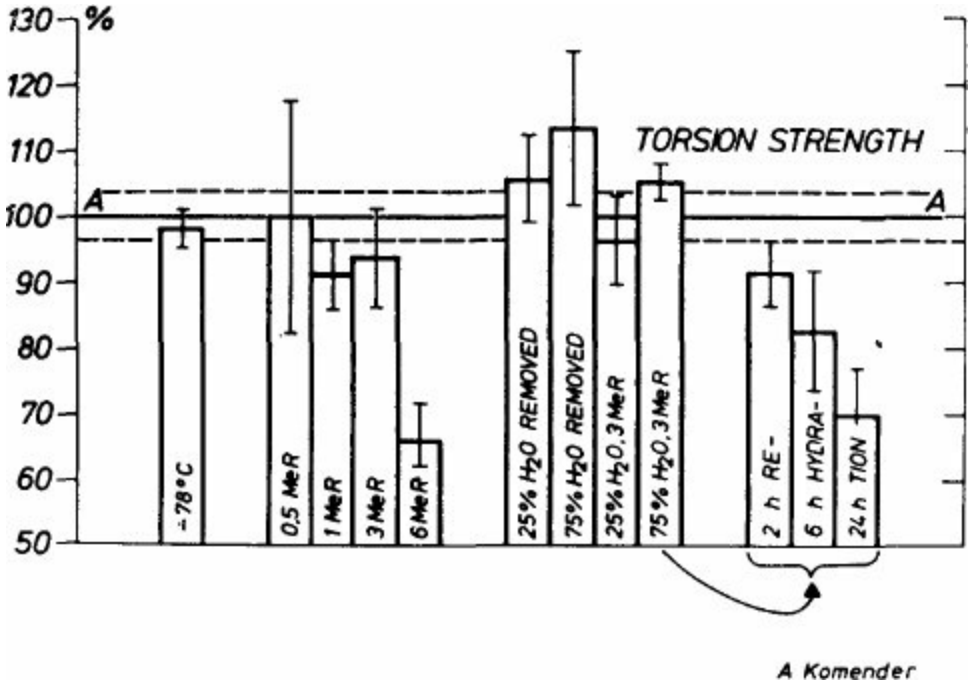

Figure *7.* Relative torsion strength values (mean ± SE) for bone samples treated in different manners (Komender, 1976).

Changes in the mechanical characteristics of radiation sterilized bone grafts. It is known that preservation procedures affect the mechanical properties of biological tissues, which is particularly important in the case of bone grafts.

A. Komender (70, 71) performed detailed studies on the effect of the particular steps of the preservation procedure, such as deep freezing, lyophilization and radiation sterilization on the different mechanical parameters of cortical bone tissue. Bending, compression and torsion strengths were investigated. The results are summarized in Figures 5, 6 and 7. It was found that deep-freezing caused no changes in the measured mechanical parameters of bone. The changes evoked by the preservation procedure were connected mainly with lyophilization. The damage evoked by ionizing radiation was dose-dependent. It has been observed that doses in the range up to 3 Mrads caused slight changes in the mechanical parameters, while 6 Mrads evoked significant irreversible damage. It also has been reported that rehydration of preserved bone improves slightly the bending strength, brings compression strength to the normal level and diminishes the torsion strength.

Triantafyllou and Karatzas (72) studied the modulus of elasticity and tensile strength of cortical bone preserved by freezing, lyophilization and irradiation with a dose of 2.5 Mrads. It was observed that the lyophilization procedure contributed significantly to the overall deterioration of the mechanical properties. A further decrease of mechanical values was reported after irradiation.

Klen and Pacal (73) tested the resistance to bending of cancellocortical bone lyophilized and irradiated with a dose of 2.5 Mrads. They found a decrease in the elasticity of irradiated bone.

Recently Sell et al. (74) confirmed the previously discussed data. After irradiation of cortical bone with a dose of 2.5 Mrads significant changes in the modulus of elasticity, the ultimate strain and the plastic modulus were observed. When radiation sterilization was combined with freeze-drying, these alterations were more pronounced.

The effect of radiation sterilization on the remodelling and osteoinductive capacity of bone grafts. Phemister

in 1914 gave to the complex process of remodelling, which occurs in bone grafts, the name of creeping substitution. Basically, remodelling consists of two processes; namely, resorption followed by accretion. Remodelling of bone grafts has been studied in numerous experimental sites (orthotopic and heterotopic) using various types of bone prepared in different manners. Excellent review papers concerning the basic problems connected with bone grafting, as well as experimental and clinical results obtained with the use of a variety of grafts, have been published by Burwell (75, 76).

The process of bone graft remodelling is balanced when resorbed grafted tissue is subsequently substituted by the newly formed bone. The formation of new bone is obligatory in order to achieve union between a bone graft and the part of skeleton with which it is placed in contact. The osteogenesis of repair is desired both from the graft and from its bed, but the relative contribution of each depends largely upon the type of bone grafted and on the methods of graft preservation and sterilization (76).

We will not discuss here the immunological problems connected with bone grafting, because they were outlined in the paper by Dr. Ostrowski. Only some experimental data concerning the influence of ionizing radiation on the remodelling process and osteoinductive properties of preserved bone grafts will be presented.

As stressed by Burwell (75), the influence of irradiation on remodelling of freeze-dried bone is uncertain. While Chalmers et al. (77) have found it to marginally accelerate resorption, another group of investigators (78) claims that it delays incorporation. De Vries (79, 80) found that lyophilized irradiated bone implanted into dogs and rats stimulated new bone formation in fracture sites.

Dziedzic-Gocławska (81) and Ostrowski et al. (56) applied the ESR technique based on measurements of stable radiation-induced defects in bone hydroxyapatite for quantitative evaluation of the rate of resorption of lyophilized bone grafts sterilized with a dose of 3.3. Mrads. Bones of the calvaria were implanted orthotopically in an allogeneic system into rabbits. The progressive resorption, proportional to the time intervals after grafting, was observed. Histological examinations showed that the graft remodelling was balanced and that the resorption process was followed by substitution by newly formed bone (Figure 8).

Sell et al. (74) studied the remodelling of freeze-dried and freeze-dried, irradiated (2.5 Mrads) cortical bone grafts implanted into dogs. They reported, that whereas 90% of lyophilized bone grafts went on to complete healing, at the same time, only one of six freeze-dried, irradiated grafts was completely incorporated.

The possibility to induce bone formation in heterotopic sites, principally in skeletal muscles, is a convenient experimental model for study of the contribution that the bone graft itself makes to osteogenesis. Although the exact nature of the factors responsible for the osteoinductive properties of nonviable bone matrix still remains unknown, many attempts have been made to establish the influence of various chemical and physical agents, including ionizing radiation, on the osteoinductive capacity of preserved bone grafts (76).

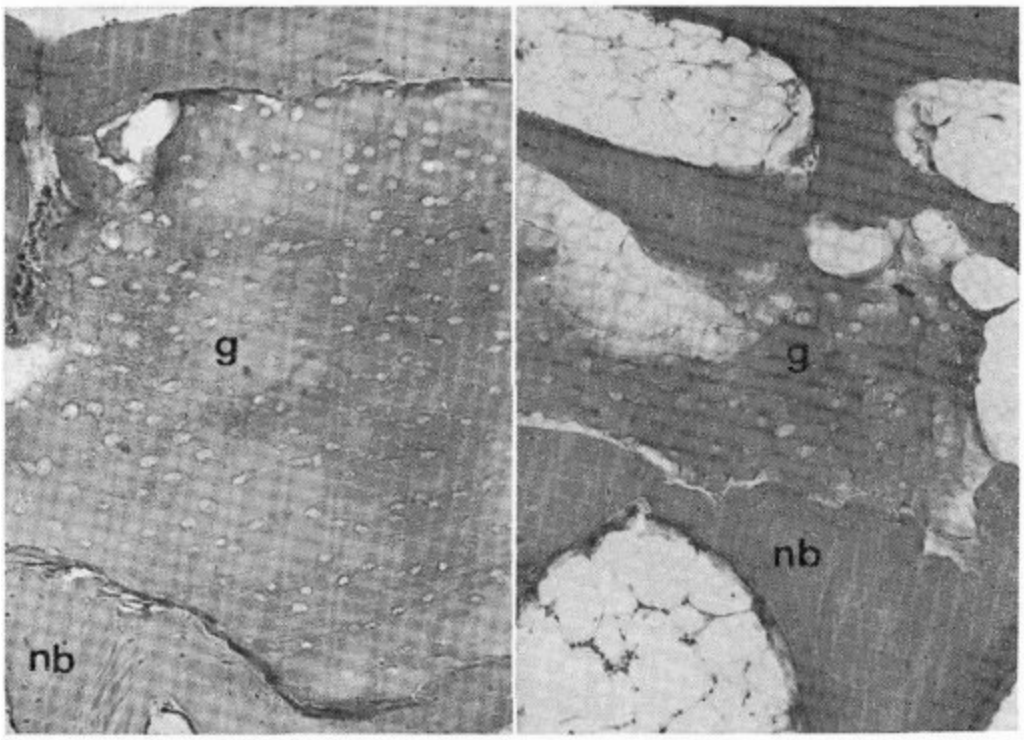

Figure *8.* Orthotopic allogenic lyophilized irradiated (3.3 Mrads) grafts implanted into rabbit calvaria illustrate the progress of resorption and creeping substitution processes; left — 8 weeks and right — 16 weeks after implantation; g — grafted tissue, nb — newly formed bone (Dziedzic-Gocławska et al., 1977).

Using the model of heterotopic bone induction, Buring (26) studied the effect of irradiation with doses ranging from 0.5 up to 10 Mrads on the osteoinductive capacity of decalcified lyophilized bone matrix implanted intramuscularly in an allogenic system in rabbits. He reported that, after a dose of 2.0 Mrads or more, the osteoinductive properties of bone matrix were completely destroyed. Histological examination showed infiltration of the implants with round cells and an increase of resorption of the irradiated grafts. These findings concern osteoinduction in heterotopic sites. Bone grafts implanted orthotopically usually have in their vicinity the host's own osteogenetic tissue responsible for graft resorption and creeping substitution.

Recently, a new methodological approach based on ESR spectrometry was developed to study the remodelling process of radiation sterilized bone grafts. This method allows one to evaluate quantitatively and separately the rate of graft resorption and its substitution by newly formed bone, as well as to estimate the crystallinity of the mineral of newly formed callus. The detailed results will be published elsewhere (66).

Further studies are necessary to optimize the preservation procedure of allogeneic bone grafts and to find a suitable quantitative method for their biological evaluation.

Heart valves

Various methods, including radiation sterilization, have been used for the preservation of heart valves (82–89).

Welch (83), using an arbitrary scale of evaluation, found that neither gamma irradiation nor electron beam sterilization induced any changes in frozen aortic valves, but he concluded that by "over-

irradiation" the structure of valves is completely altered. Unfortunately, he did not describe how high a dose of irradiation he used.

Malm et al (84) studied the mechanical strength of aortic valves sterilized by various methods. They found that neither the normal architecture nor the aortic wall tensile strength showed changes after irradiation at an average dose of 2 Mrads. Other methods of sterilization caused significant reduction in tensile strength.

Gibbons and Alladine (85) reported that irradiation with 2.5 or 3.2 Mrads caused little changes in the histological picture and no changes in the tensile strength of valves.

Trimble and coworkers (86) concluded that deep-frozen and irradiated (2.5 Mrads) aortic valve allografts resemble in function fresh valves.

Kurnatowski and Gidynski (87) performed experimental studies concerning the influence of various methods of preservation on the mechanical properties of porcine aortic valves (Figure 9). A highly significant decrease of tensile strength was observed after irradiation with 2.5 and 3.3 Mrads following lyophilization. The changes were less pronounced when valves were irradiated in saline solution or prefixed in 70% ethanol and then irradiated in saline.

While in the earlier papers (83–86) successful function and low complication rate in clinical use of frozen, irradiated valve allografts were reported, after long-term evaluation, an alarming incidence of lethal allograft failure related to degenerative changes and calcification of grafts has been noted (88). Moore et al. (89) reported that chemically sterilized or irradiated valves and freeze-dried storage result in valve failure in about 1/3 of the cases. They concluded that these methods of preservation and sterilization render the allograft acellular and lead to a high number of failures in long-term evaluation. Fresh, viable allografts are now recommended in clinical practice (90).

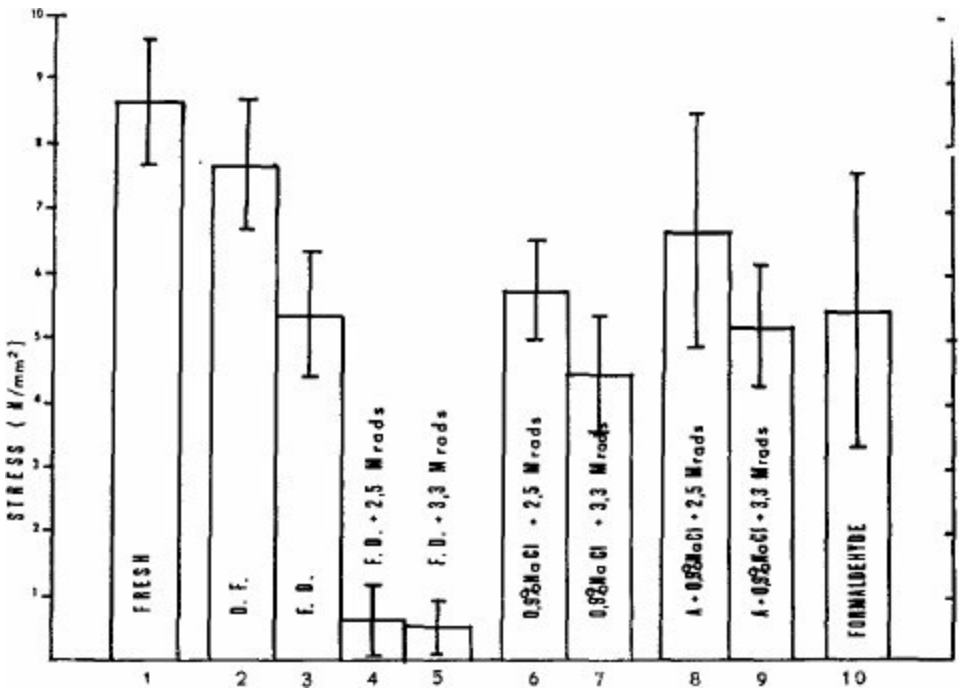

Figure *9.* The comparison of mechanical strengths of aortic valves preserved in different manners: DF — deep frozen, FD — freeze dried, 0.9% NaCl — irradiated in saline, A — prefixed in 70% ethanol and then irradiated in saline (Kurnatowski and Gidyński, 1977).

In some clinics xenograft porcine valves fixed and sterilized in glutaraldehyde are used. Glutaraldehyde treated valves should be mounted on a frame or stent. According to the opinion of Dr

W. H. Wain from the National Heart Hospital in London (personal communication) it has not yet been possible to find a satisfactory method for fixing and inserting valve grafts after glutaraldehyde fixation. There are also problems of sterility when allogenic human post-mortem tissues are used due to much higher levels of initial contamination.

Skin

Nonviable, preserved skin allografts are used as a biological dressing for extensively burned patients (91, 92). Freeze-dried skin is mainly used and lyophilized irradiated (3.3 Mrads) allogenic and xenogenic skin are also prepared in tissue banks (49).

Ranu et al. (41) studied the effect of X-ray irradiation with doses ranging from 1000 up to 3000 rads on the mechanical properties of skin. They found that the load extension curve, which was plotted to the point of rupture, exhibits two portions. The elastic properties associated with fibre alignment were little affected and the authors conclude that the ground substance is radiation resistant. On the other hand, the stretching and rupture of aligned collagen fibres showed a marked radiation dependence and stiffness of the collagen itself decreased with increasing dose.

Recently Kwarecki et al. (93) compared the solubility and mechanical properties of fresh, lyophilized, and lyophilized and irradiated (3.3 Mrads) skin. They found that the tensile strength of lyophilized and then rehydrated skin was reduced to about 80% of its original value, while lyophilized, irradiated and rehydrated skin revealed a reduction to about 25% of the control. The solubility of collagen of lyophilized skin was almost unchanged, while a slight increase of solubility (5-15%) was observed after irradiation.

Brinkman et al. (94) studied the permeability of X-ray irradiated fresh membranes from rat skin stretched across the end of a Lucite tube. A rapid increase in permeability was observed after very low doses ranging from 10 to 100 rads. This effect was interpreted as being due to depolymerization of mucopolysaccharides of the connective tissue matrix. However, the authors suggest that stretching may increase the pore size, allowing larger fragments of depolymerized mucopolysaccharides to pass through the membrane.

Klen and Pacal (73) compared the permeability of dermoepidermal grafts lyophilized and irradiated with 2.5 Mrads with unirradiated controls. They found that the permeability of irradiated specimens decreased to about one half of the control values.

These controversial results concerning the permeability of irradiated skin may be due to the various experimental conditions, wide differences in the doses used and different methods of evaluation.

It should be pointed out, that since the preserved skin serves as a temperary biological dressing, its mechanical properties and permeability are of minor importance from the practical point of view. More important is the evaluation of the duration of "stay" of such implants and immunological problems involved with their multiple use (91, 95).

Dura mater

Although preserved allogenic dura mater is widely used in clinical practice (48, 49, 96), the experimental data concerning the influence of the preservation procedure on its properties are scarce.

Dura mater is preserved by lyophilization (96), lyophilization and radiation sterilization with doses from 2.0 to 2.5 Mrads, or it is prepared in balanced salt solution and irradiated with 3.3 Mrads (48, 49,

92).

Rosomoff and Malinin (96) stated that the physical strength of freeze-dried dura mater does not differ significantly from the fresh tissue.

Triantafyllou and Karatzas (72) studied the mechanical properties and permeability of dura mater preserved by lyophilization and subsequent irradiation with a dose of 2.5 Mrads. Changes in mechanical properties and a decrease of permeability were observed in lyophilized irradiated specimens. The authors conclude that freeze-drying significantly contributes to the overall observed changes.

Fascia grafts

Fresh and preserved allogenic and some times preserved xenogenic fascia grafts are used in reconstructive surgery (91, 92).

Various methods of preparation including freezing, freeze-drying and radiation sterilization are used for preservation of fascia grafts.

Dexter (92) prepares the fascia grafts in balanced salt solution and then sterilizes by gamma radiation with a dose of 2.5 Mrads. In our tissue bank a similar method of preservation is used, but the sterilizing dose is 3.3 Mrads (48, 49).

Since preserved, nonviable fascia grafts serve as a supporting material, their mechanical properties are important from the practical point of view.

Gresham et al. (91) compared the tensile strength of fresh, frozen and freeze-dried fascia taken from cadavers. There were no significant differences of average tensile strength between fresh, frozen or freeze-dried fascia. Significant differences have been found, however, between individuals, which apparently are due to the age, sex, and cause of death of the donors.

Klen and Pacal (73) compared the permeability of fresh and freeze-dried fascia irradiated with a dose of 2.5 Mrads. The coefficient of mass permeability for nonirradiated samples was 1.21×14^{-4} cm/s, and for irradiated specimens 0.83×10^{-4} cm/s, respectively. The time needed to reach steady state was 5 minutes for non-irradiated grafts, and rose to 10-30 minutes for irradiated ones.

Tendon grafts

Allograft tendons have been used in reconstructive surgery when the use of autografts was impossible.

The experimental data concerning the evaluation of preserved tendon grafts are scarce. Seiffert [cited in (82)] preserved allogenic tendons in Cialit solution (a mercurial antiseptic). Using carbon-14-labelled proline he showed that collagen of grafted tissue was replaced by the host in six to nine months.

Recently, Feher et al. (97) studied the replacement of tendon allografts preserved by lyophilization and radiation sterilization with a dose of 2.5 Mrads. They observed that at six months after implantation the histological pattern of grafted tendon did not differ from that of the control. The authors suggest that lyophilized radiation sterilized tendon grafts seem to be of the same value for surgical use as fresh ones.

Since the research activity of tissue banks is to provide fundamental data which could be used in practical applications for the production of tissue grafts of standardized and well defined biological value, the discussed data as well as those which will be obtained in the future should serve for optimization and standardization of the preservation procedure.

Further efforts are necessary to elaborate an adequate way and to find the proper methods for objective evaluation of preserved tissue used for grafting.

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Evaluation of Radiation Sterilized Tissues in Clinical Use

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Abstract: *A short review of the clinical results of preserved tissue transplantation is presented. Most of the authors' experience is based on the activity of the Central Tissue Bank at Warsaw. Radiation sterilization of grafts is the routine method used in this bank. The clinical evaluation of biostatic graft application to bone, cartilage, fascia, dura mater, heart valves and skin is discussed. Important evidence, supported by clinical data, shows that transplantation of devitalized tissues has become the acknowledged way of treatment in some medical branches.*

Transplantation of devitalized tissues

Two types of tissue transplantation can be distinguished in modern transplantology. The first consists in grafting living tissues and organs which after transplantation should resume their physiological function in the recipient. In the second type grafts of devitalized tissue are introduced which play a supporting role in the organism and in time are substituted or sometimes they stimulate the tissue of the recipient to regeneration. Owing to the limitations resulting from this second type of transplantations, the latter were used only in some fields of medicine. At present, however, devitalized grafts are applied commonly and the percent of healings obtained is high (1–9). Preserved grafts are prepared and distributed by tissue banks. As an example a list of grafts prepared in one of these banks is given in Table I.

The general procedure of graft preparation in this bank is shown in Figure 1. It results from this scheme that the routine method for sterilization is treatment with a high dose of ionizing radiation. It has been established in basic investigations that this method achieves sterility without major unfavourable side effects on the prepared material (10–13). Sterilization of tissues with ionizing radiation has long been known and the pertinent literature is abundant (14–18). Other methods of tissue sterilization are also used (19–20).

Table I. — Preserved grafts prepared in the Warsaw Central Tissue Bank in 1963 — 1975.

Graft	Number	%
Human compact bone	3,999	18.8
Human spongy bone	9,319	43.8
Xenogenic bone	889	4.2

Human cartilage	1,813	8.5
Calf cartilage	1,757	8.3
Dura mater, tendons, fascia	2,387	11.2
Skin (allogenic and xenogenic)	1,100	5.2
Total	21,264	100

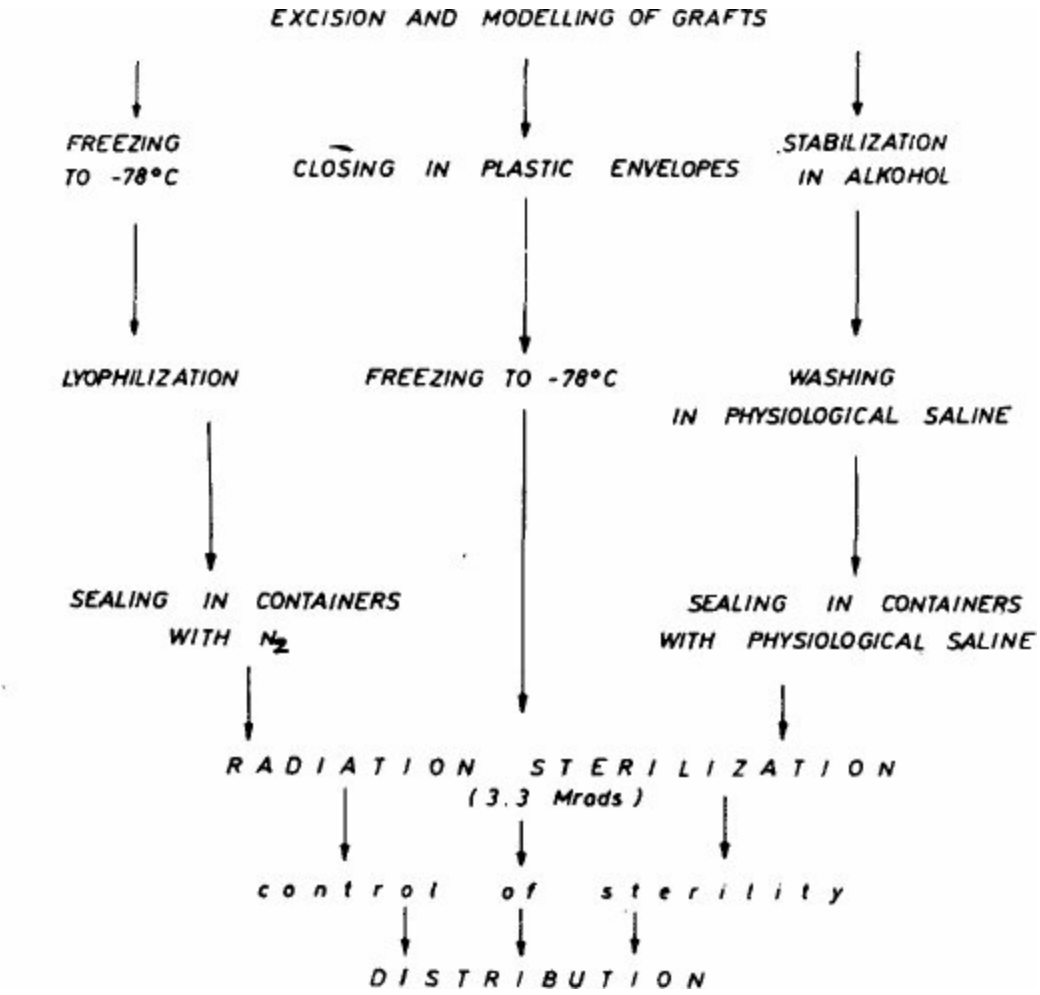

Figure *1*. Schematic presentation of the methods of tissues preservation, applied in the Central Tissue Bank at Warsaw.

Transplantation of preserved bone

Devitalized bone transplantation has long been applied in orthopedic practise and with each year new publications appear on this subject (22–26). The essential principles on which the efficacy of therapeutic transplantations is based are:

1. Bone tissue preserved in an allogenic system does not cause, after transplantation, reactions of tissue incompatibility owing to its low immunogenicity.
2. Bone is, as a rule, a devitalized transplant. After transplantation of a freshly excised bone fragment only a very small number of cells survive and enter the subsequent cell cycle even in an autogenic system. Most osteocytes die on account of the specific conditions of

vascularization of bone tissue.

3. Devitalized bone tissue undergoing gradual resorption induces a process of bone regeneration which is the essence of the phenomenon of "creeping substitution".

All preservation procedures cause a remodelling of the transplanted bone structure. Freezing and lyophilization reduce greatly the rate of reconstruction of the transplant according to Carr and Hyatt (26) and Burwell (1). Carnesale and Spanks (27) and Arden (28), who used merthiolate as preserving agent, describe a considerable delay in reconstruction of the transplant and numerous cases of failure. Bone preserved by means of high temperature (boiling or autoclaving) is, according to Burwell (29), not immunogenic, but its reconstruction is greatly retarded. This was confirmed by Chalmers et al, (30). Decalcified bone is rapidly reconstructed (31). It cannot, however, serve as supporting material in orthopedic operations. Neither is the influence of radiation on the properties of bone tissue negligible. Details on this problem may be found in the paper of Gocławska (32).

Lyophilization and sterilization of bone with ionizing radiation has long been practised in orthopedic hospitals and the clinical results are considered good. Beside clinical evaluation (1, 4, 7, 24–26), the material is also subjected to analysis in the tissue banks. Their evaluation is based on reports from numerous clinics and hospitals. Evaluation of transplants, however, on the basis of the final results of therapy, is difficult and not always objective. For instance, Table II shows early therapeutic results of treatment with the use of lyophilized and radiation sterilized transplants.

Table II. — Results of transplantations of preserved bone grafts 6-12 months after grafting.

Diagnosis	No. of Transplants Performed	Successful transplants	
		Number	%
Scoliosis	520	489	94
Benign tumors	316	277	87
Club foot, Valgus foot and Ollier's disease	282	245	87
Postraumatic deformations	379	271	72
Luxations	181	156	86
Tuberculosis	142	112	79
Perthes' disease	67	54	81
Arthrodesis	53	44	83
Spondylolisthesis	37	24	65
Abscesses and necrosed bone	23	21	91
Malignant tumors	12	2	17
Total	2,012	1,695	84

In many groups the per cent of good therapeutic results is very high and may be, in general, evidence of the efficacy of treatment by this method (including many variable factors). It is difficult, however, to evaluate a transplant exclusively on the basis of the clinical result of the entire treatment. In a selected group of cases clinical evaluation of the patients was done after a lapse of at least two years

after the transplantation. This evaluation is presented in Table III. In more than 40 per cent of cases the results were evaluated as very good by the clinicians, in a further 45 per cent of cases the result was satisfactory. If we, on the other hand, consult the opinions of physicians concerning the role of the transplant in the same material, and order them according to a 4-grade score (Table IV), it appears that in more than 80 per cent of cases the expected role of the transplant was fulfilled. This lack of parallelism with the data in Table III results from the fact that the outcome of therapy is affected by many factors such as the general condition of the patient, the extent of the changes requiring transplantation, accompanying diseases and others. Therefore, the result of treatment and the therapeutic efficacy of the transplant should be separately evaluated, even when the same tests are used for the evaluation; for instance, the reconstruction of the transplant as seen by X-ray, shortening of the time of treatment or the degree of disablement.

Table III. — Evaluation of therapeutic effect of bone transplantation.

	Total	Very good	Satisfactory	Difficult to estimate	Unsatisfactory	No information
Number of transplantations	338	138	155	19	23	3
%	100	40.8	45.9	5.6	6.8	0.9

The efficacy of treatment with the use of preserved radiation sterilized bone transplants is also reported by other authors (2, 3, 12, 24, 25). In view of such abundant positive clinical evidence concerning transplantation of radiation sterilized grafts and of the known convenience of this method of sterilization, the latter should be considered recommended practice in tissue banks.

Table IV. — Evaluation of the role of transplants in orthopedic operations.

	Total	Effective	Satisfactory	Difficult to estimate	No good effect
Number of transplantation	338	273	32	16	17
%	100	80.8	9.5	4.7	5.0

Preserved cartilage

Straight and Slaughter (33) published in 1941 the clinical results of the application of allogenically preserved cartilage in plastic operations of the face. The patients were followed for one to four years and no strong tissue reactions to these grafts were noted, while their resorption was very slow. In later years there appeared further positive reports on cartilage preservation and transplantation (34–37). As the result of many years of clinical experience, the following facts were established as imperative in cartilage transplantation:

1. Fragments of living autogenic or allogenic cartilage may be accepted by the recipient of the transplant and play a mechanical role for a long time.
2. Fragments of allogenic preserved cartilage undergo such slow resorption that they can fulfill

mechanical functions for years in the recipient.

3. Transplanted preserved allogenic cartilage has a very low biological influence on the recipient's tissue.

In 1956 Lynch and coworkers (37) published the results of their studies on the possibility of sterilization of cartilage for transplants with ionizing radiation. He found that irradiated tissue is not only sterile, but it also is less susceptible to resorption after transplantation. At present on the basis of numerous communications (38–40) it appears that radiation sterilization of cartilage immersed in physiological saline is a very good method of preservation for plastic operations. Rib cartilage is prepared before sterilization according *to* the method of Moskalewski (38), based upon the procedure developed earlier by Davis and Gibson in 1956. The cartilage is placed in a glass container in physiological saline. Then it is exposed to radiation and, after sterility control, the preparation may be used for transplants within a year. After this period the consistency of the transplant changes considerably so that it is no longer suitable for application (39). Fragments of preserved cartilage can be shaped according to need before the operation; smaller fragments can even be glued together into larger blocks with the use of cyanoacrylate glue (40).

Such preparations are most frequently used in operations of the face and skull. Więcko published in 1974 (39) the results of her investigations on the application of allogenic preserved cartilage in plastic surgery. The different uses are listed in Table V.

Table V. — Application of preserved human cartilage in operations. (After J. Więcko, 1974) (39)

Site	Number of cases
Nose	97
Ear concha	42
Chin	5
Forehead, mandible, maxilla and orbit	27
Total	171

More than one half were cases of trauma or deformations caused by other operations, congenital and post-inflammatory deformations. The age of the patients varied from 5 to 68 years. The follow-up time after the operation ranged from 1 to 10 years. Evaluation of the transplants according to clinical criteria was good, as seen from Table VI.

The quoted results and investigations provide evidence of the full usefulness of preserved cartilage for reconstructive operations, although the demand for such transplants is lower than that for preserved bone (Table I).

Noteworthy also is the fact that, besides compact transplants, cartilage also finds clinical application in the form of powder (41). Xenogenic cartilage used as dusting powder on surfaces deprived of epidermis or ulcerations stimulates the development of granulation and epithelium. This may probably be due to the biological action of n-acetyl-glucosamine polymers on connective-tissue and epithelial cells (42).

Table VI. — Evaluation of clinical result of cartilage transplantation in 128 cases. (After J. Więcko, 1974) (39)

Observation period	Number of cases		
	Effective	Satisfactory	Unsatisfactory
1 — 3 years	51	15	5
4 — 6 years	9	15	0
6 — 10 years	11	18	4

Fascia and dura mater

In surgery sometimes xenogenic or allogenic connective tissue membranes such as fascia, tendons or dura mater are necessary (43, 44). After grafting, the role of these transplants is to supplement a part of the body integuments, while undergoing very slow remodeling. Usually such transplants do not produce defence reactions in the recipient tissues. The biological neutrality of this material is due to the low immunogenicity of collagen, which is the main component of these tissues, and to the preservation procedure in which a large part of the tissue components is denaturated (45).

A frequently applied method of preservation is the placing of the material in physiological saline and sterilization with gamma radiation (43, 46, 47). A preparation thus obtained may be used for transplants within one year.

In this Centre within the last few years more than 2000 such connective tissue transplants were prepared (Table I). They were used in laryngological, ophthalmic, neurosurgical and oncological operations. Up until now no complaint has been received of failure as a result of these transplants. It is known that even infection of the operative field is not a contraindication of the application of these grafts (48, 49).

Heart valves

In the world literature numerous reports may be found on the clinical application of heart valves, both allogenic and xenogenic, preserved by various methods. The most common methods are: 1. sterilization with ethylene oxide, lyophilization and storage at room temperature, 2. sterilization and storage in buffered formalin or glutaraldehyde, 3. sterilization and preservation in various combinations of antibiotic solutions, 4. sterilization for one-half hour in 0.2 per cent acetic acid and storage in phosphate buffer at pH 7.2 – 7.4 and 5. sterilization with gamma radiation or fast electrons and storage at low temperature. Various combinations of these methods have also been described (51–52).

We have no experience in this tissue bank in preservations of heart valves, but this problem is dealt with in numerous scientific publications and should be discussed briefly here.

Beach et al. (53) used in clinical practice irradiated human heart valves in 50 cases. The material was

taken from cadavers within 12 hours after death. The valves were calibrated and, after being placed in polyethylene containers, were sterilized with fast electrons by the methods described by Meeker and Gross (54). Transplants prepared in this way were stored at — 70°C until use. The clinical results were not too good. Six patients died in the course of the operation, this having no connection with the transplanted material. A further 15 patients died after an average time of 2 years following the operation. The reason for their death was damage to the implanted valves owing to a state of chronic inflammation, mechanical injury or other unexplained causes. In earlier years the clinical results after application of radiation sterilized valves were described more optimistically (55, 56).

Ionescu et al. (57) claim that exposure of the valves to radiation energy destroys the tissue structure since a decrease in their tensile strength was noted as compared with that of fresh valves. The histological and histochemical investigations of Welch (58) on valves preserved and sterilized by various methods revealed that valves preserved by rapid freezing and storage in buffered formalin and those irradiated with fast electrons or gamma radiation did not show changes. On the other hand, valves preserved in beta-propiolactone exhibited wide changes consisting in obliteration of the laminar pattern of collagen fibres and a decrease in Periodic acid Schiff-positive material content in the graft.

Although sterilization of heart valves with ionizing radiation is effective, in view of the relatively large number of clinical failures in transplantation of allogenic valves, it is difficult at present to foresee that this method of preservation in the banks will be further developed or to foresee the type of preservation which will be adopted finally.

Allogenic and xenogenic skin

Skin transplantation may be considered from two different viewpoints. First, in the case of an autogenic or an isogenic living graft the skin is transferred directly from one subject to other or from one site on the patient to another site. In such cases preservation of skin is not necessary. In the case of skin transplantation in an allogenic system, the donor and recipient should be matched by taking into account the histocompatibility antigens (HL-A) so as to give a chance of survival to the graft. Allogenic skin can be stored for a short time in conditions similar to those of tissue culture *in vitro.* In such cases the possibility of sterilization is very limited.

On the other hand, allogenic and xenogenic skin is used also as a biological dressing and can be exposed to ionizing radiation. In surgical practice such skin is very useful (6, 59, 60).

The purpose of a biological dressing is the protection of a wound from drying, protein and salt loss, and the creation of an appropriate medium for granulation. The use of preserved allogenic or xenogenic skin is as a rule a preparation for an isogenic graft.

In surgical practice various skin preparations are used, both allogenic and xenogenic (60), fresh skin which has not been subjected to any preservation treatment and lyophilized skin which has been sterilized by various methods (59). Skin graft sterilization with ionizing radiation is a method used frequently (19, 21). The Central Tissue Bank in Warsaw prepared 1200 lyophilized and radiation sterilized skin grafts all of which were used as biological dressings. They were applied in trauma (scalping), extensive burns, trophic ulceration and extremity amputation wounds refractory to healing. In 80 per cent of cases the use of such dressings accelerated the patient's preparation for a transplantation of his own skin and greatly abbreviated the period of hospitalization.

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Ionizing Radiation in the Preparation of Vaccines and Antigens

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Abstract: *Gamma radiation from a Cobalt-60 (Co-60) medical products irradiator has been used for more than ten years to sterilise the containers and administration sets of multidose animal vaccines.*

More recently radiation has been suggested as an answer to the problem of contamination by mycoplasma of working cell seed. This leads to a reduction of virus yield and can result in a contaminated end product. The source of mycoplasma is frequently animal byproducts introduced during manufacture, particularly bovine serum albumin and trypsin. Irradiation of these two products, as received from the manufacturer, with 2.5 Mrads in a medical products irradiator results in no apparent loss of activity, no gross effect on the cell lines used, and no reduction in virus yield.

Further uses postulated are in the production of a leptospiral vaccine which will produce a low systemic reaction while still protecting against the renal carrier state. The possibility of end sterilisation of viral vaccines contaminated with low levels of radiation sensitive bacteria is discussed.

Brief mention is made to the considerable volume of work which has been done in the field of irradiated larval vaccines.

Introduction

This paper reports the influence of an on-site Co-60 medical product irradiator on the commercial manufacture of vaccines. As such, it represents the use made of the irradiator in the solution of specific practical problems; it does not therefore describe a planned approach to research into the subject, either generally or specifically.

It is interesting to record that the only truly indigenous mammals in New Zealand are two species of bat (pekapeka). During the great Maori migrations in the first four hundred years of this millennium the Polynesian rat *(Mus exulans)* arrived in the voyaging canoes, probably as stowaways. A dog was also introduced at this time, although the much prized pig of the Polynesian people did not make an appearance until left by Captain James Cook in 1769. Sheep and cattle were introduced during the early days of European settlement about 130 years ago, and so well has the climate favoured their development that at the last animal census in June 1976, there were 55.8 million sheep and 9.7 million cattle. The meat, wool and dairy products of these animals produce 85% of the total export earnings for the country's human population of 3.2 million people.

It is thus not difficult to understand why a company such as ICI Tasman Vaccine Ltd., should direct its efforts towards the control of animal disease. Biological products such as vaccines and toxoids became a central feature in this programme. The ready availability of a wide variety of plastic resins and

an increasingly proficient plastic fabricating industry in the early 1960's allowed a significant change to be made in the presentation of vaccines and toxoids. The combination of a soft collapsible pack of 500 or 1000ml, and a cheap yet accurate vaccine gun allow the rapid inoculation of large numbers of animals by a single operator (Figures 1 and 2). It was this proposed system of storing and administering vaccines, in fact, which led to the decision to construct a Co-60 irradiator at the Upper Hutt site, a decision reinforced by a quite independent decision by the New Zealand Department of Health to change the bulk of their in-hospital sterilisation to commercially produced irradiated disposable packs.

Sheep and cattle losses can be high in a temperate climate with relatively intensive farming. As an example, lamb losses from tetanus after docking can be as high as 35% in some areas in un-vaccinated flocks, and vaccination can largely prevent this. If this can be rapidly performed, an adequate regular vaccination programme becomes a relatively simple procedure in flocks of say 2 - 10,000. The relatively simple procedure of irradiating the administration sets with 2.5 Mrads gamma radiation prior to sterile filling achieved this purpose and represents the first and probably most significant use of radiation in the preparation of these biological products.

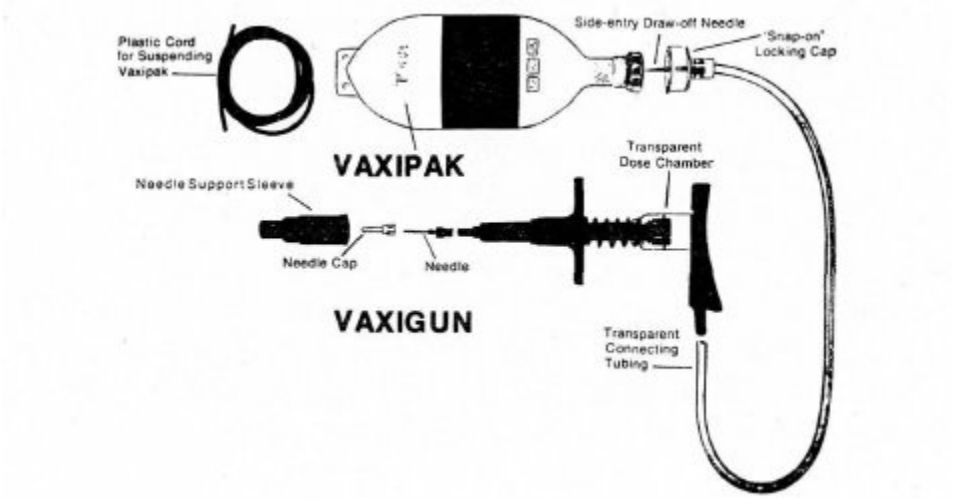

Figure *1.* Vaccine gun for rapid inoculation of a large number of animals by a single operator.

Figure *2.* Details of vaccine gun assembly.

A concomitant use has been the sterilisation of plastic syringes prefilled with diluent for reconstitution of freeze dried viral vaccines. These are irradiated with 2.5 Mrads. This has been found to be an easier and safer method than the filling, by terminal filtration, of empty irradiated syringes.

It would, however, be unreasonable to leave this important, yet somewhat prosaic, use of radiation without commenting on the occasional untoward effects obtained with radiation sterilised plastic containers. Medical diagnostic laboratories report an unexpected bactericidal effect from time to time when known contaminated cultures are placed in radiation sterilised containers made from a wide variety of polymers. These include polyvinyl chloride, high density polyethylene, polypropylene and polystyrene. In those cases where the container can be sterilised by autoclaving, this bactericidal effect is usually, but not always, eliminated. It is not the intention to discuss the reasons for this. It is simply important to recognize that each polymer and each batch of containers should be separately tested to eliminate this undesirable effect.

Vaccine Production

The desirable end result of vaccine and antigen production is an agent retaining as high a level of antigenicity as the naturally-occurring organism or its toxin, but causing little or no virulence. Virulence may be eliminated by a number of methods including heat and formalin, or it may be reduced by attenuating the virulence of the organism by frequent subculture in an unnatural environment. It is not unusual to find that while a vaccine may offer protection to the recipient in the

form of a significant rise in antibodies, infectivity may still be obvious with the persistence of a subclinical carrier state. An excellent example of this phenomenon is the renal carrier state which may develop in an animal previously immunised with a formalin treated leptospiral vaccine.

Ionizing radiation has been studied to determine its effect on bacterial and viral vaccines, particularly with a view to achieving the twin goals of high residual antigenicity and low infectivity. Pollard (1) gave an extensive review of the physical chemistry of the subject and the efficacy of gamma irradiated viral vaccines has been reported by Gruber (2), Polley (3) and Kulevich (4) and bacterial vaccines by Hubbert and Miller (5) and Tumanian (6). These reports dating from the early 1950's through to the present day, indicate that under experimental conditions suitable vaccines can be prepared using ionizing radiation to achieve the goal of high antigenicity and low infectivity. It is, therefore, of interest that vaccine manufacturers have not widely used this tool in a practical manner, although there is no doubt that it offers advantages in some aspects of the process.

It is difficult to be certain how much of the work to be described would have been carried out if there had been no on-site irradiator. The anecdote of the man who was given a pair of water skis and who wasted a whole summer looking for a lake with a slope, comes to mind. So frequently one has a problem — the water skis — the solution to which — a sloping lake — looks unattainable. However, we had the lake and I suspect we began to look for some water skis.

A simple flow diagram for virus vaccine production and the relevant quality control procedures before and during production is shown in Figure 3.

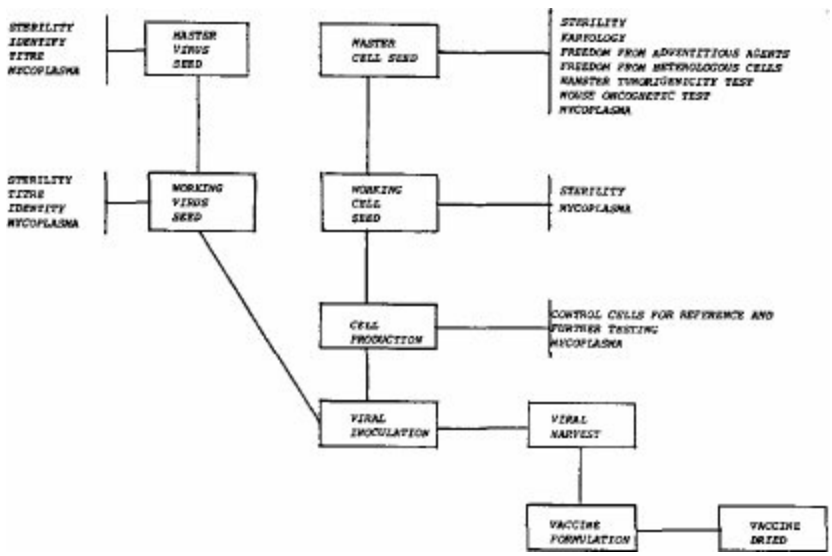

Figure *3.* Basic virus vaccine. Production flow chart and in-process quality control procedures.

It is not the purpose of this paper to describe the process in detail but the general principles of cell production from the master cell seed, virus inoculation, harvest and formulation are shown. The in-process quality control procedures are based largely on the United States Department of Agriculture's requirements.

It is during certain of the steps in the process that ionizing radiation has proved to be of value. The background to the problem is given and the work performed in attempting to overcome it is described.

Infection by mycoplasma of agents used in vaccine production

A significant recent problem has resulted from a better understanding of organisms of the group

mycoplasma, now known to be widely present in many normal mammalian populations (7–9), and to be the cause of human and animal disorders. The organism has been found in our laboratory and reported elsewhere (9) in many biological products of animal origin.

Some characteristics of this group of organism are briefly mentioned. *Mycoplasma mycoides* was shown in 1898 to be the causative agent of contagious bovine pleuro-pneumonia (10), although its true nature was not then understood and it was simply known as the pleuro-pneumonia organism (PPO). Similar organisms, referred to as pleuro-pneumonia-like organisms (PPLO) were subsequently found to be the cause of respiratory disease in many species, including sheep *(M. ovipneumoniae),* goats *(M. mycoides)* and pigs *(M. suipneumoniae)*.

In 1961 *M. pneumoniae* was proved to be the causative agent of primary atypical pneumonia in humans. Of interest to vaccine manufacturers was the finding in 1956 of mycoplasma contamination of cells in culture and Stanbridge (8) describes the group as being present in a significant number of cell lines cultured throughout the world.

McCormack et al (11) in an extensive review of mycoplasmal genital disorders in humans, suggested this organism as a possible causative agent in non-specific urethritis, Reiter's disease, rheumatoid arthritis, vaginitis, septic abortion, post-puerperal infections, infertility, spontaneous abortion, stillbirth, prematurity and low birth weight and even chromosomal abnormalities such as Down's syndrome.

General characteristics of the group relevant to vaccine production, are listed in Table I. Stanbridge (9) states that contamination of cell cultures in individual laboratories ranges from 12-98%, and predicts that the majority of continuous cell lines in use throughout the world have been, or are, contaminated with mycoplasma. Contamination of primary cell cultures on the other hand is reported to be rare. Addition of bovine serum albumin as a growth supplement for animal cells is a likely major source of contamination, along with faulty handling techniques, airborne transmission despite laminar flow techniques and infection from technicians in whom the organism may be part of the normal flora of the oropharynx.

Table I. — Some general characteristics of mycoplasma:

1. Lack a true cell wall and are bounded by a simple trilaminar membrane.
2. Size range 300-1000nm (8), although some authorities suggest smallest forms 100nm or less (7).
3. When grown on agar, form characteristic 'fried egg' colonies 0.05-6.0 mm in diameter.
4. Growth inhibited by antibiotics.
5. All mycoplasma except the genus *Acholeplasma* require sterol for growth.
6. Is the smallest known organism at present capable of free living existence.

Mycoplasma contamination of cells in culture leads to interference with growth rate of the cells up to complete disintegration of the cell; increases in growth have also been recorded (12). Chromosomal aberrations are induced in cultured cells by the organism, probably due both to the depletion of arginine and interference with host cell DNA synthesis (9). In the same article, Stanbridge indicates that virus yields in mycoplasma-contaminated cultures can either be decreased or increased. The former can often, but not always, be reversed by the addition of arginine. Increase in virus yield may be due to mycoplasma-induced inhibition of interferon production or activity.

Quite obviously the detection of mycoplasma in cell cultures, in the additives and in the end product is critical, and if found to be present, their elimination is essential. They are fastidious in their growth requirements and a simple, single medium cannot be relied upon to grow all mycoplasma. As a consequence, a whole host of cultural and non-cultural (13) methods has been devised. These latter methods include electron microscopy, autoradiography, enzyme assays, fluorescent assays, physical separation by centrifugation and separation of mycoplasmal nucleic acids.

Mycoplasma, although insensitive to penicillin, have been shown to be sensitive to tetracyclines, lincomycin, kanamycin, streptomycin, gentamycin, novobiocin and tylosin. Increasing resistance to these antibiotics has been encountered with increasing use; as well, it appears that living cells with which mycoplasma are in contact often give protection to the organism, so that the organism in contact with cells may be resistant to as much as twenty-fold higher concentrations of antibiotic than in a cell-free culture.

Other possibilities for decontaminating cell cultures are chemical agents, specific antisera and heat treatment. These methods all have their particular drawbacks. Having obtained a mycoplasma-free primary culture, careful attention to technique must be followed in order to allow production of organism-free subcultures.

a) ***Bovine Serum Albumin***

A specific problem, only recently recognized, is the difficulty in culturing mycoplasma. This may lead for instance, to quality control release of vaccine batches subsequently proved by non-cultural methods to be contaminated by mycoplasma. Present-day commercially obtained cell lines are usually sterile. Mycoplasma has been however, detected in prepared solutions of bovine serum albumin (BSA) used in cell culture medium (14).

BSA is purchased as bovine albumin fraction V and is described as “an amorphous protein supplied as a homogeneous off-white powder obtained from the fractionation of fresh plasma by the classical Cohn low temperature-alcohol method”. It has an ash content and moisture content of less than 2% and 3% respectively and is said to be easily sterilised by filtration.

BSA is used at two points in the production process; firstly in the production of the cell culture, and secondly as an additive after virus inoculation. It is also used in some bacterial vaccine culture media, particularly that used in the culture of leptospira. It has been assumed until recently, that filtration of a 0.2% solution in water rendered the solution at least bacteriologically sterile and that an inability to culture mycoplasma in the end product indicated the truth of this assertion. Some of the now well known characteristics of mycoplasma have shown this assumption incorrect. Even if one accepts that the minimum size of the organism is 300nm, its pliability (because of the lack of cell wall) is likely to allow it to pass through the 0.22μm filter used in preparing the BSA solution. Hence sterilisation of the BSA powder by radiation seemed to be worth attempting, particularly as regulations for animal products, world-wide, will shortly include testing to require serum and other ingredients of animal origin to be tested to exclude bacteria, fungi, mycoplasma and viruses.

Bovine albumin fraction V as received from the manufacturer in polythene containers of 500g was packed into cardboard outers in an exactly similar way to medical products. The product received a dose of 2.5 Mrads over a period of 21 hours from an Atomic Energy of Canada Ltd. commercial Co-60 irradiator. A 4% solution was then prepared in the usual manner and final filtration was by 0.22μm

Millipore® membrane filter. The 4% solution was further diluted in the cell culture medium down to 0.2%.

The test cell used in the experiment was Vero. No gross effects on the cells were noticed after culturing for three days. When a 2.0% concentration of BSA was used some cell rounding was noticed after three days incubation, but this is a severe test, and indicates that there was no gross toxic effect.

The 0.2% solution was used in a titration of canine distemper virus, (CDV) standard virus, by exposure of the virus dilution overnight at 4°C. A normal control medium preparation (prepared in an exactly similar way, except that the BSA had not been irradiated) yielded $10^{3.8}$ Tissue Culture Infective Doses $(TCID)_{50}$/0.1ml., and the test fluid $10^{4.2}$ $TCID_{50}$/0.1ml.

Culture for mycoplasma was not performed on the test material either before or after irradiation. The work was simply performed to indicate whether any gross changes were observed in cells grown with irradiated BSA in suitable dilution and whether a normal virus harvest could be produced. The radiation sensitivity of mycoplasma has not been investigated nor were estimates made of the degree of contamination of the BSA as received from the manufacturer. Schimmel, Ahlendorf and Burger (16) in an English translation summary of their paper, state that inactivation of mycoplasma is possible with 300,000-400,000 rads of X-radiation but do not state in the summary the level of pre-irradiation contamination.

The results are summarised in Table II. The results, therefore, give a lead towards a further potential method for the sterilisation of BSA.

Table II. — Effects of using irradiated bovine serum albumin in the manufacture of viral vaccines.

	On cell condition[a)b)]		On virus yield[e)]
	0.2%	2.0%	$T.C.I.D._{50}$
Unirradiated	Nil	Some rounding	$10^{3.8}$/0.1ml
Irradiated[d)]	Nil	Some rounding	$10^{4.2}$/0.1ml

a) Vero
b) After 3 days incubation
c) Canine distemper virus
d) 2.5 Mrad gamma radiation

b) ***Trypsin***

Trypsin is a proteolytic enzyme and is an exocrine secretion of the pancreas. It is used in the production of viral vaccines during the process of cell production. During growth, cells become firmly attached to the surface of the culture vessel and are detached by the use of several agents, including trypsin. This process is repeated, several times, as the cells are transferred to larger bottles. As the enzyme is manufactured from pancreas, and collected during the processing of animals in freezing works, the chances are high that the substance is contaminated. Because of the wide distribution of mycoplasma in the animal kingdom the organism is a likely contaminant.

Wills (17) in a review article states that there is an exponential relationship between radiation dose

and the degree of enzymatic inactivation of trypsin. She makes the general observation that enzymes in the dry and natural state are fairly resistant to radiation. She quotes Huber (18) as saying that trypsin is resistant to a sterilisation dose of ionizing radiation.

An attempt was made to determine whether trypsin irradiated with 2.5 Mrads retained its activity insofar as the requirements for vaccine production were concerned. Trypsin as received from the suppliers in polythene containers of 450g was packed into cardboard outers and irradiated in the manner described under the previous section. The product received the normal dose of 2.5 Mrads gamma radiation over a period of 21 hours. For the purpose of this test its ability to digest unfixed photographic film emulsion was investigated. Ordinary photographic film is used and a solution of trypsin is dropped on to the film; the area and depth of "digested" gelatin can be readily measured and compared.

After sterilisation a trypsin solution of 0.25% in Hanks balanced salt solution was prepared. The control solution was prepared in a similar method from unirradiated trypsin. The pH of both solutions was adjusted to 7.6 and the film was incubated at 37°C for 15 minutes and left overnight at 4°C. The activity of both solutions appeared identical.

Subsequent use of an 0.25% solution in the digestion of tissue fragments of dog kidney was effective with no gross changes obvious in the cell condition nor in subsequent virus yield.

Suggested future uses of irradiation

It must be stressed that this survey has recorded the use made of ionizing radiation in our laboratory. So too, will it now record potential avenues for investigation as seems most relevant to us, manufacturing as we do animal vaccines for use largely in temperate climates.

It would not be reasonable to suggest that radiation is a panacea to the many problems that beset a vaccine manufacturer. The careful techniques currently in use must continue to be carried out, with radiation appearing to offer, at most, fringe benefits at varying points in the process. Nevertheless, the well documented fact that viral and bacterial vaccines can be terminally irradiated, with loss of infectivity but without loss of antigenicity, has already been mentioned. This finding suggests two possible uses with specific problems.

a) *Leptospiral Vaccines*

Leptospirosis is recognised in New Zealand as a disease of some significance in both humans and animals. A peak incidence of 30 cases/100,000 in 1971 (19) was considerably higher than in USA, UK or Australia (20) Its high incidence among dairy farmers, in whom infection is contracted in the milking shed (21), is probably due in large part to the renal carrier state in cattle (5). The renal carrier state can exist in vaccinated asymptomatic animals and to date formalin inactivated vaccines do not protect against a leptospiruria (5). While control of the disease lies in control of the animal carrier state, a human vaccine may well offer protection for what can be a prolonged and unpleasant illness.

In manufacturing leptospiral vaccines, bovine serum albumin fraction V is an essential ingredient in culture mediums for this fastidious organism. Contamination with mycoplasma, as mentioned earlier, can lead to a slowing down of the growth of the leptospira with the possibility as well of producing a mixed antigen.

Hubbert and Miller (5) indicated in 1965 that *L. copenhageni,* gamma irradiated with 70,000 rads or greater (from a Co-60 source) failed to multiply *in vitro,* although they still retained their ability to induce agglutination lysis antibody. It has been shown (21), however, in more recent trials that irradiated vaccines were no more effective than formalised vaccines in preventing either death or persistent renal carrier infection in guinea pigs. It may well be that the finding by Faine (22) of a lipopolysaccharide antigen and by Auran (23) of the protective value of the outer leptospiral sheath, presents a more fruitful line of approach, although the methods of extraction do not appear to be easily adapted to a commercial process.

b) ***End sterilisation of contaminated vaccine***

The known general difference in radiation sensitivity between viruses on the one hand, and many bacteria on the other, suggest the possibility of selectively eliminating bacterial contamination from a live viral vaccine. Despite careful technique, the use of appropriate antibiotics and laminar flow techniques during filling, the production of vaccines on a commercial scale occasionally results in a contaminated end product. Frequently under these circumstances the level of contamination is low, with organisms of known high radiation sensitivity.

While it is attractive to postulate the use of ionizing radiation to "clean" a contaminated attenuated lyophilised viral vaccine, the potential for change in the residual viral population would currently rule out the possibility. Nevertheless, the technique should not be dismissed out of hand simply on the grounds that radiation is involved in the process. Many agents and practices are tolerated in this field which would not perhaps meet the criteria which are now applied to radiation.

Attenuation alone can lead to unexpected consequences. Vaccination of pregnant ewes with attenuated blue tongue vaccine can result in cerebral lesions in Iambs subsequently born to them (25). Both live and dead SV 40 virus as a contaminant of a Salk vaccine, has been incriminated as the cause of an upsurge in the numbers of cases of subacute sclerosing panencephalitis in New Zealand following the administration of Salk vaccine (26). It has been suggested that an imbalance in the immune response to the virus could be responsible. Formalin and beta-propiolactone are both used to kill virus during vaccine manufacture, yet both are known to have mutagenic properties.

These few examples are mentioned simply to present the point of view that currently accepted practices are not necessarily devoid of undesirable side effects.

Radiation, both with attenuated vaccines for "end decontamination" and possibly with killed vaccines in place of an agent such as formalin, should at least be considered as an alternative. With relatively small doses, e.g. 1.0 Mrads, loss of virus activity as measured by the $T.C.I.D._{50}$ is not reduced below a level acceptable to registering authorities.

c) ***Antitetanus Serum***

Antitetanus serum, also contaminated with cocci, has been irradiated in an endeavour to sterilise the antiserum. A marked loss of activity, of 40%, after 1.0 Mrad gamma radiation has led to abandonment of the work. This confirms the findings for antitetanus serum by Wills (17). She also records loss of activity of up to 40% for anti-gas gangrene serum at 1.5 Mrads and a loss of 10% after the same dose with diphtheria antitoxin.

Discussion

Twenty-five years ago there appeared to be great promise in the use of ionizing radiation in the production of vaccines. This potential has been exploited experimentally, and numerous products have been prepared in the laboratory which fulfil the criteria of a safe and adequate vaccine. This potential, however, does not appear to have carried over into commercial production.

Loss of activity of bacterial antitoxins seems to be a general rule and toxoids, too, lose their antigenicity when compared with products prepared by the standard procedure of using formalin (17). Although Wills (17) reported no effect on antigenicity of gamma irradiated typhoid vaccine at 1.5 – 2.0 Mrads, it has recently been reported that thermally inactivated typhoid vaccine has a higher protective activity than gamma irradiated vaccines (27). Both the frankly adverse as well as the conflicting experimental evidence has obviously not persuaded commercial vaccine producers to use this tool widely with bacterial preparations.

Viral vaccine production appears, from experience mentioned earlier, to be a more likely avenue for the use of gamma radiation. Here, however, the law of demand and supply appears to operate. Whether a viral vaccine is presented as a live attenuated or killed vaccine depends on the demand of the end user. Despite the known examples of danger of attenuated vaccines both in human and veterinary practice, attenuated vaccines are frequently preferred because of the longer immunity they confer.

As a consequence of this, and because careful technique during production would continue to be obligatory, the use of ionizing radiation for terminal sterilisation, or for producing a vaccine with a low generalised reaction, does not appear to offer much over current methods for a commercial manufacturer. Nevertheless, benefits such as those described, represent a considerable advance over methods otherwise available.

Any review of this subject would be incomplete without making mention of the use of ionizing radiation-attenuated larval vaccines. Work on this interesting immunological problem has taken second place within our laboratory due in part to the concentration on the current practice of parasite control on New Zealand and Australian farms. Farm animals are born directly on to pasture and it is common for them to become infested with a parasite within a short time of birth. Subsequent drenching with an anthelmintic twice yearly usually keeps the parasite load down to minimal levels, and the animal after 18-24 months, develops resistance which protects if from subsequent massive infestation. This measure, along with current stock rotation and resting of pasture, has led to a reasonable level of control.

As greater and more intensive use of land is made, the adequate resting of infected pasture becomes more difficult. As well an increasingly successful early use of anthelmintics seems to be creating more and more unprotected adult animals; this will lead to a need to continue anthelmintic treatment regularly. This continuing and therefore expensive treatment, particularly as land becomes more intensively used, will certainly lead to a demand for the use of animal larval vaccines.

Reviews of the extensive work into this subject, and the problems involved, have been written by Miller (28), Varga (29) and Mulligan (30). The sensitivity to radiation of the various species of parasite varies considerably. Males are generally more sensitive than females and the worm population of an effective vaccine will generally contain few motile males, with a larger number of motile, sterile females. The pathogenic effect of the parasite is usually reduced while an immunogenic effect is produced if an adequate number of parasites are present for an adequate period. Vaccines have been produced against cattle lung worm, and sheep lung worm, and have been available commercially for a

number of years. The dose of radiation used was between 20-40 Krads (X-ray) and protection has been created by one dose of vaccines. More recently (1973) a vaccine against canine hookworm has been registered in the USA.

The lung worm vaccines described have many drawbacks, namely a short shelf life, together with a provision that vaccination should be performed before exposure to natural infestation can occur. The canine hookworm vaccine is described as having at least a six month shelf life.

The measurement of attenuation, the radiation source, the dose rate and the effect of variables such as temperature, larval concentration and oxygen, are all quoted by Mulligan (30) as factors which can influence the effectiveness of a vaccine. The narrow range of absorbed radiation necessary for proper attenuation would be difficult to attain accurately with a medical products irradiator.

Conclusion

This paper presents a survey on the application of ionizing radiation to vaccine production by a laboratory with access to a Co-60 medical products irradiator delivering a fixed dose of 2.5 Mrads to product of low density. Delivery of the small doses frequently required, for example for the attenuation of parasites, is time consuming and difficult to administer accurately. Hence those projects where 2.5 Mrads is a feasible dose have attracted most interest. These uses are summarised:

1. Sterilisation of collapsible plastic containers and administration sets prior to filling.
2. Sterilisation of plastic syringes prefilled with diluent for reconstitution of lyophilised viral vaccines.
3. Sterilisation of products of animal origin used in vaccine production, particularly with a view to the elimination of mycoplasma. Two products studied have been bovine serum albumin and trypsin.

Subjects of possible future interest are:

4. Attempts to produce a leptospiral vaccine which will protect both against disease and against the renal carrier state in cattle.
5. The technique of end sterilisation of contaminated live attenuated viral vaccines by doses tailored to the individual case should not be discarded as a potential tool simply because the agent used is radiation.
6. Because of a likely demand from veterinarians and farmers and despite the difficulty of administering the relatively low dose required with accuracy, work is likely to proceed with the preparation of larval vaccines.

Acknowledgements

The bulk of the work reported in this paper was done in the Research & Development Department of ICI Tasman Vaccine Ltd. under the direction of Dr. L. B. Fastier. I thank him for his permission to publish his work and for his advice given during preparation of the paper. I also thank Mr. D. Liardet and Mr. S. Chifney of the same Department for their help, Mr. J. Hill, Biological Production Manager, Mr. L. Reynolds of the Quality Control Division, Mr. H. Robson of the Technical Services Division and Mr. S. Curry from the Viral Production Unit, for their helpful advice and instruction on

various aspects of the paper.

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Irradiation of Drugs with Co-60 and Electrons

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Abstract: *The application of Cobalt-60 (Co-60) or electron radiation sterilization of drugs is discussed considering the developments during recent years in the technology of high dose rate electron accelerators.*

The effects of both gamma and electron radiation on the chemical and microbiological properties of chloramphenicol, tetracycline, ampicillin, atropine and pilocarpine are compared after treatment with 2.5 and 5.0 Mrad.

Observations are presented indicating that electron radiation does not change the substances as much as gamma radiation, but the differences are not of any major significance for the substances in dry form.

It was also noted that after irradiation with 2.5 Mrad from either a Co-60 source or an accelerator, ampicillin, tetracycline and pilocarpine still met the official pharmacopoeia quality requirements, whereas atropine and chloramphenicol did not.

Ever since ionizing radiation was first used for industrial sterilization some twenty years ago, the method has attracted considerable attention in the pharmaceutical sector for the sterilization of drugs. Numerous investigations of the effect of ionizing radiation on different kinds of drugs are available (for reviews, see references 1–6, 13).

Above all, the method is of interest in treating pharmaceutical products requiring sterility and which are difficult to sterilize by conventional methods, for example by heat, because of their thermolability. Although some of the pharmaceutical substances in question have proved to have good resistance to radiation, the occurrence of any changes is duly noted, such as changes in colour of the substances, their partial decomposition, and other possible changes. Consequently, there are at present only a relatively limited number of radiation sterilized pharmaceutical products on the market.

As a result of the increased knowledge that has been gained of the effect and influence which ionizing radiation has on both microorganisms and the object of irradiation under different experimental conditions, such as the presence of oxygen, water, etc., various approaches have been discussed and studied with the aim of reducing or eliminating the destructive effects of radiation on the object subjected to it.

Blackburn et al. (5) have studied this area more closely and have suggested various measures, such as the use of suitable additives or radical scavengers to protect the drug from harmful radiation effects.

Either of two methods are currently employed for the sterilization with ionizing radiation, namely exposure to gamma radiation from a Co-60 source, or treatment with high-energy electrons from accelerators. The action exerted on microorganisms is, in principle, similar in both methods. This

means that, on the whole, the same sterilizing effect is attained when equally large radiation doses are used, regardless of whether they originate from Co-60 or electrons from an accelerator.

Barring some earlier investigations, in which accelerator treatment of several kinds of drugs was studied, the results of the effect of radiation on drugs that have been published in recent years are in large measure based on observations made with Co-60 as a radiation source. This stems from the fact that from a practical viewpoint this method has been easier and more convenient to use.

One dissimilarity between accelerator sterilization and Co-60 sterilization is the vast difference in the rate of energy absorption. While a dose rate of up to 10^2 rads/sec is attained by using Co-60, the dose rate achieved by accelerator treatment amounts to 10^6 rads/sec and over.

Only few systematic studies have been made of how this difference in radiation intensity could influence the susbstance in question in terms of degree of degradation. However, some recorded observations could be of interest in this connection.

Pandula et al. (7) have recorded results indicating that for solutions of atropine, morphine and lidocaine irradiated with Co-60, the destruction of the substances decreases with increasing dose rate. Proceeding from these observations and from the increasing degree of process reliability resulting from improved construction techniques of recent years in the area of electron accelerators for sterilization, Nablo (8) has made comparative studies of Co-60 and accelerator treatment. The results he recorded indicate that sterilization with electrons from accelerators is less harmful than treatment with the same dose from a Co-60 source.

Three per cent solutions of alkyldimethyl benzalkonium chloride, treated in doses up to 3 Mrad either with high dose-rate accelerator (10^{13} rads/sec) or Co-60 (10^2 rads/sec) were compared. It was shown that the structure of the substance had been almost completely destroyed by the Co-60 irradiation but remained unaffected after the high dose-rate treatment. Similar results were obtained with an insulin preparation.

Furthermore, reference may be made to studies conducted by Bradbury (9) who on cellulosic material found that electron irradiation did not reduce strength as much as gamma irradiation when similar doses were used.

Present investigation

The object of this study has been to compare the effect of radiation sterilization on drug substances by treatment with Co-60 and electron accelerators. The pure unformulated drugs in powder form have been used for the study. The tested substances were selected among those which have been used for sterile preparations but which, due to their sensitivity, have not readily lent themselves to sterilization treatment through heating.

Experimental

Materials

The following substances were studied:

Chloramphenicol
Ampicillin trihydrate
Tetracycline hydrochloride

Atropine sulphate
Pilocarpine hydrochloride

All substances met the quality requirements of the Nordic Pharmacopoeia (Ph Nord).

When exposed to radiation the substances were filled in well sealed polyethylene tubes about 50 mm high and with an outer diameter of 15 mm.

Radiation Treatment

The samples of the substances were given 2.5 and 5.0 Mrad, respectively. The gamma irradiation was administered by using a Co-60 radiation source at dose rates of about 30 rads/sec. In the electron treatment, a linear electron accelerator HRC 712 was used, at dose rates of $2 \cdot 10^7$ rads/sec.

Methods

Such methods were used as could provide data on the changes which the radiation caused, thereby giving a basis for determining the quality level of the substances after the irradiation treatment.

After radiation treatment all substances were examined in accordance with the monographs in the Nordic Pharmacopoeia. This was done in order to ascertain whether the substances, after treatment, could still meet the pharmacopoeia requirements and be used for the manufacture of drugs.

In establishing the pharmacopoeia quality demands, no account has been taken of the special reactions that could arise in conjunction with radiation treatment and the particular degradation products that might be formed as a result of such treatment.

In addition to the tests conducted according to the pharmacopoeia requirements, the following specific methods have been employed in order to shed further light on the changes that have occurred in conjunction with radiation treatment.

Infrared Spectroscopy (IR)

The infrared absorption spectra from 4,000 to 250 cm^{-1} have been recorded on a Perkin Elmer 521 grating spectrophotometer. All substances have been prepared as potassium bromide discs using about 1.5 mg of the substance and 300 mg of KBr.

Thin-layer Chromatography (TLC)

Chloramphenicol: Precoated silica gel plates (Merck Silica gel 60 F-254) were used, and a mixture of 3 volumes of chloroform and 1 volume of methanol served as mobile phase.

Tetracycline hydrochloride: According to procedure described in Ph Nord.

Atropine sulphate: Precoated silica gel plates (Merck Silica gel 60 F-254) were used, and a mixture of 9 volumes of chloroform and 1 volume of diethylamine served as mobile phase. 200 μg of each sample were applied as aqueous solutions. Quantitative evaluation: The chromatograms were scanned by a Zeiss KM 3 chromatogram spectrophotometer, recording the reflectance at 254 nm. Two atropine sulphate samples, containing known amounts of apoatropine were used as references.

Pilocarpine hydrochloride: Precoated silica gel plates (Merck Silica gel 60 F-254) were used, and a mixture of 5 volumes of chloroform, 4 volumes of acetone and 1 volume of diethylamine served as mobile phase. 200 μg of each sample were applied as aqueous solutions. The chromatograms were examined (a) under UV light, 254 nm, (b) after exposure to iodine vapour, (c) after spraying with Dragendorff reagent, and (d) after spraying with potassium permanganate solution 0.025 mol/l.

Thermal Analysis

Thermal methods, both Differential Scanning Calorimetry (DSC) and Differential Thermal Analysis (DTA), have been used to determine the chemical purity and also to detect the presence of polymorphic forms. The basis for the purity determination is the relation between the melting point depression and the amount of impurity present.

To obtain a reliable purity estimate by this method it is important that the substance does not decompose, at least to any appreciable extent near the melting point. In the event some decomposition should occur, it is in many cases possible, however, to obtain a sample-to-sample comparison if the experimental conditions are carefully defined.

The thermograms for all substances, from 40°C to above the expected melting points, were recorded with a Perkin Elmer DSC-IB using a scan speed of 16°C/min and a nitrogen purge.

Quantitative thermograms were recorded for chloramphenicol and pilocarpine hydrochloride. These determinations were carried out with a Mettler TA 2000 system operated on line with a Hewlett Packard 9810A calculator programmed to calculate the mole per cent impurity as described by Mettler (10). The heating rate used was 2°C per minute.

High Pressure Liquid Chromatography (HPLC)

Ampicillin Trihydrate: A modification of a method described by Hartmann and Rödiger (11) was used. The column was a 30 cm × 4 mm I.D μ-Bondapak C_{18} prepacked column (Waters Assn.) and the mobile phase was a mixture of 85 volumes of 0.05 M phosphate buffer pH 7.0 and 15 volumes of acetonitrile, which was pumped through the column at a flow rate of 1.2 ml/min. A Perkin Elmer LC-55 spectrophotometer equipped with an 8 μl flow cell and operated at 220 nm was used as a detector.

Light-absorbing Impurities

Ampicillin Trihydrate: The method described by Seitzinger (12) in a work dealing with determination of degradation products of penicillins was used. The UV-spectrum in the range of 300 to 350 nm of a 0.25 per cent aqueous solution was recorded and the extinction at the maximum wavelength was determined.

Impurities Containing Free SH-groups

Ampicillin Trihydrate: The method described by Seitzinger (12) was used.

UV Absorption

Atropine sulphate: Spectra of aqueous solutions containing 1.00 mg/ml were recorded in 10.00 mm quartz cell using a Zeiss DMR 21 recording spectrophotometer.

Microbiological Assay

The quantitative microbiological determination of ampicillin, chloramphenicol and tetracycline has been performed by using a conventional two-dose agar diffusion technique according to the provisions in Ph Nord. The same substances not exposed to irradiation treatment were used as reference standards.

A summary review of all the tests that have been conducted in this study is presented in Table I.

Table I. — List of substances and methods used

Chloramphenicol	Ampicillin trihydrate	Tetracycline hydrochloride	Atropine sulphate	Pilocarpine hydrochloride
Ph Nord	Ph Nord	Ph Nord	Ph Nord	Ph Nord
TLC	HPLC	TLC	TLC	TLC
IR	IR	IR	IR	IR
DTA	UV-absorption	Light absorption	UV-absorption	UV-absorption
Microbiological assay	Free SH-groups	DSC		DTA
	Assay B.P.-73 Microbiological assay	Microbiological assay		

Results

A compilation of the results obtained for each substance is given in Tables II-VI, which also show the requirements and permitted limits for pharmacopoeia tests.

Although all the pharmacopoeia tests were performed, the tables do not give those results in which no differences have been noted between irradiated and non-irridiated samples and which in other respects were of no significant interest.

It might also be mentioned that for all substances tested by the IR spectroscopy technique, no differences in spectra were obtained between irradiated and non-irradiated samples.

Table II. — Tests and results for chloramphenicol

Test \ Sample	Untreated	Acc 2.5 Mrad	Acc 5 Mrad	Co 2.5 Mrad	Co 5 Mrad
1 Description.....	Almost white	Yellowish	Yellow	Yellowish	Yellow
2 Melting range 148 - 153°	148 - 150°	147 - 149°	147 - 149°	146 - 148°	146 - 148°
3 $(\alpha)_D^{20}$: + 18.5° to + 21.5°.....	+ 19.6°	+ 19.5°	+ 19.1°	+ 19.5°	+ 19.1°
4 Light absorption.... E (1%, 1 cm): 300 at 278 nm	297 at 277.5 nm	295 at 277.5 nm	295 at 277.5 nm	294 at 277.5 nm	295 at 277.5 nm
5 Colour: 2.5 % sol. in ethanol.....	Complies	1.25 % complies	1.0 % complies	1.0 % complies	0.5 % complies
6 Chlorides.....	Complies	Complies	Does not comply	Complies	Does not comply
7 Assay 97.5 to 100.5%.....	99.7%	99.5%	99.8%	99.1%	99.4%
8 DTA, mole % impurity, melting-point.....	3.6% 150.1°	4.4% 149.7°	5.0% 149.1°	4.1% 149.2°	5.2% 148.6°
9 Microbiological assay.....	(100 %)	99.6 %	97.6%	98.3%	97.0%

Chloramphenicol, Table II and Figure 1

Ph Nord. Tests No. 1 – 7.

It is clear from the table that the samples do not in all respects comply with the pharmacopoeia tests after irradiation. This is true both in the case of treatment with Co-60 as well as accelerator. The change in colour, however, is somewhat more marked after treatment with Co-60.

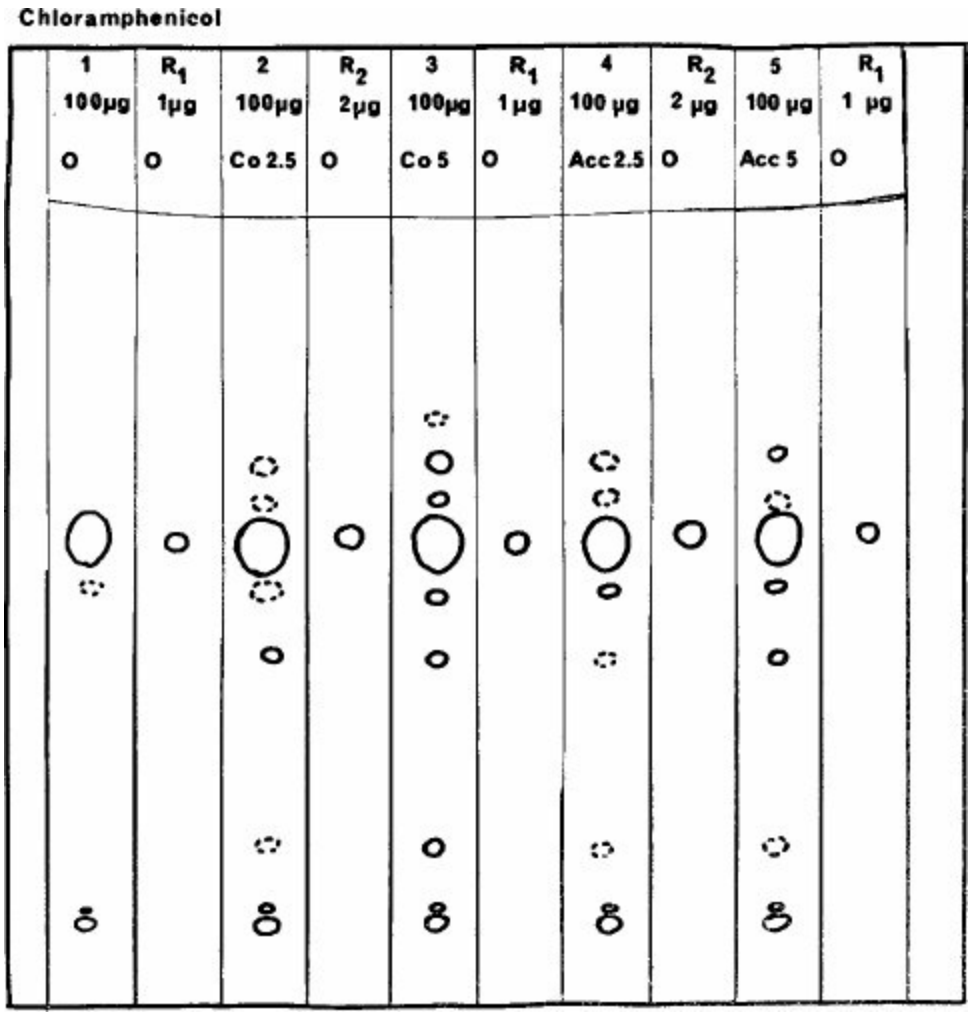

Figure *1*. TLC of Chloramphenicol. O = Untreated sample. R_1 and R_2: 1:100 and 1:50 dilutions of solution 1. All secondary spots are less intense than the spot obtained with solution R_1 (1 μg).

TLC: The chromatograms of the irradiated samples (Figure 1) all show the same pattern of secondary spots. The impurity spots, however, are somewhat more intense in the chromatograms of the Co-60 irradiated samples than in those of the accelerator treated ones when comparing corresponding dose levels. This indicates a higher degree of degradation in treatment with Co-60. The degree of degradation also increases with the irradiation dose level. All secondary spots, however, are less marked than the spot obtained with reference solution R_1 (1 μg, corresponding to one percent).

DTA: The impurity values obtained are reported in the table. The difference between samples irradiated at similar dose levels is within the limits of error under the method employed, whereas the difference between samples treated with 2.5 Mrad doses and those treated with 5 Mrad doses is more distinct. It is obvious from the figures shown that no difference is obtained between Co-60 and accelerator treatment.

It was noted that the thermogram for all chloramphenicol samples showed two endotherms, one at about 140°C and the other, the melting endotherm, at about 149°C. The latter is recorded in the table. It has not yet been clarified whether the first peak indicates the presence of a polymorphic form or an impurity undetected with other methods used in the investigation. The values for molar impurity shown in the table should be used only for comparison between the samples.

Microbiological Assay: No significant decline in microbiological activity has been obtained.

Ampicillin Trihydrate, Table III and Figure 2

Ph Nord. Tests No. 1 – 4.

All irradiated samples met the established requirements, with the exception of the demands relating to colour of the substances.

Table III. — Tests and results for ampicillin trihydrate

Test \ Sample	Untreated	Acc 2.5 Mrad	Acc 5 Mrad	Co 2.5 Mrad	Co 5 Mrad
1 Description.....	White	Yellow	Yellow	Greyish-yellow	Greyish-yellow
2 $(\alpha)_D^{20}$: + 280° to + 310°.....	+ 299.4°	+ 297.0°	+ 294.2°	+ 296.8°	+ 293.4°
3 Assay 95.0 to 100.5% Penicillinase method.....	100.6%	100.3%	100.2%	100.2%	99.9%
4 UV-absorption E(1 %, 1 cm)					
at: 256.5 nm.	7.8	8.7	9.1	8.7	9.6
261.6 nm.	6.6	7.5	7.9	7.5	8.4
268 nm ..	4.4	5.1	5.5	5.2	6.0
5 Light-absorbing impurities					
λ_{max},	323 nm	314 nm	315 nm	314 nm	315 nm
extinction.....	0.03	0.12	0.23	0.14	0.31
6 Impurities with free SH-group µmol/g...	0.5	5.8	11.5	7.4	20.7
7 Assay B.P. 73 Ref: untreated sample...	(100%)	99.3%	99.0%	99.0%	97.9%
8 Microbiological assay.....	(100%)	97.6%	99.1%	97.5%	100.5%

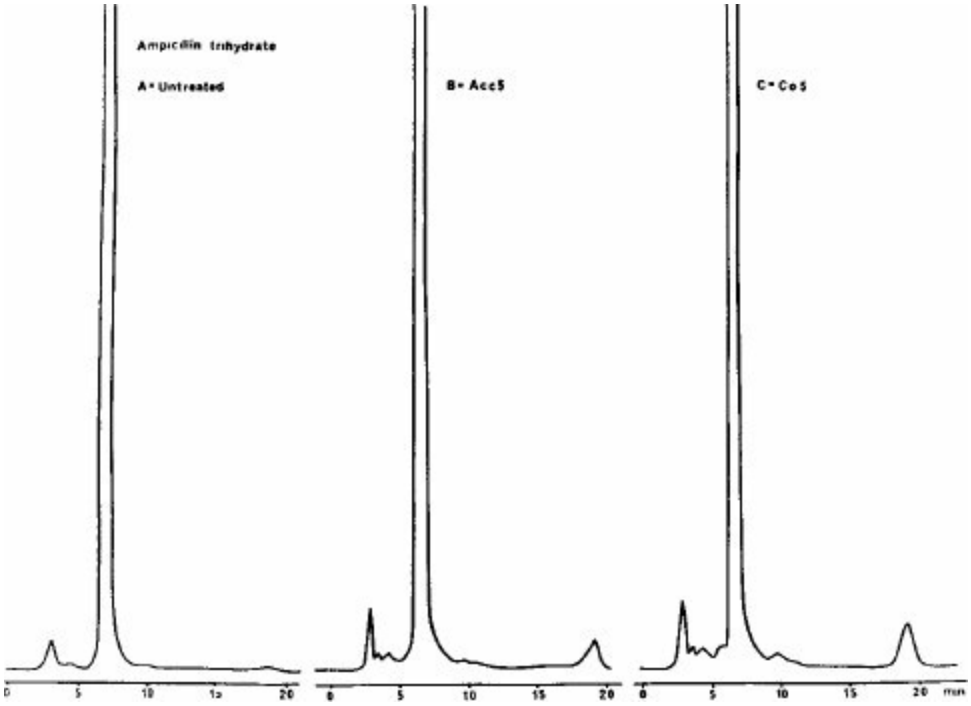

Figure 2. HPLC of Ampicillin trihydrate. 40 μg of each sample were injected. The area of the peak at 19 min in chromatogram C is about 0.5 per cent of the area of the main peak.

HPLC: It is apparent from Figure 2 that the height of the impurity peaks increases with the irradiation dose and is slightly higher after treatment with Co-60 compared to accelerator treatment. As shown in Table III, this is also in accordance with the results obtained in other tests (UV absorption, light absorbing impurities, and amount of free SH-groups), in which the degree of decomposition is shown to be somewhat greater after Co-60 treatment.

Microbiological Assay: No decline in microbiological activity has been observed.

Tetracycline Hydrochloride, Table IV

Ph Nord. Tests No. 1 – 3.

All irradiated samples were well within the limits required in the monograph, including the TLC test. In this test, the same number of secondary spots were obtained from irradiated and untreated samples. Two of the impurity spots, one of which could be identified as anhydrotetracycline, located above the main spot were somewhat more intense in the case of irradiated samples. No difference was recorded between Co-60-treated and accelerator treated samples.

Table IV. — Tests and results for tetracycline hydrochloride

Test \ Sample	Untreated	Acc 2.5 Mrad	Acc 5 Mrad	Co 2.5 Mrad	Co 5 Mrad
1 $(\alpha)_D^{20}$: −240° to −255°	− 246.0°	− 246.0°	− 246.1°	− 245.6°	− 246.4°
2 Light absorption E (1%,1 cm): 400 at 269 nm	390	390	386	392	389
E(1%, 1 cm): 180 at 299 nm	181	182	179	181	179
E(1%, 1 cm): 300 at 356 nm	306	306	302	308	305
3 Assay 98.0 to 102.0 % Perchloric acid titr.	100.2%	99.7%	99.5%	99.5%	99.5%
4 Light absorption at 430 nm (Ph Europ).	0.186	0.201	0.213	0.207	0.220
5 Microbiological assay	100.0%	98.5%	101.7%	99.2%	100.0%

Light Absorption according to Ph Eur: (Test for anhydrotetracycline and epianhydrotetracycline). Only minor differences resulting from radiation were observed, but all treated samples were well within permissible limits.

DSC: Two exotherms were obtained, one at 239°-242°C and the other at 243°-245°C. The ratio between the two peaks varied with the irradiation dose but there was no indication of any differences between Co-60 and accelerator treatment. The changes could be due to crystal transformation.

Microbiological Assay: All irradiated samples had the same microbiological potency as the untreated one.

The tests made with tetracycline hydrochloride showed that this substance revealed only insignificant changes resulting from irradiation treatment. No difference between Co-60 treatment and accelerator treatment was noted.

Atropine Sulphate, Table V and Figures 3, 4 and 5

Ph Nord. Tests No. 1 – 5.

None of the irradiated samples complied in all respects with the pharmacopoeia tests. The lowering of melting range and pH, and the increase in reducing impurities are more pronounced in the Co-60 treated samples. The potency is also somewhat lower after Co-60 treatment.

Table V. — Tests and results for atropine sulphate

Test \ Sample	Untreated	Acc 2.5 Mrad	Acc 5 Mrad	Co 2.5 Mrad	Co 5 Mrad
1 Description.....	White	Yellowish	Yellowish	Yellowish	Yellowish
2 Melting range 191 - 198°	191 - 193°	190 - 192°	190 - 192°	187 - 189°	183 - 185°
3 pH (2% aq. solution).....	4.90	4.40	4.28	3.93	3.64
+ NaOH.....	6.57	6.00	5.77	4.43	3.85
+ HCl.....	4.02	3.89	3.85	3.71	3.52
4 Reducing substances (colour stability > 5 min).....	> 5 min	1 min 45s	1 min 30s	20 s	10 s
5 Assay 97.5 - 100.5%	98.5%	98.1%	98.3%	97.5%	96.6%
6 UV absorption E (1%, 1 cm) at 257 nm.....	5.8	6.1	6.3	7.5	9.1

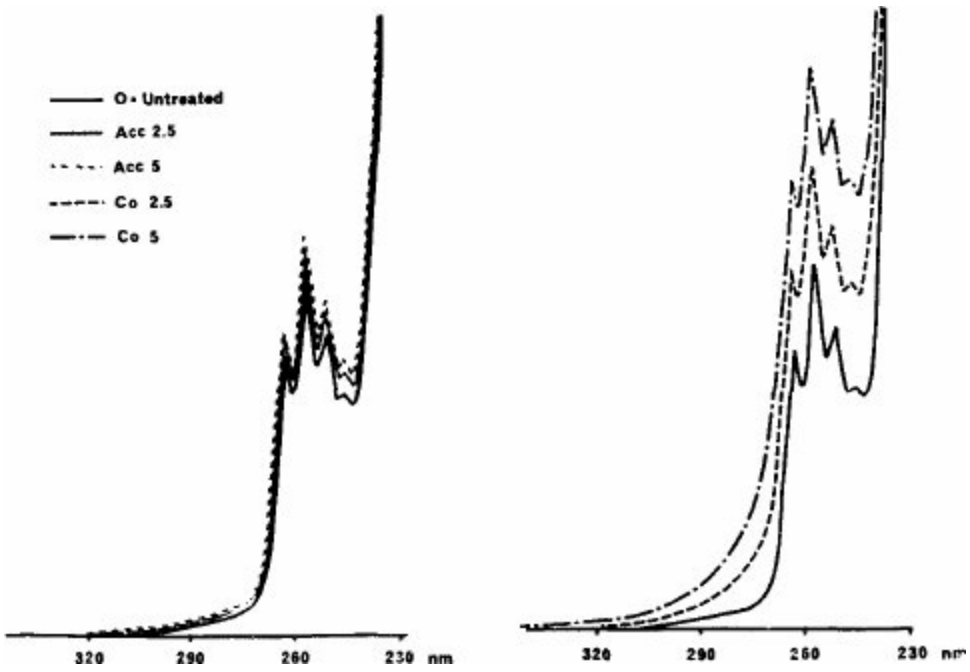

Figure *3.* UV absorption of the Atropine sulphate samples. 1.0 mg/ml, aqueous solutions. Path length = 10.00 mm. Left group: accelerator treated samples, compared to untreated substance. Right group: Co-60 treated samples, compared to untreated substance.

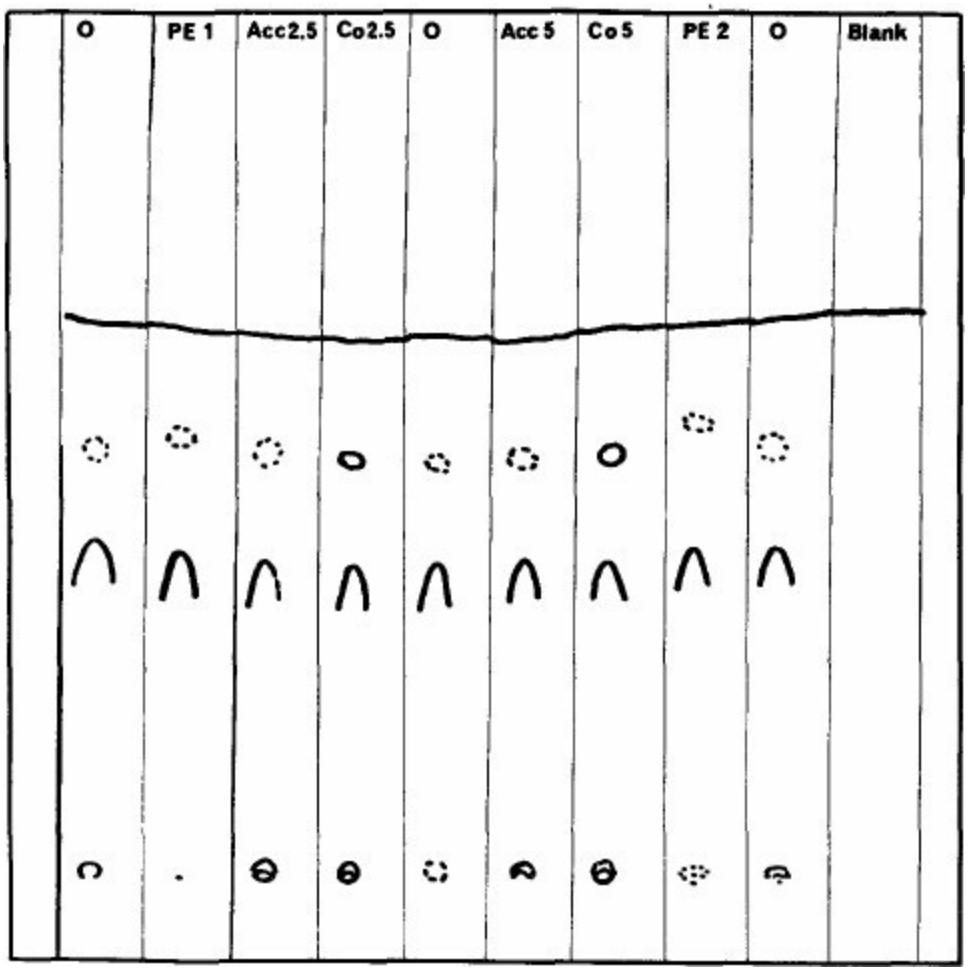

Figure *4.* TLC of Atropine sulphate samples. Chromatographic conditions: see text.

The results from the other tests (UV absorption — Figure 3 — and TLC — Figures 4 and 5) also indicate more marked changes in the Co-60 treated samples.

From the TLC it can be seen that the amount of impurity at Rf = 0.05 increased by about 50 per cent during accelerator irradiation and by about 200 per cent during Co-60 treatment. Similarly, the amount of the impurity at Rf = 0.8 (possibly apoatropine) increased by about 100 per cent during the accelerator treatment and by about 800 per cent during the Co-60 treatment.

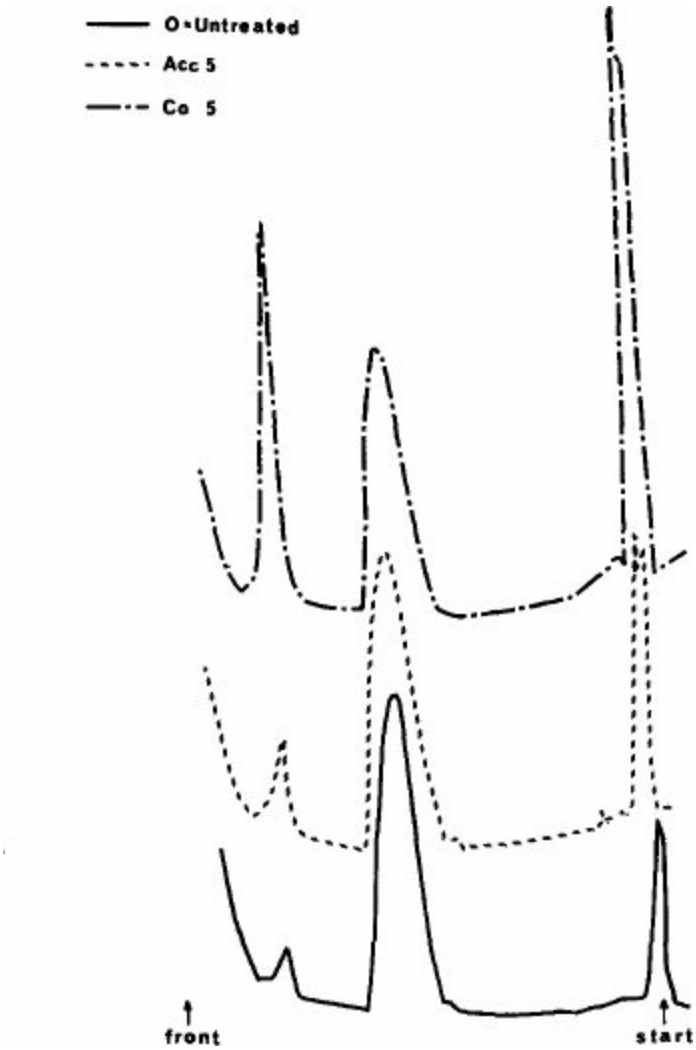

Figure 5. Quantitative evaluation of the TLC of Figure 4. Instrument: Zeiss KM3 Chromatogram Spectrophotometer. Wavelength: 254 nm. Operation mode: reflectance. Ordinate: relative reflectance (arbitrary units).

Pilocarpine Hydrochloride, Table VI

Ph Nord. Tests No. 1-4.

Aside from some discoloration of the irradiated samples, they all met the pharmacopoeia requirements and no differences have been noted between Co-60 and accelerator treated samples.

Similar observations were made in connection with the other tests. Thus, there were no differences in UV and IR spectra, TLC chromatograms or the DTA tests between samples subjected to Co-60 or accelerator treatment, and the sample that was untreated. These observations agree with earlier one which show that pilocarpine has good resistance to radiation.

Table VI. — Tests and results for pilocarpine hydrochloride

Test \ Sample	Untreated	Acc 2.5 Mrad	Acc 5 Mrad	Co 2.5 Mrad	Co 5 Mrad
1 Description........	White	Yellow	Yellow	Yellow	Yellow
2 Melting range 201 - 205°C	205 - 206°	204 - 205°	204 - 205°	204 - 205°	204 - 205°
3 $(\alpha)_D^{20°C}$ + 89.0 to + 93.0°	+ 91.9°	+ 91.3°	+ 90.7°	+ 90.9°	+ 90.8°
4 Colour of 2% aq. solution............	complies	complies ?	does not comply	does not comply	complies ?
5 DTA, mol % impurity	0.8%	1.0%	1.1%	0.8%	0.8%
melting point	202.2°	201.5°	201.8°	201.8°	201.8°

Conclusion

In recent years, development within the sphere of linear accelerator technology has progressed at an impressive pace. High dose rate linear accelerators of good reliability and suitable for sterilization purposes are now available.

Observations have been made earlier indicating that some substances of pharmaceutical interest, after having been sterilized with such accelerators, showed a lesser degree of degradation than when they had been irradiated with an equal dose of gamma radiation.

These observations, according to the findings of this study covering five drug substances irradiated in dry form, have been confirmed. The differences which have been noted are not very significant.

The study also showed that three of the substances tested (ampicillin, tetracycline and pilocarpine), after having been subjected to sterilization doses under cobalt and accelerator treatment, still met the pharmacopoeia quality requirements, whereas two of the substances (chloramphenicol and atropine sulphate) did not meet those requirements because of changes the treatment caused.

Acknowledgement

The authors wish to convey their thanks to B. Österberg, Johnson & Johnson Ltd., Sollentuna, Sweden and K. Sehestedt, Danish Atomic Energy Commission, Research Establishment, Risö, Denmark for most generous help with the irradiation treatments.

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Physical and Chemical Studies on Some Irradiated Drugs

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Abstract: *In order to estimate the possibilities for the radiation sterilization of some drugs (semisynthetic penicillins, anaesthetics and cordiaminium) we made radiation and chemical studies aimed at clarification of the processes occuring on radiolysis of these substances by the methods of ESR, NMR, IR and UV-spectroscopy, fluorescence and chromatography.*

Irradiation was performed both in the solid state and in water solution. Radical radiation yields were measured. Some products of radiolysis were isolated and identified. It has been established that the rupture of the C-N bond is the principal radiation and chemical process. The effect of radical concentration on the rate of radiation processes was investigated and the peculiarities of radiation and chemical processes depending on the chemical structure of compounds was considered.

The ionizing radiation affecting the drug causes not only death of microorganisms but also various damage in the molecular structure and the formation of compounds whose structure differs from the initial one. These compounds can possess different pharmacologic properties and sometimes they may be toxic. This paper describes the results of radiation and chemical processes in the different classes of drugs: antibiotics (semisynthetic penicillins), anaesthetics and diethylamide of nicotinic acid (DEANA) (25% aqueous solution of diethylamide of nicotinic acid is the drug cordiaminium).

The investigation was carried out by the methods of electron spin resonance (ESR), nuclear magnetic resonance, infrared and ultraviolet-visible spectroscopies, fluorescence and chromatography.

The ESR spectra and the kinetics of radical accumulation were registered by a device made under a fast electron beam with an energy of about 1 Mev. The input of a fast electron beam into the spectrometer resonance cavity permits the study of formation, transformation and disappearance of free radicals directly at the moment of irradiation. The radical radiation yield was measured by the initial part of the free radical accumulation curve. If the ESR method characterizes the dynamics of primary radical processes, the other above-mentioned methods can be used for studying the final change of the individual group or the molecule as a whole. When the optical and nuclear magnetic resonance spectroscopy methods are used, it is necessary to obtain the new changed fragments in amounts large enough from the point of view of sensitivity. Therefore, in a number of cases in order to "accumulate" the damages, dose values much larger than the values of sterilizing dose were used. Identification of the admixture is facilitated considerably if they are first separated by chromatography or other methods.

The experimental values of radiation radical yields in the investigated drugs are shown in Table I.

Table I. — Radiation yield value of radicals of irradiated semisynthetic penicillins, their fragments, and

anaesthetics

COMPOUND	1/100ev.
SEMISYNTHETIC PENICILLINS	
Sodium oxacillin, crystal	0.8±0.1
Sodium oxacillin, amorphous	4.0±0.5
Sodium or potassium benzylpenicillin, crystal	0.8±0.1
Sodium methicillin, crystal	2.0±0.2
Sodium dicloxacillin, crystal	3.3±0.3
Sodium dicloxacillin, amorphous	7.1±0.5
Sodium ampicillin, amorphous	11.0±1.0
Ampicillin, acid	15.0±1.5
Phenoxymethylpenicillin, acid	3.0±0.5
FRAGMENTS OF SEMISYNTHETIC PENCILLINS	
6-aminopenicillanic acid	3.4±0.3
5-methly-3 phenyl-4-isoxazol carboxylic acid	0.1±0.03
Penicilloic acid	0.7±0.1
Thiazolydine	0.5±0.1
ANAESTHETICS	
Trimecaine (α-diethylaminoacetyltrimethylamilide hydrochloride)	0.7±0.2
Novocaine (β-diethylaminoethyl ester of p-aminobenzoic acid hydrochloride)	0.1±0.03

Semisynthetic penicillins

It is seen from Table I that the radical radiation chemical yields of the semisynthetic penicillins are greatly changed, depending on the chemical and crystalline structure; the largest values being observed for the amorphic sample and for the samples in which the regular structure form includes water molecules (1).

The essential role in this case seems to belong in radiolysis to the water molecules or to the oxygen of air.

The study of the peculiarity of the super-fine structure of oxacillin and its fragments by ESR spectra in a wide temperature range permits identification of possible types of radical states occurring at deamination, decarboxylation and at dehydrogenation of an H-atom from a CH bond adjacent to the carboxyl group of the molecule (2).

These radical states cause a number of secondary reactions which result in a rupture of the thiazolidine ring and peptide linkage in the molecules. Similar reactions according to IR spectroscopy are characteristic of the whole investigated class of semisynthetic penicillins and result in formation of a new molecular constituent of the initial antibiotic. Comparison of the radiation radical yields of the

substance, penicillin fragments, and penicillin itself shows that an aromatic part has a protective effect.

Eventually, after irradiation, the general concentration of the radicals decreases due to their recombination. The storage of the irradiated crystalline oxacillin reduces the concentration of the free radicals by about 10 times during 7 days.

The anaesthetics and cordiaminium

The low values of anaesthetic radiation radical yields indicate their considerable radiation stability. This is confirmed by the absence of any essential changes in IR spectra of anaesthetics irradiated in the solid state at a dose of about 100 Mrad.

The ESR spectra of irradiated anaesthetic are unresolved signals which, though indicating the difference in the nature of paramagnetic centers, do not permit unequivocal identification of their structure.

The storage of irradiated (25 Mrad) polycrystalline samples of novocaine at room temperature reduces the concentration of free radicals by about 3 times during 8 months.

The study of water solutions of anaesthetics revealed some peculiarities of their radiation and chemical conversions. It has been established that the principal radiation chemical dissociation product of trimecaine is acetomesidine which is already fixed in 1% solution as a suspension at dose values near to the value of the sterilizing dose. According to the data of IR spectroscopy, the radiolytic chemical decay of 1% trimecaine solution is 3.5 ± 0.5 molecule/100 ev and the acetomesidine yield is 1.5 ± 0.5 molecule/100 ev. Diethylamine hydrochloride was also identified with other products of trimecaine radiolysis. It is interesting to note the fact of post-radiated accumulation of acetomesidine in the irradiated solution during the long time of its periodic filtration. This fact demonstrates an inducing effect of irradiation. Acetomesidine, as a radiolysis product, was isolated from trimecaine irradiated in a solid state by the dose of about 150 Mrad. This testifies to the single type process of radiolytic decomposition in the solid state and in solution. The irradiation of trimecaine solutions with the addition of tert-butanol shows that the decrease of OH-radical concentration does not affect kinetics of radiation chemical processes, whereas the increase of OH-radical concentration changes these processes by decreasing acetomesidine yield.

The study of a water solution of novocaine hydrochloride shows that the products of radiolysis include: anaesthesine (ethyl ester of p-aminobenzoic acid) and p-aminobenzoate hydrochloride as the identified products and aromatic compounds containing methoxy- and oxy- groups as unidentified products. Thus the study of the secondary products of radiolysis demonstrates that the rupture of the C-N bond is a principal radiation chemical process in anaesthetics.

Irradiation of the water solution of DEANA results in changes of initial IR spectra, fluorescent characteristics and in the appearance of a color reaction of the carbonyl group. Chromatographic separation of the irradiated solution made it possible to isolate the following products: high molecular weight compounds (i), nicotinic acid (ii), heterocyclic compound (iii) reacting to carbonyl group, and heterocyclic compounds (iv) having characteristic IR absorption and characteristic fluorescence, different from the corresponding properties of DEANA. The latter two substances are the main products of radiolysis. The qualitative evaluation of radiation chemical decay of cordiaminium was not performed because its IR and UV spectra overlapped with the spectra of dissociation products. Irradiation of DEANA solutions with addition of tert-butanol decreases the formation rate of the latter

(iv) products by 3 times and the rate formation of the carbonyl product (iii) by 6 times. Saturation of DEANA solution with N_2O increases the radiation decay rate. These data indicate that the OH radicals, whose interaction with DEANA results in a rupture of C-N bond and in the oxygenation of the heterocyclic ring, play the principal role in the process of radiolytic cleavage.

Therefore, the study of the irradiated drugs by the methods of radio and optical spectroscopy made it possible to elucidate the mechanism of their radiation damage and identify some products of radiolysis.

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Physical-Chemical Changes in Irradiated Drugs

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Abstract: *Radiation sterilisation of pharmaceuticals is accompanied by chemical degradation which must be eliminated or minimised if the method is to be successfully applied. In order to find ways of protecting a drug the identity, yield and mechanism of formation of the decomposition products must be established, and the rate-constants for the reactions involved measured. Such data has been previously reported for a variety of drugs including penicillins, sulphonamides, arvin and heparin. The corresponding data is presented here for the effects of ionising radiation on mepacrine methane sulphonate, chloroquine diphosphate and triamcinalone acetonide.*

Introduction

The major difficulty to be overcome in applying a sterilising dose of ionising radiation is the prevention of decomposition of the biologically active agent and its vehicle or excipient. Earlier studies of the effects of ionising radiations on pharmaceutical systems suffered from the limitations that they were principally concerned with superficial changes such as, acid production, gas formation, loss of potency, change of colour and spectral properties. Moreover, complete formulations were often irradiated in which the pharmaceutical comprised only a very small percentage of the total. Insufficient attention was given to the presence of oxygen and other potential radical scavengers. The data obtained from such *ad-hoc* investigations was therefore often confusing. The studies demonstrated certain of the disadvantages of radiation sterilisation without suggesting any procedures for its improvement and possibly had a detrimental effect on the development of the technique. In the last decade, radiation chemistry has been applied to the study of pure drugs. As a result, the possibility of radiation sterilisation can be more confidently assessed. The effects of ionising radiations on medicines and pharmaceutical base materials both in the solid-state and in solution has been reviewed (1). Here, emphasis will be placed particularly on irradiation of aqueous solutions of certain drugs, in order to show how radiation chemical techniques can be applied.

In aqueous solution, free radicals derived from the water are usually responsible for solute decomposition. The effect of high energy radiation on neutral deaerated water (2) may be summarised as:

$$H_2O \rightarrow 2.7\ (e_{aq}^-) + 2.7\ (^{\bullet}OH) + 0.55(H^{\bullet}) + 0.45(H_2) + 0.71(H_2O_2) + 2.7(H^+)$$

The numbers before the chemical symbols represent G-values, i.e. the number of molecules of each

species formed per 100 eV of energy absorbed. Addition of suitable radical scavengers can provide protection by removing the primary radicals and converting them into non-reactive species.

1. *Effects of γ-irradiation on Benzylpenicillin (penicillin G)*

Extensive degradation of sodium benzylpenicillin accompanies γ-irradiation of dilute aqueous solutions (3). The G(-benzylpenicillin) value, 3.8 in argon saturated solution indicates both e^-_{aq} and ·OH participate in the degradation. Pulse radiolysis confirms the high reactivity of penicillin G towards e^-_{aq} ($k_2 = 2.7 \times 10^9$ dm^3 mol^{-1} s^{-1}) and ·OH ($k_2 = 3.4 \times 10^9$ dm^3 mol^{-1} s^{-1}). The total yield of primary radical species formed during irradiation of water is approximately 6.0. Values for G(-benzylpenicillin) in excess of this value were obtained at benzylpenicillin concentrations > 10^{-3}M. This observation indicates some kind of limited chain reaction. The products formed during irradiation of benzylpenicillin are shown in Table I. Thus, reaction of benzylpenicillin with ·OH radicals yields o, m and p hydroxybenzylpenicillins in the ratio 2:1:1. The rate constant for the reaction is 3.4×10^9 dm^3 mol^{-1} s^{-1}. Benzylpenicilloic acid is formed exclusively by ·OH attack.

Table I. — Yields of major products formed during γ-irradiation of benzylpenicillin in dilute aqueous solution (10^{-3}M)

	G Value	
	Argon	N_2O
Benzylpenilloic acid	1.5	1.2
Benzylpenillic acid	0.5	<0.05
Benzylpenicilloic acid	0.47	0.97
o-Hydroxybenzylpenicillin	0.24	0.46
m-Hydroxybenzylpenicillin	0.13	0.23
p-Hydroxybenzylpenicillin	0.12	0.23
CO_2	1.0	0.6
H_2S	0.06	<0.02

Benzylpenillic acid is formed only by reaction with e^-_{aq}. The major product, benzylpenilloic acid, is formed by either ·OH or e^-_{aq} attack. Therefore, sterilisation of aqueous solutions is impracticable. In contrast the molecule is most stable in the solid state. No detectable decomposition occurred below a dose of 3 Mrad. Sterilisation of the solid should therefore present no problem.

2. *Effect of γ-irradiation on Sulphonamides*

In aqueous solution, five sulphonamides were found to be extensively degraded (4). G(-sulphonamide) values varied from 3.5-5.1 under anoxic conditions. Accordingly, both e^-_{aq} and ·OH must be involved in degradation. When the N_4 substituted compounds thalamyd and sulphasuccidine are irradiated scission of the N_4-CO-NH- bond occurs yielding sulphacetamide (G+ = 1.4) and phthalic

acid (G+ = 1.0) from thalamyd and yielding sulphathiazole (G+ = 0.7) from sulphasuccidine (Table II). These products are not formed in N_2O saturated solutions following γ-irradiation. Attack by the hydrated electron e^-_{aq} is therefore responsible for their formation. Both e^-_{aq} and ·OH react rapidly with sulphonamides and their degradation products (Table III). The site of ·OH attack is the S-N sulphonamido bond.

Table II. — Effects of γ-irradiation on aqueous solutions of sulphathiazole, sulphasuccidine and thalamyd (10^{-4} M)

Compound	−G-value Argon	−G-value N_2O	Irrigant Component R_f value (reference (4))			Irradiation conditions[a] A	B	C	mpt	Identity	G (value)
Sulphathiazole	5.1	4.3	iii	I	0.58	+	+	+	165	sulphathiazole	
				II	0.49	+	−	+			
				III	0.43	+	+	−	165	sulphanilamide	
				IV	0.12	+	+	−		phenolic compound	
				V	0.06	+	−	+	286	sulphanilic acid	0.6
				VI	0.02	+	+	−			
Sulphasuccidine	5.1	4.0	iv	I	0.94	+	−	+	165	sulphathiazole	0.7
				II	0.22	+	+	+	190	sulphasuccidine	
				III	0.14	+	+	−		phenolic compound	
				IV	0.05	+	−	+			
Thalamyd	4.4	4.6	iii	I	0.98	+	−	+		acetamide	
				II	0.91	+	−	+		phthalic acid	~1.0
				III	0.73	+	−	+	183	sulphacetamide	1.4
				IV	0.3	+	+	+	202	thalamyd	
				V	0.09	+	+	−			
				VI	0.04	+	−	+	286	sulphanilic acid	
				VII	0.02	+	+	−			

[a]A = argon saturated solution, B = nitrous oxide saturated solution, C = argon saturated solution containing ethanol (10^{-1} M).

Table III. — Rate of reaction of several sulphonamides and related compounds with hydrated electrons (A) and hydroxyl radicals (B)

	A $k_2 \times 10^9$ dm^3 mol^{-1} s^{-1}	B $k_2 \times 10^9$ dm^3 mol^{-1} s^{-1}
Sulphanilamide	7.4	1.6
Sulphaguanidine	8.6	3.1
Sulphathiazole	11.7	7.8
Sulphasuccidine	14.2	4.6
Thalamyd	7.4	6.3
Sulphanilic acid	5.9	2.9
Phthalic acid	4.8	3.0
Benzoic acid	3.0	2.5
Benzenesulphonamide	9.8	2.8
Sulphacetamide	41.0	4.7

Sodium sulphacetamide also reacts rapidly with both e^-_{aq} ($k_2 = 4.1 \times 10^{10}$ dm^3 mol^{-1} s^{-1} and ·OH ($k_2 = 4.7 \times 10^9$ dm^3 mol^{-1} s^{-1}). Attack by e^-_{aq} yields sulphanilic acid as a major product (5). A very similar reaction is observed with sulphathiazole where sulphanilic acid is formed by scission of the S-N bond. G(-sulphacetamide) is maximal and = 4.9 at 1.2 M, i.e. 30% solution (w/v). The decomposition of such concentrated solutions by a dose of 5 Mrad is therefore not >4% (Table IV), which compares favorably with other more conventional procedures of sterilisation (6). Provided that the products of γ-irradiation are not harmful therefore, radiation sterilisation of such concentrated solutions might be a viable proposition. (The major degradation product of sulphacetamide irradiation is sulphanilic acid.) For more dilute solutions, radical scavengers would be required, calling for a thorough knowledge of mechanism and kinetics.

Table IV. — Effect of γ-irradiation (5 Mrad) on concentrated aqueous sulphacetamide in oxygen

Sulphacetamide	(M)	2×10^{-1}	8×10^{-1}	1.2
Degradation	(%)	13	4	3

3. *Effects of γ-irradiation on antimalarial drugs*

The antimalarial drugs tested react rapidly with e^-_{aq} (Table V). Both mepacrine and chloroquine contain 4-amino-1-diethylamino-pentane side chains. These compounds can accept two protons in the range from pH 6 to pH 11, one proton attached to the ring nitrogen and one to the diethylamino nitrogen. The ionisation equilibria exhibited by the drugs involve two processes:

Table V. — Rate of reaction of antimalarial drugs with hydrated electrons

	$k_2 \times 10^{10}$ dm^3 mol^{-1} s^{-1}
Mepacrine methanesulphonate	4.6
Chloroquine diphosphate	3.7
Primaquine diphosphate	3.2

$$H^+B\text{-}B'H^+ \rightleftharpoons H^+ + B\text{-}B'H^+ \quad \text{— (1)}$$
$$B\text{-}B'H^+ \rightleftharpoons H^+ + B\text{-}B' \quad \text{— (2)}$$

where B represents ring nitrogen and B′ the diethylamino nitrogen. The acid dissociation constants for processes (1) and (2) for chloroquine are $pK_1 = 8.08$, $pK_2 = 10.16$. It is not surprising therefore that the reactivity of chloroquine towards e^-_{aq} varies enormously with pH (Figure 1). The reactivity of mepacrine towards e^-_{aq} is also pH dependent, at pH 6 $k_2 = 4.6 \times 10^{10}$ dm^3 mol^{-1} s^{-1} and 2.4 dm^3 mol^{-1} s^{-1} at pH 10.0.

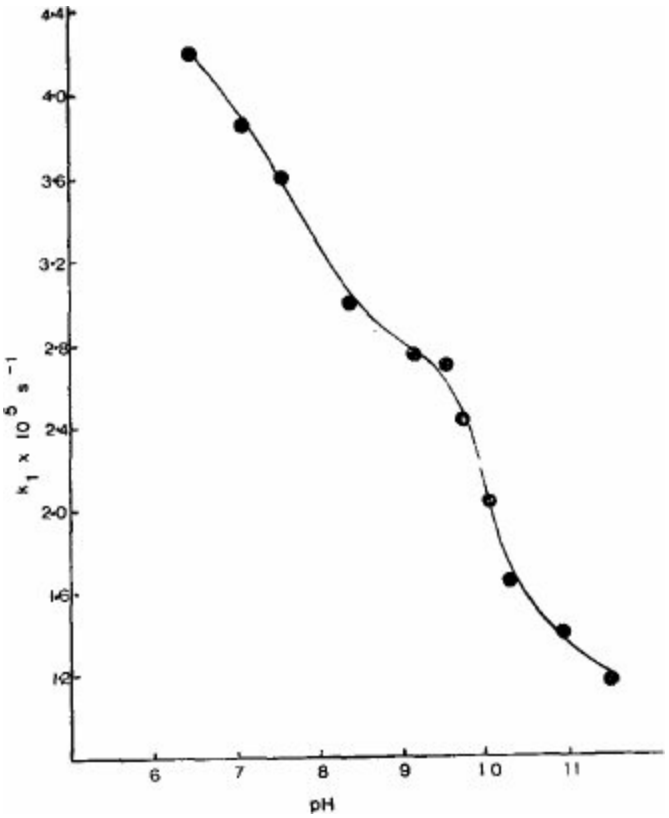

Figure *1.* The effect of pH on the rate of reaction of e^-_{aq} with a 10^{-5} M aqueous solution of chloroquine diphosphate.

Table VI. — Effects of γ-irradiation on aqueous solutions of mepacrine

Irradiating Conditions	Solute Concentration (M)	G (–mepacrine)
Argon saturated	10^{-4}	1.7
Argon saturated	5×10^{-4}	1.8
Argon saturated	10^{-3}	2.0
Argon saturated	2×10^{-3}	2.2
Argon saturated	10^{-2}	2.7
Argon saturated	10^{-4}	0.5 (a)
Argon saturated	10^{-3}	0.6 (a)
Argon saturated	10^{-4}	1.5 (b)
Oxygen saturated	10^{-4}	1.2
N_2O saturated	10^{-4}	2.6
N_2O saturated	10^{-3}	3.1
N_2O saturated	2×10^{-3}	3.3
N_2O saturated	10^{-2}	3.8

a = solutions contain 2-methylpropan-2-ol (10^{-1} M)

b = pH = 2.0

The reaction of mepacrine and chloroquine with ·OH leads to the formation of hydroxycyclohexadienyl radicals. The rate of formation of which were used to evaluate a rate constant for the reaction of ·OH with mepacrine ($k_2 = 1.7 \times 10^{10}$ dm^3 mol^{-1} s^{-1}) and chloroquine ($k_2 = 6.2 \times 10^9$ dm^3 mol^{-1} s^{-1}). The G(-mepacrine) values obtained under a variety of conditions are shown in Table VI. Drug destruction is more marked in N_2O saturated solution than in argon saturated solution. Hydroxyl radicals therefore contribute to destruction of mepacrine. The G(-mepacrine) value of 1.5 in argon saturated solutions (10^{-4}M) at pH = 2 in which G (H) = 3.2 infers little contribution by H· atoms to the destruction of mepacrine. Destruction of chloroquine also results principally from OH· attack (Table VII).

Table VII. — Effects of γ-irradiation on aqueous solutions of chloroquine 10^{-3} M.

Irradiating Conditions	G (–chloroquine)
Argon saturated	2.2
Argon saturated	0.6 (a)
N_2O saturated	3.5

a = 2-methylpropan-2-ol (10^{-1} M)

A product of the attack of e^-_{aq} on aqueous mepacrine solutions (5×10^{-3}M) is 2-chloro-6-methoxy-9,10 dihydroacridine, G values for its formation are shown in Table VIII. The values are very low in the presence of an effective electron scavenger, e.g. N_2O or H^+ (10^{-2} M). This confirms that the product is formed by e^-_{aq} attack. The G + (2-chloro-6-methoxy-9,10 dihydroacridine) value is 0.6 in argon saturated solutions containing 2-methylpropan-2-ol, an effective ·OH scavenger. A much lower yield (G = 0.04) is obtained following irradiation in argon saturated solution in the absence of any OH· scavenger. Therefore, ·OH radicals prevent the formation of 2 chloro-6-methoxy-9,10-dihydroacridine.

Table VIII. — Yields of products formed during γ-irradiation of aqueous mepacrine solutions (5×10^{-3} M)

Irradiation Conditions	G(+A)	G(+B)
N_2O saturated	0.04	<0.02
Argon saturated	0.03	0.04
Argon saturated at pH = 2	0.03	< 0.01
Argon saturated plus a	0.02	0.6

G(+2-chloro-6-methoxyacridone) = G(+A) and G(+2-chloro-9, 10-dihydro-6-methoxyacridine) = G(+B)

a = 2-methylpropan-2-ol (10^{-1} M)

Irradiation of aqueous mepacrine solutions also led to the formation of 2 chloro-6-methoxyacridone in low yield.

4. *Effects of γ-irradiation on Triamcinolone acetonide*

The hydrated electron reacts extremely rapidly with corticosteroids (Table IX). The high e^-_{aq} reactivity is probably due to the carbonyl groups in positions 3 and 10 common to all the corticosteroids. The G(-triamcinolone acetonide) values summarised in Table X suggest that both e^-_{aq}, ·OH and H· contribute to solute degradation in aqueous solution.

Table IX. — Rates of reaction of hydrated electrons with various corticosteroids at pH 6.0.

Solute	$k_2 \times 10^{10}$ dm^3 mol^{-1} s^{-1}
Triamcinolone Acetonide	3.5
Triamcinolone	3.5
Fluocinolone Acetonide	3.0
Hydrocortisone	3.5
Hydrocortisone Acetate	3.2
β-Methasone Valerate	3.7

Table X. — The effects of γ-irradiation on triamcinolone acetonide (10^{-4} M)

Irradiation Conditions	G – (triamcinolone acetonide)
Argon saturated	3.8
Argon saturated at pH = 2.0	3.5
N_2O saturated	4.2
Argon saturated plus a	1.5

a = 2-methylpropan-2-ol (10^{-1} M)

5. *The effect of γ-irradiation on Heparin*

Heparin is a glycosaminoglycan found in the presence of mast cells and has a medical application because of its anticoagulent properties. In solution, in common with all polysaccharides, heparin is unreactive towards e^-_{aq} (7) but it reacts rapidly with ·OH, $k_2 = 2.2 \times 10^8$ dm^3 mol^{-1} s^{-1}. Thus, the addition of N_2O results in a marked increase in the destruction of the solute with the formation of reducing products and acids. Loss of dye binding capacity (8) and anticoagulent properties (9) accompany these chemical changes.

6. *The effects of γ-irradiation on Arvin activity*

Arvin, an enzyme present in the venom of the malayan pit viper *(Agkistrodon rhodostoma)* is an anticoagulent used in patients with venous thrombosis (10). γ-irradiation of arvin in dilute aqueous

solution (11) leads to a loss of enzymatic activity (Table XI). The dose required to reduce arvin activity to 37% of the original is only slightly greater in N_2O than in argon. This observation suggests that both e^-_{aq} and OH· radicals play a part in the radiation inactivation of arvin. The observation (Table XI) that decreased values for K_m are obtained following irradiation is unexpected. Generally irradiation increases K_m values (12). Such increases are taken to indicate a decrease in the affinity of the irradiated enzyme for its substrate, since $1/K_m \approx K_{affinity}$, where $K_{affinity}$ is the affinity constant. Thus, the decrease in K_m observed for arvin could be indicative of an increased affinity of the irradiated enzyme for its substrate. However, strictly, $K_{affinity} = 1/Ks$ where $Ks = k{-}1/k{+}1$ and is the true equilibrium dissociation constant of the ES complex. Thus, $1/K_m$ is not a true measure of the affinity of the enzyme, except under conditions where $k - 1 >> k + 2$. Since no safe conclusions can be drawn as to the relative magnitudes of $k - 1$ and $k + 2$, it would be unwise to attach too rigid a significance to the present observations.

Table XI. — Changes in K_m and V during irradiation of arvin in argon saturated solution.

Dose eV $ml^{-1} \times 10^{-17}$	Arvin activity (per cent original)	K_m M $\times 10^{-4}$	V μ mole min^{-1}
Unirradiated	100	5.2	61
9.2	75	3.2	48
22.4	50	2.3	37
32.2	37	1.5	21.4

Gel filtration of arvin irradiated to 50 per cent original activity in argon revealed two major peaks 1 and 2.

The kinetic behaviour of the enzyme from peak 1 showed it to be indistinguishable from the unirradiated sample. On the other hand, values of K_m and V obtained for the material in peak 2 (mol. wt ~ 15,000) were considerably less than those of the unirradiated control. Little esterase activity was detected in any of the remaining fractions.

Discussion

Most pharmaceuticals tested to date suffer degradation when irradiated in dilute aqueous solution. However, if the degradation pathways are known, considerable protection can often be afforded by the addition of suitable radical scavengers. From a kinetic competition view point, the protective effect of these scavengers will be greatest when the pharmaceutical is present in low concentration. Unfortunately, it is never possible to achieve complete protection because of side reactions and small losses are of course proportionately more serious in dilute solutions. Moreover, the radical scavengers commonly employed by radiation chemists would probably not be practicable or acceptable in a pharmaceutical formulation. An additional factor which must be taken into account when using radical scavengers is the possible protective effects of such compounds on the microbial population. This of course depends on whether the products of radical scavenging are themselves capable of killing microbes and on the extent to which indirect action contributes to lethality as opposed to direct effects.

For concentrated solutions (e.g. sodium sulphacetamide) percentage losses can be kept small.

However if the products of irradiation are toxic any degradation whatsoever would be unacceptable.

In the solid-state little degradation of most pharmaceuticals tested accompanies a dose of 2.5 Mrad. Accordingly, separate sterilisation of solid drug and water followed by asceptic mixing would be a more acceptable procedure.

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On the Theoretical and Practical Aspects of the Use of Radiation Sterilization and Radiation Pasteurization in the Pharmaceutical and Cosmmetic Industry

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You will probably agree with the statement that the recent years have brought a new impulse to the application of radiation sterilization in the cosmetic and pharmaceutical industry. The reasons for the upswing are the following: —

— more rigorous microbiological safety standards have been introduced,

— the legislation requirements are becoming equally rigorous; therefore, some of the conventional methods (for instance, heat and ethylene oxide sterilization), which enjoyed undeserved advantages in this respect, are also being revised, and occasionally replaced, and

— progress in the technology of radiation sterilization, including the development of large radiation sources, which call for economical industrial applications.

Let us examine the outlooks of the industrial application of radiation sterilization considering the present conditions and possibilities.

In spite of the world-wide research of more than two decades in this field, the scope of the application of radiation is rather limited in the pharmaceutical and cosmetic industry. According to the available information, not more than several dozen products are sterilized by radiation; mainly ointments, powders, talc and basic materials. Results of the research on radiation/pasteurization have not been employed in these fields as yet in spite of the increasing application of this very promising technique in the food industry and in the production of premixes. However, the statistics are considerably improved when the results of the treatment of packaging materials also are included; radiation sterilization of metal tubes, plastic flasks, droppers and rubber stoppers is conducted on an industrial scale in many countries.

In order to encourage the industrial application of radiation sterilization a revision of the principles of earlier research seems to be inevitable. Instead of the previous attitude considering the different details, we favor the *comprehensive* approach as a principle. The results of the microbiological model experiments, the physico-chemical investigations on stability and the technological developments must not be analysed separately, but they should be considered in connection with each others. Moreover, the examination should include all the ways and means by which the efficiency of the procedure can be increased.

One of the most important points is the *dose* of radiation. According to the accumulated research experience, the wide-scale application of radiation sterilization cannot be introduced unless the applied

dose is *reduced.* The unjustified adoption of the 2.5 Mrad dose used in the sterilization of medical equipment, and the suggestion of even larger doses up to 4.5 Mrad, based on an incorrect microbiological concept, did much to retard the growth of radiation sterilization.

We propose a revision of the applicability of radiation sterilization by considering three main aspects:

1. Re-examination of the biological monitoring of efficiency.
2. Critical survey of the other methods of sterilization (heat and ethylene oxide).
3. Elaboration and application of combined procedures.

In support of the claims made, we present our results and several reports from the literature.

Re-examination of the biological monitoring of efficiency.

It is an untenable practice that the determination of the effective dose for the sterilization and pasteurization by radiation of various products has generally been based on the radiation resistance of artifically propagated strains like *Bacillus pumilus* and *Streptococcus faecium.* Instead, the evaluation of the effectiveness of the procedure has to be based on the sensitivity of the "natural" flora of the actual preparation by determining the total microbial count and the species present. If this is feasible, the effective sterilizing or pasteurizing dose can accurately be determined. The likely effective dose can be estimated, in most cases, using the available documentations on the usual common microbial contaminants of various basic materials and products. Such investigations have been performed by Dony (1), Penso (2), Speiser (3), Wallhäusser (4) and Thonke (5). Our results are shown in Table I, which includes the data obtained in the course of the examination of a total of 330 lots of 18 types of ointment base materials.

Table I. — Microbiological contamination of ointment basic materials

Base Materials	No. of Batches Tested	Percent Contam. Samples	Total Bacterial Count/gram					*E. coli* Contam. Batches	Mold Contam. Batches
			0	10^{1-2}	10^{2-3}	10^{3-4}	10^{4}		
Emulsifiers:									
Tween 60	13	85	2	9	2	—	—	—	2
Span 60	12	33	10	2	—	—	—	—	2
Arlacel C	10	40	7	3	—	—	—	1	—
Tegin	13	30	11	2	—	—	—	—	2
Natural Products:									
Stearyl Alcohol	19	74	8	9	2	—	—	—	2
Cetyl Alcohol	17	41	11	3	3	—	—	—	1
Beeswax	32	57	19	6	5	—	2	—	3
Lanolin	35	66	13	17	5	—	—	—	—
Cocoa Butter	3	100	—	—	3	—	—	—	3
Synthetic Products:									
Isopropyl Myristate	10	50	6	3	—	1	—	—	1
Miglycol	18	28	15	3	—	—	—	—	2
Propylene Glycols	30	55	14	13	3	—	—	—	3
Polyethylene Glycols	3	66	1	1	1	—	—	—	2
Glycerin	30	67	11	16	3	—	—	—	3
Others:									
Petroleum Oil	30	66	12	11	7	—	—	—	4
Petroleum Jelly	27	50	13	12	1	1	—	1	1
Borax	11	82	2	3	6	—	—	—	6
Nipagin	17	23	13	4	—	—	—	—	—
Total:	330	491	168	117	41	2	2	2	35

Following the stated comprehensive approach, we remark here on two points:

a) a part of the contamination is secondary; the amount of which can be decreased considerably by proper storage and aseptic processing, and

b) in the course of the generally applied warm technology of ointment production (90° C in certain phases, and 70-80° C at mixing), a marked reduction of the microbial count can be expected.

Remarks on Table I.

As shown by the Table, about half of the lots were not contaminated (according to the procedures, this means less than 3 bacteria per gram), whereas the other lots contained less than 10^3 bacteria per gram.

Secondary contamination was indicated by the presence of *Escherichia coli,* which, however, is characterized by a low resistance to heat, as well as to radiation.

With regard to the species of microorganisms contaminating different materials, several reports already are available in the literature. We refer to the results of Abdou (7) from the Boehringer Company, who has published a complete list of microorganisms occurring in the pharmaceuticals investigated.

Table II. — Radiation resistance of the microorganisms in ointments and ointment basic materials

Microorganisms	Periodicity	D_{10} - Value		
		dry aerobic	wet aerobic	wet anaerobic
Bacillus pumilus E601	–	0.192	–	0.300
Bacillus subtilis and other aerobic spores	+	–	0.025–0.190.	–
Gram/+/micrococci	+ + +	–	0.016	0.044
Staphylococcus aureus	+	0.141	–	–
Escherichia coli	+	0.013	0.020	0.040
Klebsiella sp	+	–	0.016	–
Micrococcus tetragenus	+ +	–	0.010	–
Bacillus coagulans	+	–	0.119	–
Salmonella sp	(+)		0.013–0.158	0.039
Pseudomonas aeruginosa	(+)	0.013	–	–
Molds	+ +	–	0.061–0,180	–
Aspergillus niger	+	–	0.115	–

Table II demonstrates the results of our investigations on ointments and ointment base materials showing the most frequent microbial contaminants as well as their D_{10}-values according to the data of Burt (9), Ley (8), Bartha (10) and others.

Remarks on Table II.

The frequency of the occurrence is indicated by the number of crosses for each species. Radiation resistance of *Bacillus pumilus* is shown for the sake of comparison.

The circumspect survey of the antimicrobial efficiency of radiation requires the thorough analysis of the condition of the treatment, especially the nature of the suspending medium, which can modify markedly the effect of radiation. Our previous investigations in collaboration with Haraszti and Czéh (11), and with Bartha (12) have shown that the resistance of *Staphylococcusaureus, Escherichia coli, Streptococcus faecalis* and *Pseudomonas aeruginosa* strains is diminished when they are suspended in a fatty ointment, while the resistance of the spores of *Bacillus pumilus* is increased in the same fatty ointment because of the partial anoxia (Figure 1).

When the identification of the contaminating microorganisms is not feasible, then the sterilizing (or pasteurizing) dose should be determined by trial treatments. Generally, the survey performed according to the above guidelines result in a *decrease of the necessary dose to 0.5-1.5 Mrad.* This circumstance is very important because it facilitates the further applications of the procedure.

According to the comprehensive view, we should also consider:

— the aseptic or hygienic manufacturing of the products,

— the effect of several processing procedures during which the bacterial count is reduced, for instance the mixing of ointments at 70-80° C, and the drying of tablets and powders by heat, etc.

One has to reckon with such antimicrobial effects inherent in the technology and, at the same time, they present the first stages of the combined treatment which will be dealt with later.

Further, the presence of antiseptic and bactericidal compounds (antibiotics and preservatives) should also be considered.

On the other hand, one must not dismiss the possibility of the presence of extremely resistant microorganisms.

We have found highly resistant strains in ointments only rarely, and even these exhibit reduced viability in ointments. However, in biochemical products we have detected spores with D_{10}-values of 0.3 (13, 14) and similarly resistant microorganisms have been found by Lüssi-Schlatter and Speiser (15). Moreover, recently we have isolated and cultured an unidentified anaerobic spore, the D_{10} of which is 0.41 (16). The inactivation curve of this species is shown in Figure 2.

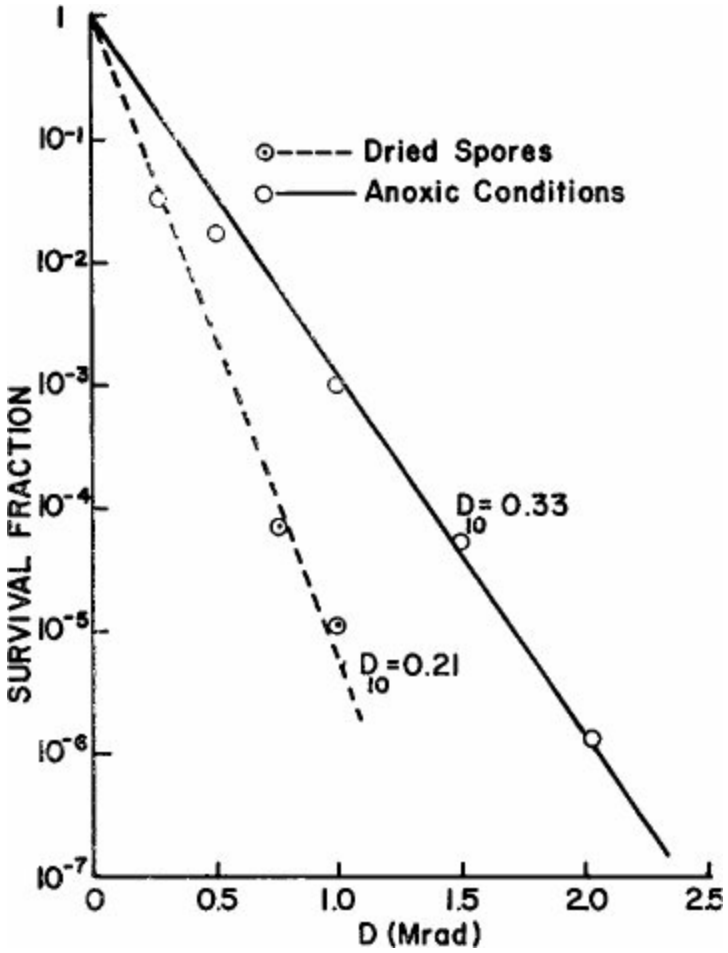

Figure *1.* The effect of anoxy on the resistance of *Bacillus pumilus* spores.

In the case of these products, a less pessimistic impression results when it is considered that the processing involved several chemical procedures (extraction with isopropyl alcohol or butanol, recrystallization, treatment with hydrogen peroxide, etc.) which cause the reduction of the bacterial count to a value which is only one to two orders of magnitude higher than the prescribed count (10^3-10^4 bacteria/gram of product). Thus, the setting of the pasteurizing dose between 0.5-1.0 Mrad seems to be sufficient.

If more efficient treatment is required (17) and the elevation of the radiation dose is undesirable because of the physico-chemical or biochemical instability of the material, then the application of combined procedures is recommended.

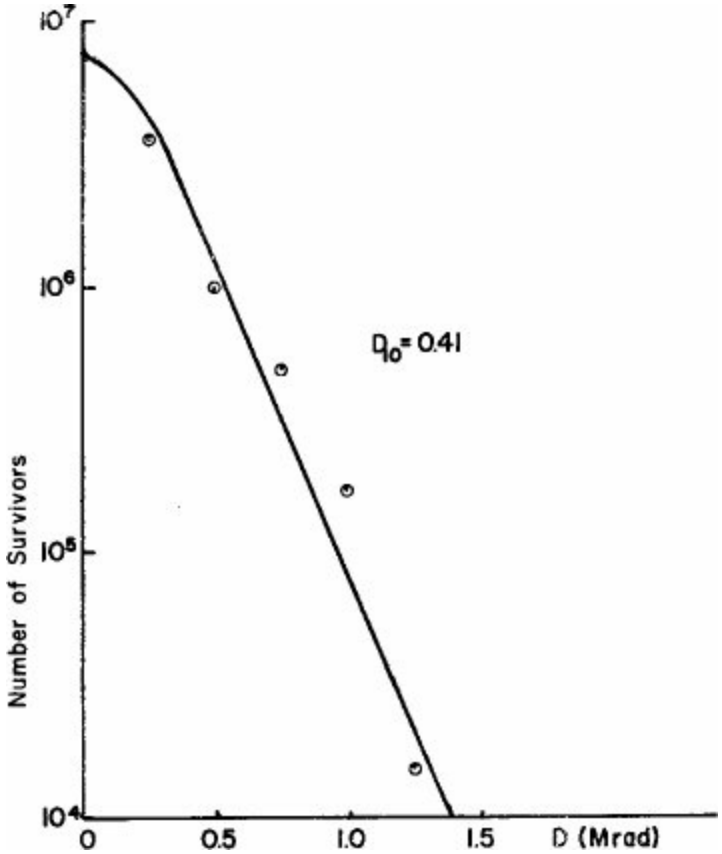

Figure *2.* Inactivation curve of highly resistant anaerobic spores of the strain isolated from biochemical products.

Evidently, the choice of the dose has to be specified in each case according to the requirements concerning the actual product; that is sterility or a prescribed bacterial count per gram. It is important to note that the standards are changing all over the world, generally towards rigorous limits (18–22). The present values are the following:

Premixes	1.5×10^4-10^5 bacteria per gram
oral and biochemical products	10^3-10^4
ordinary cosmetics	10^3-10^4
refined cosmetics	10^2-10^3
therapeutic ointments	10^2-10^3

Some maximalists are demanding the introduction of even more rigorous standards: 10^2 bacteria/gram for ophtalmic and wound ointments, and other externally used pharmaceuticals, and 10^3 bacteria/gram for oral products.

Further rules prescribing more effective disintegration techniques, the application of more specific culturing methods and other measures not detailed here are also increasing the requirements indirectly.

Combined procedures

General development resulted in the reconciliation of different techniques which appeared to be in conflict with each other earlier. The food industry also was pioneering in this field (23–25), but some

research experience also has been gathered in the pharmaceutical industry. The most promising combinations are those of irradiation plus heat and irradiation plus chemical treatment.

1. The combination of chemical treatment with irradiation has a relatively limited scope of application. From the technical and economic point of view simultaneous radiation and chemical treatment followed by chemical treatment seem to be impracticable.

   Chemical treatment followed by irradiation seems to be much more favorable. The latter combination has already been, applied unintentionally, because several processing procedures involved chemical treatment, which results in the reduction of the bacterial count of the raw material. Therefore a reduction of the dose of the final irradiation could be allowed.

   According to our opinion, this combination is advantageous and economical only in those cases where the chemical treatment can be integrated into the manufacturing process or it can be performed as the last step of the procedure. We refer to the investigations of Lüssi-Schlatter and Speiser (15), who examined each of the three combinations in connection with the sterilization of pancreatin.

2. Irradiation and heat can effectively be combined. Theoretically, three variations are possible:
   a) irradiation, then heat
   b) simultaneous treatment with radiation and heat
   c) heat, then irradiation

   The simultaneous treatment is not feasible because of technical reasons.

   The pertinent literature (mostly reports from the food industry) favors the first combination, that is irradiation followed by heat. This sequence of treatment results in a synergetic effect (24, 25) and in the opinion of several authors this is the only way to achieve a synergetic action. On the other hand, the sequence heat then irradiation is much more favorable from the technological point of view and this is why we have investigated this possibility.

   We obtained good results with heat followed by irradiation, and even some synergism could also be demonstrated. In connection with the examination of pancreatin samples, we observed that a pretreatment with dry heat at 70-90°C sensitized the samples with respect to radiation (6). The D_{10}-value of 0.33, determined prior to heat treatment, was reduced to 0.21 in response to dry heat (70 ± 2°C).

   No significant difference has been found in the effect of the treatment by varying the temperature between 70° and 90°C; therefore, we recommend the lower value (70°C), which entails other advantages. Further, it should be pointed out that the reduction of the bacterial count by heat was insignificant; therefore, the decrease of the D_{10}-value had to be attributed to a synergetic effect.

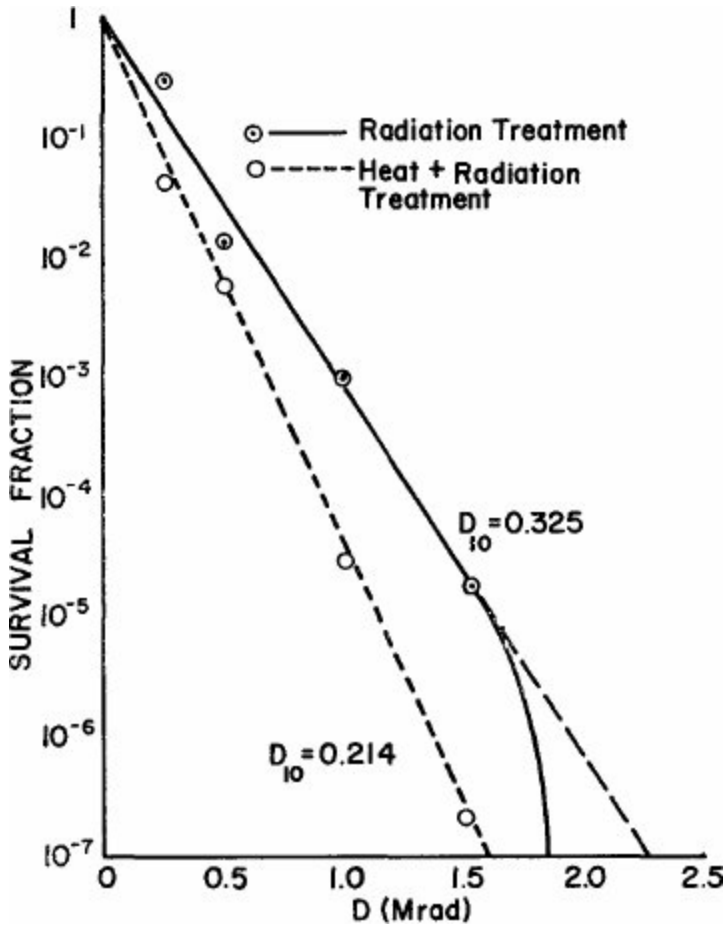

Figure *3.* Synergetic effect of dry heat and irradiation.

The impact of the dose reduction and the combined procedures on the physico-chemical stability of different products in comparison with the conventional methods of sterilization.

The separate treatment of the microbiological and physicochemical problem is due to purely didactic reasons and it does not mean that we have abandoned the comprehensive approach emphasized before.

The requirement of the high "general sterilizing dose" of 2.5 Mrad and occasionally even higher doses up to 4.5 Mrad presented an unsurmountable obstacle for the development of radiation sterilization, as mentioned in the introduction. Relatively few substances remain unchanged after receiving a dose as high as that. Among the rare examples, we mention the steroids (prednisolone, hydrocortisone acetate, fluocinolone acetonide, etc.) We present in Figure 4 the thin-layer chromatogram of prednisolone samples irradiated with 1 and 2.5 Mrad for the demonstration of the irradiation resistance of this substance.

However, several of the substances which were considered as radiation resistant (e.g. carbohydrates and glycols) have been re-classified as unstable compounds on the basis of recent examinations with refined methods.

The methods applied most frequently are the following: instrumental measurement of color and pH, and gas chromatography. In addition, electron spin resonance measurements and the analysis of contaminants are performed in some cases.

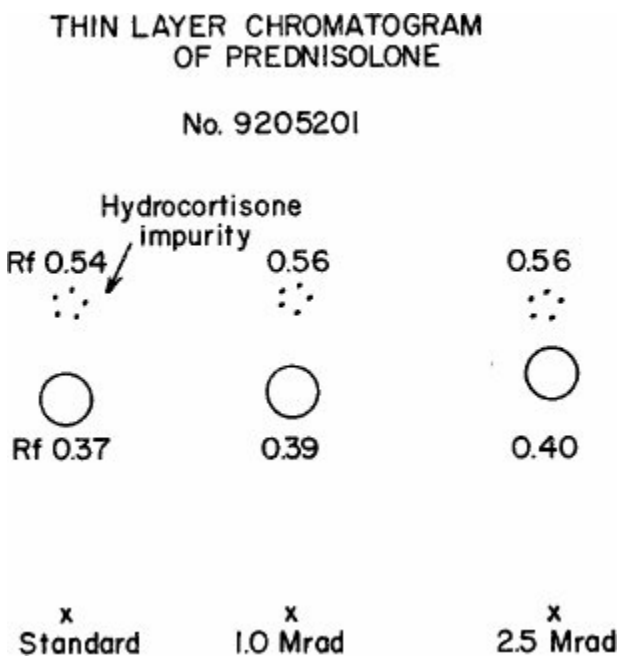

Figure *4.* Thin-layer chromatogram of prednisolone samples irradiated with 1 and 2.5 Mrad doses.

The decrease of the dose of radiation is most significant since thereby a number of substances are classified among the stable compounds. A series of possible applications became realistic in view of the reduction of the necessary dose to 0.25-1.5 Mrad. Among the ingredients of various ointments we mention the following examples (29, 30).

Discoloration of some substances is an undesired effect of radiation; however, the extent of the color change generally does not surpass the tolerable limits at doses of 1.0-2.5 Mrad. We examined the color changes of 50 basic materials after irradiation with doses between 0.5-1.5 Mrad, which resulted in critical changes in 18 cases. The results of the instrumental color measurement of Tween 60® (polyoxyethylene 20 monostearate) after irradiation and after treatment with heat are presented in Figure 5.

On the other hand, after irradiation even with doses of 2.5-5.0 Mrad the values were within the permissible limits represented by the ellipse showing the "two-fold discernibility" region. However, color brightness was changed more than the allowed ±2% by doses higher than 1.5 Mrad. Therefore, the example presented is typical, as far as irradiation with 2.5 Mrad was found inapplicable, whereas 1.5 Mrad proved to be suitable. We will comment later on the effect of heat treatment at 140°C.

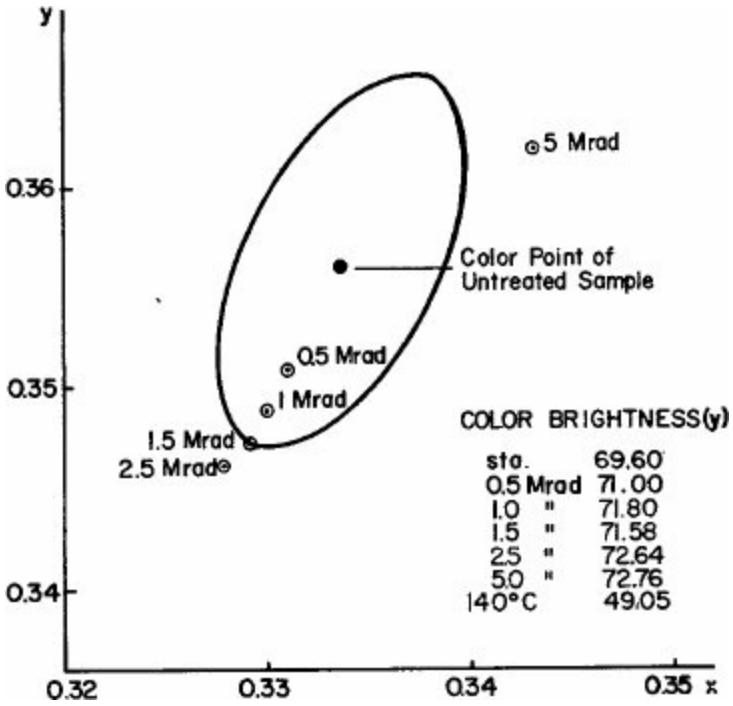

Figure 5. Discoloration of Tween 60 in response to radiation and heat.

The change of pH is also a frequent finding after irradiation. The reduction of the dose is beneficial also in this respect. The radiation treatment of eight products out of a total of 50 became possible by reducing the dose to 0.5-1.5 Mrad. The pH changes observed after the irradiation of 23 ointment ingredients with various doses are shown in Table III.

Another undesirable effect of radiation is the formation of gas and free radicals, which can be observed with the majority of organic materials (31). The upper limit of tolerance is a matter of dispute. Since both processes are proportional to the absorbed dose, the reduction of the amount of radiation decreases these effects also. For example, in the case of hydrocortisone acetate (32) the yield of radicals was as follows: at 2.5 Mrad, 0.3392 mole-percent; at 1.5 Mrad, 0.1459 mole-percent.

We have demonstrated that the reduction of the dose proved to be useful in every respect. Further, the application of the combined procedures has many advantages. Therefore, they are recommended especially in those cases where they fit into the technology of processing. In some instances the combinations provide the only suitable method because of the sensitivity of the substances.

Table III. — Change in pH of the ointment base materials after radiation or heat treatment

Materials:	Radiation treatment Dose (Mrad) /						Heat treatment (°C and hours)	
	0.0	0.5	1.0	1.5	2.5	5.0	80°C 1 hrs.	140°C 3 hrs.
Polysorbate 60®	4.5	4.35	4.5	4.55	4.7	5.0	4.4	4.4
Polysorbate 80®	6.65	6.6	6.8	6.8	6.85	6.9	6.5	6.9
Arlaton 983®	5.05	4.9	4.45	4.4	3.8	3.6	4.4	4.2
Tegin® *.....	7.95	—	7.8	7.85	7.75	7.8	8.3	8.35
Miglyol. 812®	5.0	—	5.0	5.05	5.0	4.55	4.5	4.4
Isopropyl myristate.....	5.8	—	5.8	5.8	5.85	5.1	5.35	4.0
Lanolin*.....	5.55	—	5.5	5.35	5.3	4.6	5.3	4.9
Beeswax, white.....	3.5	—	3.5	3.5	3.55	3.65	3.5	3.8
Stearic acid.....	6.2	4.6	4.45	4.3	4.2	4.0	6.2	6.25
Cetostearyl alcohol.....	7.0	6.9	6.8	6.85	6.8	6.7	6.8	6.85
Silicon oil.....	4.5	4.4	4.45	4.4	4.3	4.4	4.35	4.8
Petrolatum oil.....	6.85	6.8	6.7	—	6.75	6.6	6.6	6.4
Petrolatum jelly.....	6.35	6.3	6.25	6.3	6.0	6.05	5.9	6.3
Petrolatum wax.....	6.8	6.9	6.8	—	6.85	6.5	6.55	5.9
Polyethylene glycols.....	4.5	4.65	4.55	—	4.25	3.8	4.25	4.0
β-phenylethyl alcohol....	3.7	3.55	3.55	3.45	3.35	3.3	+	+
Propylene glycol.....	6.5	—	6.4	6.35	5.8	5.8	6.25	5.7
Glycerin.....	6.5	—	6.5	6.4	6.45	5.3	6.3	6.3
Nipagin M.®	4.7	—	4.5	—	4.55	4.7	5.0	+
Texapon K 12®	7.65	7.45	7.2	7.1	7.0	7.0	6.3	2.4
Carbopol 940® **.....	3.2	3.4	3.4	3.3	3.35	—	+	+

Ratio between the material and shake solution (1+9)
*(1 + 19), **(1 + 500)

According to the data in the relevant literature, as well as our results, the possibilities of the radiation treatment of various materials and products can be summarized as follows:

a) Semi-solid and solid materials, and the saturated oils are generally resistant to radiation, although the magnitude of the tolerated doses awaits determination.

b) Tablets and powders (especially talc) are likely objects for radiation sterilization.

c) Polar solvents and solutions prepared with polar solvents (injections, etc.) do not seem suitable for this procedure.

d) Ointments can partly be sterilized by radiation directly, for the introduction of the combined procedures appears to widen the scale of the products which can be treated by radiation.

e) Biochemicals and premixes constitute a special group of solids the radiation treatment of which also becomes feasible by the application of the combined procedures.

The preservation of the desired effect of the product, that is the stability of the active ingredient, is a precondition of the irradiation in each case. The interactions of the different agents have to be examined in the case of combined preparations. Finally, we conclude that the prospects for radiation pasteurization are much better due to the lower dose requirements.

Re-examination of the efficiency of earlier methods

According to the experience of several experts, the legal requirements for radiation sterilization procedures is generally more difficult and circumstantial than for other methods, since much more detailed documentation is required.

Ethylene oxide gas sterilization was readily accepted. However, the problems which arose later are familiar to many experts present in this meeting. Therefore, they will not be discussed here.

Let me comment on the data presented before in connection with the effects of conventional heat sterilization (33).

a) We have shown in Figure 5 that the discoloration of Tween-60 is significantly greater after the treatment at 100-140° C, which is the usual range of temperature, than after the irradiation with even 5 Mrad.

b) The third column of Table III has demonstrated that the generally recommended heating of 140° C for 3 hours, as well as the very mild treatment at 80° C for 1 hour, caused a significant change in the pH of the majority of the materials, the extent of which exceeded the magnitude of the effects of irradiation with 2.5-5.0 Mrad.

c) Another example is presented in Figure 6 showing the changes of the absorbtion spectra of polyoxyethylene 1500 between 250-400 nm. Irradiation with 2.5 Mrad had no effect, whereas heating at 140° C changed the spectrum considerably.

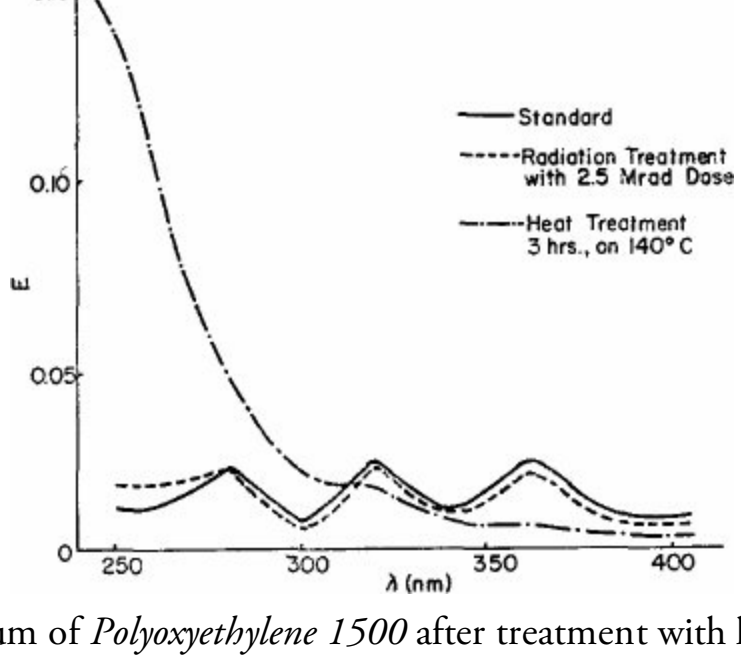

Figure *6.* Ultraviolet spectrum of *Polyoxyethylene 1500* after treatment with heat and irradiation.

The aim of the presentation of the selected data has not been to criticize heat sterilization, but rather to demonstrate the relative virtues and suitability of radiation sterilization as compared with the earlier methods.

Further possibilities

We wish to mention the possibility of the increased application of radiation by the use of radiation resistant materials (34). Detailed lists of materials resistant to radiation have been published. On the basis of this the manufacturers are able to select properly resistant substances for the composition of

ointments or powders, etc.

Discussion

The acceptance of radiation sterilization and radiation pasteurization in the pharmaceutical and cosmetic industry can be promoted greatly by the reduction of the 2.5-4.5 Mrad doses which have been prescribed earlier.

This can be realized by:

a) Pre-examination of the biological monitoring of efficiency with due regard to the natural flora.

b) Introduction of combined procedures, especially the most promising combination of heat and irradiation.

Further factors of the development are:

a) The comprehensive approach.

b) Development of more suitable techniques of control.

c) Adequate examinations allowing comparisons with earlier methods particularly with respect to setting the limits of tolerance.

d) Application of radiation resistant materials.

We may conclude on the basis of the results presented here, as well as in the literature, that the application of radiation in this field is passing from the experimental stage, and the introduction of the new techniques into the industry can be expected soon.

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Radiation Sterilization of Some Sulphur Containing Compounds

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Abstract: *The radiation stability of DL-methionine and of cysteamine was investigated. Radiation decomposition of methionine during its sterilization in aqueous solution mainly is characterized by oxidation, decarboxylation, deamination processes and the release of volatile products. The ESR spectra of irradiated DL-methionine indicated a radical formation mainly by hydrogen atom subtraction (at low doses of radiation) and by rupture of CH_3S-bonds and formation of volatile products like CH_3SH and H_2S (at higher doses of radiation). The behaviour of cysteamine during radiation sterilization in aqueous solution and in solid state is similar to that of methionine. It was concluded that radiation sterilization of sulphur containing compounds in solid state (crystalline) leads to minimum damage and decomposition compared to radiation sterilization of those compounds in aqueous solution. A scheme of mechanisms of the mean reactions was proposed.*

Introduction

In the application of ionizing radiation to the sterilization of pharmaceutical and biological products, the study of their radiolytic behaviour is or major importance. In a series of our previous papers we studied the effect of gamma radiation on vitamin B_6 (pyridoxine) and its derivative (pyridoxal) (1–3), folic acid (3, 4), thyroxine and its derivatives (triiodothyronine and tyrosine), Hippuran (o-iodohippuric acid sodium salt) (5) and tetracyclines (6). As a continuation of the above mentioned studies our attention was concentrated on the study of radiation stability of some sulphur containing compounds: methionine (γ-methylthio-α-aminobutyric acid) and cysteamine (2-mercapto-ethylamine). The irradiation of these compounds was performed at a Cobalt-60 (Co-60) source of 10,000 Ci, in aqueous solution and also in the solid state. After irradiation in air, at ambient temperature and at different doses, the samples were analysed for determination of the decomposition products using the following techniques: paper and thin-layer chromatography (TLC), spectrophotometry, gas chromatography and electron spin resonance. The identification of some principal decomposition products of gamma irradiated sulphur containing compounds, like methioninesulfoxide, methioninesulfone, homocysteic and α-amino-n-butyric acids, cystamine etc., was performed by specific chemical reactions with ninhydrin and also by chromatographic separation using, in the first step, a one-dimensional ascending TLC (solvent system: ethanol-water, 7:3) and, in the second step after the elution of the obtained spots, a two-dimensional ascending TLC [with solvent

systems: (I) ethanol-water, 7:3 and (II) isopropyl alcohol-butanol-water, 1:3:1]. For the determination of (carboxylated) decomposition products we used the specific reaction with 2,4-dinitrophenylhydrazine and o-phenylenediamine-trichloracetic acid and then measured it spectrophotometrically. Using the colorimetric method it was possible to determine the volatile decomposition products of the irradiated sulphur compounds by measuring the methylene blue complex formed on addition of N,N-dimethyl-p-phenylene-diamine to the -SH, trapped in cadmium hydroxide. The mercaptans were determined by a similar method after being trapped in mercury acetate (7, 8).

Radiation sterilization of DL-methionine

The radiation induced chemical reactions during the irradiation at 2.5 Mrad of methionine in aqueous solution at pH 5-7 are exceptionally complex and several studies were published (9–14). These reactions yielded a large number of decomposition products of which the majority have been identified, as we mentioned above, as follows: methionine sulfoxide, methionine sulfone, homocysteic and α-amino-n-butyric acids, decarboxylated and deaminated compounds and mercaptans.

Table I. — Thin layer chromatography (silica gel plates and Solvent I). Spot activity distribution of L-selenomethionine Se-75 irradiated in air.

Dose Mrad	Inorganic Se-75 and highly oxidized selenomethionine		Selenomethionine oxide		Selenomethionine undestroyed		Other decomposition products			
	R_f	%	R_f	%	R_f	%	R_f	%	R_f	%
0.0	0.03	2.5	0.15	8.0	0.60	89	0.97	1.0	—	—
0.1	0.02	8.0	0.15	76.0	0.52	18	0.88	2.0	—	—
0.2	0.02	11.0	0.18	55.0	0.52	27	0.82	2.0	—	—
0.3	0.02	21.0	0.15	44.0	0.52	28	0.84	2.5	—	—
0.5	0.02	12.0	0.12	23.0	0.57	35	0.96	12.5	—	—
1.0	0.02	15.0	0.12	11.0	0.57	30	0.97	27.0	—	—
1.5	0.02	15.0	—	—	0.57	17	0.88	31.0	0.65	10
2.0	0.02	24.0	—	—	0.57	15	0.88	17.0	0.65	9

For the study of the radiolytic decomposition of DL-methionine we used the radioactive labelled methionine with Sulphur-35 (S-35) and Selenuim-75 (Se-75).

The experimental results obtained on the chromatographic behaviour of gamma irradiated DL-methionine-S-35 and L-seleno-methionine-Se-75 solutions as a function of radiation dose using the thin-layer chromatographic method solvent (II) are shown in Figures 1–3 and Table I. A large decomposition effect was observed. Radiation damage to DL-methionine-S-35 or DL-methionine-Se-75 in solution leads not only to an oxidation process, but also to the rupture of the CH_3S- or CH_3Se- and -NH_2 groups accompanied by the formation of volatile decomposition products like CH_3SH or CH_3SeH and probably H_2S or H_2Se (Figure 5). The loss in activity of L-selenomethionine-Se-75 irradiated solution in air as a function of the radiation dose is due to the formation of volatile decomposition products as shown in Table II. The chromatographic analysis of irradiated L-selenomethionine-Se-75 solutions and the specific reactions of amino groups with ninhydrin, followed by colorimetric measurements, indicated an increase of the deamination process proportionally to the

absorbed dose (G_{NH_2} = 0.95). After the interpretation of the experimental data, it seems that the radiation decomposition of DL-methionine-S-35 and of L-seleno-methionine-Se-75 is a function of concentration and the absorbed dose (G(-R′SR) = 25) can be regarded as a first order reaction:

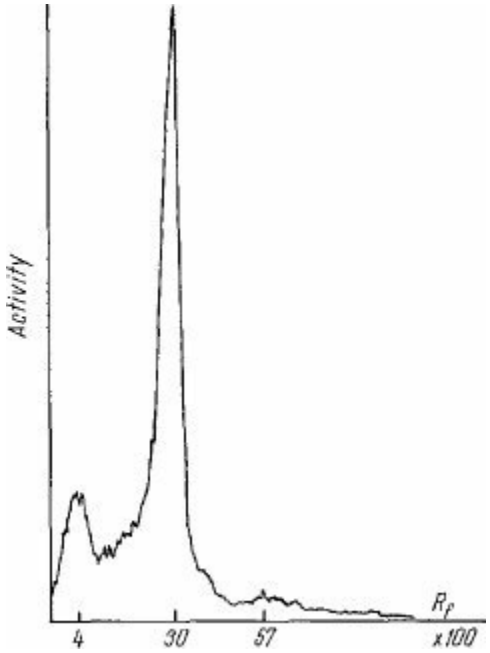

Figure *1.* Thin-layer chromatogram (solvent II) of irradiated DL-methionine-S-35 in aqueous solution at a radiation dose of 1 Mrad.

Table II. — Loss in activity of selenomethionine-Se-75 irradiated in air as function of the radiation dose due to the formation of volatile decomposition products.

Dose. Mrad	Activity after irradiation, counts/25 min	Remaining activity, % ± 10%
0.0	12.5×10^4	100
0.5	11.4×10^4	91
1.0	12.0×10^4	81
1.5	7.0×10^4	56
2.0	6.5×10^4	52
3.0	5.1×10^4	40
5.0	2.8×10^4	22
10.0	1.9×10^4	15

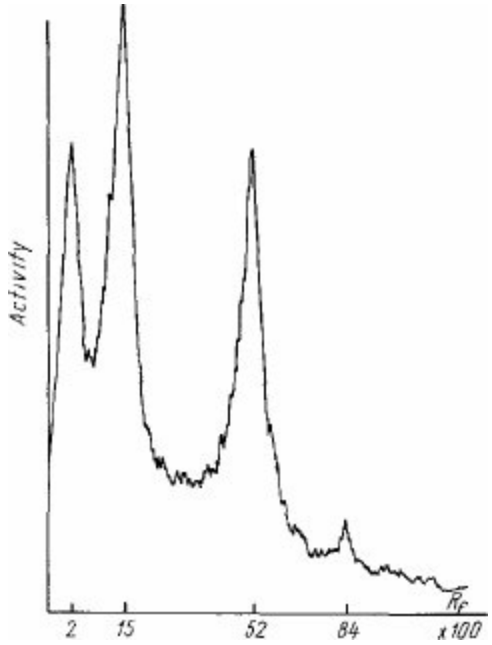

Figure *2.* Thin-layer chromatogram (solvent II) of irradiated DL-methionine-S-35 in aqueous solution at radiation doses of 1.5 Mrad.

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

where S_o and S are the initial and final concentration of methionine; k = rate constant, D = absorbed dose.

The mean reactions of the radiolytic decomposition of DL-methionine aqueous solutions are:

1. Cleavage of S-C bonds:

$$R_1SR_2 + e^-_{aq} \rightarrow R_1S^- + \dot{R}_2$$

$$R_1 = CH_3S- ;$$

$$R_2 = -CH_2-CH_2-CH(NH_2)-COOH$$

2. Formation of volatile products and α-amino-n-butyric acid:

$$R_1S^- + \dot{R}_2 + 2H \rightarrow R_1SH + HR_2$$

3. Oxidation of methionine and formation of methionine sulphone:

$$R_1S\,R_2 + O_2 \text{ (aerated solutions)} \rightarrow R_1\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}R_2$$

$$R_1S^-R_2 + 2H_2O_2 \rightarrow R_1\overset{\overset{O}{\|}}{\underset{\underset{O}{\|}}{S}}R_2 + 2H_2O$$

4. Decarboxylation of methionine:

$$R_1SR_2 + e^-_{aq} \rightarrow R_1SR_3\dot{C}O + OH^-$$
$$R_3 = -CH_2-CH_2-CH(NH_2)-$$

5. Deamination of methionine :

$$R_1SR_2 + e^-_{aq} \rightarrow R_1S\dot{R}_4 + NH_4OH$$
$$R_4 = -CH_2-CH_2-CH_2-COOH$$

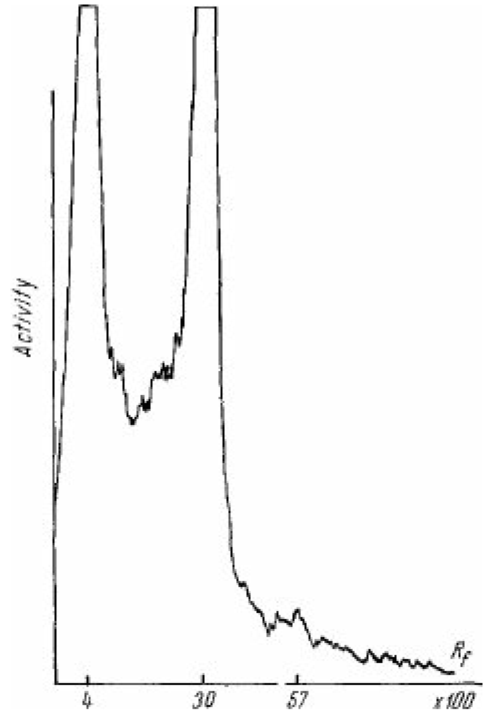

Figure *3.* Thin-layer chromatogram (solvent II) of irradiated DL-methionine-S-35 in aqueous solution at a radiation dose of 2 Mrad.

Concerning the radiation damage to methionine we studied also the formation of free radicals in gamma irradiated DL-methionine in the solid state in air and at room temperature in the range of doses up to 11.5 Mrad. It was possible to detect free radicals in this case as shown in Figure 4, only at an absorbed dose higher than 0.25 Mrad. The comparison of the ESR-spectra with those of other sulphur containing amino acids and the determination of the g value indicated a similar behaviour to that of cysteine hydrochloride (15) by formation of radicals:

Radical formed:

1. $\dot{C}H_2-S-CH_2-CH_2-CH(NH_3^+)COO^-$
2. $\dot{S}-CH_2-CH_2-CH(NH_3^+)\ COO^-$

Mean process:

1. subtraction of H atoms at lower doses;
2. by cleavage of CH_3-S bond at doses higher than 2.5 Mrad.

This is confirmed by the spectral parameters obtained for the central peak of ESR-spectra (Figure

4), g = 2.026 and the width ΔH = 60 gauss which demonstrates that the localization of unpaired electrons is mainly on the sulphur atoms of the irradiated methionine at a dose higher than 2.5 Mrad.

Comparing the radiation sterilization of DL-methionine in solution with the one in the solid state, two quite different situations were observed. Whereas, the radiation sterilization of DL-methionine in solution at 2.5 Mrad leads to a great decomposition by formation of the methionine sulphoxide, methionine sulphone, homocysteic and α-amino-n-butyric acids, decarboxylated and deaminated acids and mercaptans, the radiation sterilization in the solid state is not accompanied by an essential decomposition. In this case the oxidation, decarboxylation, deamination processes and the formation of volatile decomposition products are minimized. The only important radiation damage to the irradiated DL-methionine in the solid state is the hydrogen atom subtraction which remain as interstitial radicals. Dissolution in aqueous media of the irradiated DL-methionine leads to a proton transfer from the medium to the damaged molecule of the methionine which, thus, is repaired. This positive effect we observed first by radiation sterilization of the pyridoxine and its derivatives in the solid state (1–3), followed by folic acid (3, 4) and tetracyclines (6).

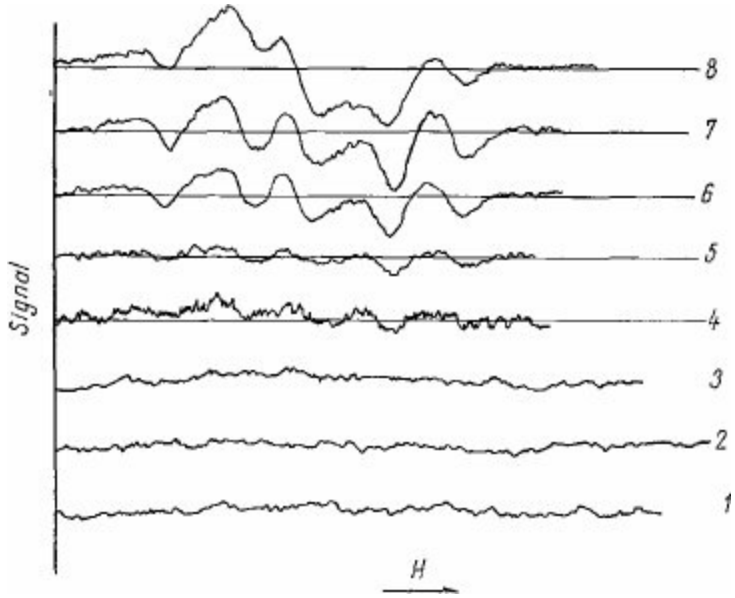

Figure *4.* ESR-spectra of Co-60 gamma-irradiated D-L-methionine measured 20 days after irradiation.

Curves: 1 — non-irradiated DL-methionine;

2 — DL-methionine irradiated at 0.1 Mrad;

3 — DL-methionine irradiated at 0.25 Mrad;

4 — DL-methionine irradiated at 0.5 Mrad;
(free radical concentration determined ≡ 0.25×10^{16} spins/g);

5 — DL-methionine irradiated at 1 Mrad
(free radical concentration determined ≡ 0.40×10^{16} spins/g);

6 — DL-methionine irradiated at 2.5 Mrad
(free radical concentration determined ≡ 1.3×10^{16} spins/g);

7 — DL-methionine irradiated at 4 Mrad
(free radical concentration determined ≡ 1.8×10^{16} spins/g);

8 — DL-methionine irradiated at 11.5 Mrad
(free radical concentration determined ≡ 2.8×10^{16} spins/g).

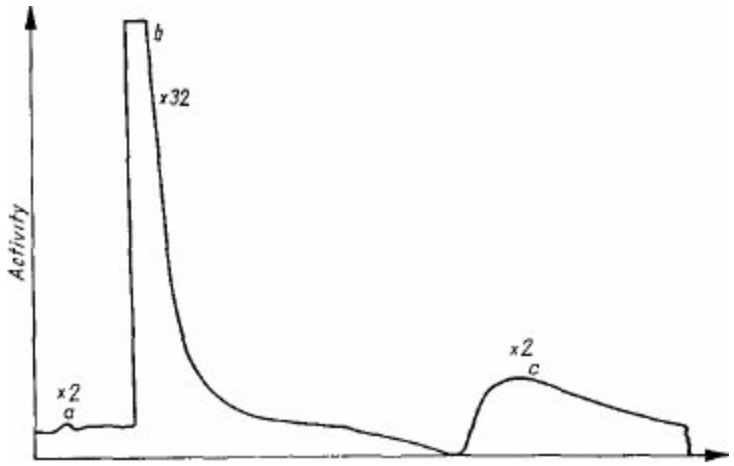

Figure 5. Radioactivity distribution on the radio gas chromatogram of the irradiated (2 Mrad) aqueous solution of L-selenomethionine-Se-75. Peaks: a) SeH_2; b) CH_3Se; c) $(CH_3)_2$ Se.

Radiation sterilization of cysteamine

The radiation sterilization of cysteamine at a dose of 2.5 Mrad in aqueous solution at pH = 5 - 8 is characterized mainly by the formation of cystamine and followed by formation of oxidation products. At pH > 8 the radiation sterilization of cysteamine in aqueous solutions leads to the formation of other decomposition products like ammonium hydroxide, molecular hydrogen and hydrogen peroxide (16).

During the radiation sterilization of cysteamine (including also methionine) in aqueous solutions, it is necessary to take into account the reaction of the hydrated electron (e^-_{aq}) with the related compounds. The reactivity of the e^-_{aq} to the cysteamine (and methionine) in the solution of pH > 7 decreases toward the reactivity of the same compounds in neutral or acid solutions. This fact can be ascribed firstly to the competition of the reaction of e^-_{aq} with OH radicals and secondly to the dissociation of the sulphydryl groups followed by formation of the negatively charged residues which are less reactive with the e^-_{aq}. This is confirmed by the reaction of e^-_{aq} with sulphur containing compounds at neutral or weak acid pH (17):

$$e^-_{aq} + RSH \rightarrow \dot{R} + SH^-$$

The main reactions in aqueous solution at pH 6-7 by radiation sterilization of cysteamine are:

1. Water radiolysis products: e^-_{aq}, H, $\dot{H}_2$, $O\dot{H}$, H_2O_2, H_3O^+
2. $RSH + OH^- \rightarrow R\dot{S} + H_2O$
3. $R\dot{S} + R\dot{S} \rightarrow$ RSSR (cystamine)
4. $RSH + H \rightarrow \dot{R} + SH_2$
5. $\dot{R} + RSH \rightarrow R\dot{S} + RH$
6. $R\dot{S} + O_2$ (aerated solutions) $\rightarrow R\dot{S}O_2$
7. $R\dot{S}O_2 + RSH \rightarrow RSO_2H + R\dot{S}$

8. $RSO_2H + 3RSH \rightarrow 2RSSR + 2H_2O$

The radiolysis yield of cysteamine in aerated aqueous solution at pH = 5 – 7 at ambient temperature is G(-RSH) = 28.5 and G(-RSH) = 6.5 in nitrogen saturated solutions.

Radiation sterilization of cysteamine in the solid state at radiation doses in the range of 2 - 3 Mrad indicated the formation mainly of the radicals:

$\dot{S}{-}CH_2{-}CH_2{-}NH_2$ and probably $\dot{S}{-}CH_2{-}CH_2{-}\dot{N}H$
by reactions of hydrogen atom subtraction similar to the DL-methionine radiation sterilization process.

Conclusions

1. Radiation sterilization of sulphur containing compounds in aqueous solutions like DL-methionine, cysteamine and other similar compounds (cysteine, cystine, etc.) leads to a large radiolytic decomposition attributed mainly to the interaction of water radiolysis products with the related compounds.

2. In the solid state irradiation of DL-methionine and of cysteamine at the sterilization doses in the range of 2.5-3.0 Mrad radiolytic decomposition of these compounds essentially does not occur. The only decomposition observed is the hydrogen atom subtraction as the main process at lower doses, and the cleavage of CH_3-S bonds and H atom subtraction at doses higher than 2.5 Mrad.

Dissolution of the irradiated compounds in aqueous solutions leads to a proton transfer from the solvent to the damaged molecules which repair themselves.

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Panel
Questions and Answers

To: A. DZIEDZIC-GOCŁAWSKA — Poland, by: G. H. LORD — U.S.A.

Q. Why do you state that glutaraldehyde tanned xenografts cannot be placed on metallic stents?

A. I cited the opinion of Dr. Wain of the National Heart Hospital in London who stated that, in his opinion, it has not yet been possible to find a satisfactory method for fixing and inserting heart valves after glutaraldehyde fixations. I would be very grateful if you could give me some references concerning the experimental and clinical results with the use of glutaraldehyde-treated xenograft valves.

Comment by

R. L. KRONENTHAL — U.S.A.

Glutaraldehyde-fixed porcine heart valves, fixed on polypropylene supports, have been used clinically and are available commercially in the United States. More than seven years of human experience has shown them to be the graft of choice in the mitral position.

To: G. HANGAY — Hungary, by: Anonymous Questioner

Q. Will you explain the purpose of analyzing the prednisolone?

A. We examined the prednisolone as a basic raw material of ointments and injections, because it does not tolerate the effect of heat sterilization; and as the active ingredient of some ointments, which we would like to radiation sterilize. The methods applied were: pH measurement, IR spectroscopy, UV visible spectroscopy, ESR spectroscopy, thin layer chromatography, and color measurement.

To: G. HANGAY — Hungary, by: T. ALPER — England

Q. If I understand you correctly, you showed a result of pretreatment by heat causing sensitization of microorganisms to radiation. Would you regard this as a general phenomenon? If so, what would the mechanism be?

A. The result presented is not a general phenomenon. We are going to examine this question. We observed the sensitization effect of the pre-treatment in many cases, chiefly with the biochemical products.

We did not concern ourselves with the theory of the phenomenon, but the mechanism would be the

following. The healthy anaerobic spores have a relatively high heat resistance. Therefore, the treatment at 70-90°C does not reduce their counts, but effects changes in the spores, which enhances the effect of the second process, i.e. the radiation treatment.

To: G. HANGAY — Hungary, by: G. E. HEINZE — U.S.A.

Q. You said you had isolated a microorganism which had a D_{10} value of 0.41 Mrad. What species was it? What medium did you use to isolate it?

A. It was an anaerobic spore. We did not identify it further. We found it chiefly in pancreatin samples.

To: H. B. RAINEY — New Zealand, by: G. H. LORD — U.S.A.

Q. In which parasitic diseases have vaccines been found to be effective? Do they prevent larval forms from developing into adults? If not, how do they work?

A. Parasite vaccines are commercially available for cattle and sheep lung worms, and for canine hookworms. The former had a shelf life of 10-14 days, the latter for 6 months or more. I believe they exert their effect by preventing larval forms developing into adults. An excellent review of the topic appears in Nuclear Techniques in Helminthology Research, IAEA publication (1975), particularly an article by Mulligan of Glasgow.

To: D. M. POWER — U.K., by: A. CHARLESBY — England

Q. The use of radiation in the solid state results in less degradation of drugs than in solution. Does this reflect an effect of the presence or absence of water?

A. Yes, one of the degradations discussed occurred in dilute solution as the result of the indirect effects of irradiation. Under these conditions, the solute is present at relatively low concentration and is attacked by the primary products of water radiolysis, namely e^-_{aq}, ·OH and H·. In the solid state pharmaceuticals are much more resistant. Thus in aqueous solution of sulphonamide G values of 3.5-5.1 are obtained, whereas in the solid state, values of 0.15-0.6 are more usual. (G. O. Phillips, D. M. Power and M.C.G. Sewart in Radiation Research 53: 204-215, 1973).

To: N. DIDING — Sweden, by: H. ROUSHDY — Egypt

Q. Attention has been focused on microbiological and chemical investigations of radiation sterilized drugs. Our preliminary results indicate that changes in the pharmacological response of irradiated drugs should be investigated as well.
Have you carried out some studies on the radiation induced changes in the pharmacodynamic characteristics of radiation sterilized pilocarpine?

A. We have not performed pharmacological studies. I agree that it is important to study pharmacological and toxicological changes of irradiated drugs if there are indications in this respect. The aim of our study, however, was primarily to find out to what degree the pure drug substances in dry form were influenced after irradiation with respect to official pharmacopeial requirements. In the case of pilocarpine hydrochloride we found no difference between the untreated and irradiated

samples.

To: N. DIDING — Sweden, by: I. GALATZEANU — Rumania

Q. Concerning the sterilization by irradiation of tetracyclines, did you determine the chemical structure modifications and the biological activity depending on the physical state (solution or solid state) of the product? What is the optimal dose of irradiation in such cases?

A. The structure modifications that we have tested for are anhydrotetracycline and epianhydrotetracycline. One of the impurity spots from the TLC was thus identified as anhydrotetracycline.

The light absorption test according to the European Pharmacopeia is also a test for anhydrotetracycline and epianhydrotetracycline and the amounts found were well within permissible limits.

The only biological tests that we have done and which are officially prescribed in the pharmacopeia are the quantitative microbiological assay and a test for nonspecific toxicity. All the irradiated samples passed these tests.

Comment by

I. GALATZEANU — Rumania

I would like to mention that ten years ago, we studied the radiation sterilization of the oxytetracycline and chlorotetracycline. We studied the irradiation in both aqueous solutions and also in the solid state. We found that in aqueous solution decomposition and loss of the antibiotic activity of the tetracyclines was great, even at a low dose of 0.5 Mrad. However, in the solid state, irradiation up to 8 Mrad resulted in a very small loss of antibiotic activity. This is very important for radiation sterilization of such compounds.

To: J. KOMENDER — Poland, by: N. TRIANTAFYLLOU — Greece

Q. What kind of criteria have been used for the classification of the results after bone allotransplantation?

A. There are several categories of criteria which we have used for clinical result classification:

1. Information from patients	— subjective troubles
	— ability for physical effort
2. Medical examination	— general state
	— local information
3. X-ray examination	— Resorption and substitution of graft
	— Regeneration of bone

4. Final conclusion of clinician who performed the operation.

To: J. KOMENDER — Poland, by: W. A. STAUB — U.S.A.

Q. Do you have any comparative data on the success rate of tissue transplants utilizing non-irradiated material?

A. I have no personnel experience with preserved, non-irradiated bone, but there are the big centers of tissue preservation (Berlin, Hradec Kralove, Kharkov, Moscow) in which chemical sterilization is used. It may be concluded from analyses done in these centers that the success rate is the same as that for irradiated material.

Often, in the clinicians opinions, the resorption rate of non-sterilized bone grafts is higher than with irradiated or chemically sterilized bone. However, this was never proved by analysis of large groups using the same criteria.

To: J. KOMENDER — Poland, by: H. B. RAINEY — New Zealand

Q. In many countries bone grafts are taken directly from the recipient. Can you give the preference of surgeons who can choose between bone bank bone and bone directly from the recipient?

A. It is obvious that the best material for plastic operations is autogenous bone. But the risk of operation is higher when bone must be excised from one place to be planted in another. In many cases, however, mainly in children, excision of large fragments of bone might be dangerous for future development. So for children and for patients in which the operation could not be performed without additional trauma, the preserved bone is recommended.

To: T. P. KLIMOVA — U.S.S.R., by: Z. P. ZAGÓRSKI — Poland

Q. What is the reason for the radiation sterilization of dry novocaine and similar compounds which can and should be produced sufficiently clean?

A. The reason for doing it with that substance and other comparable substances was to study the effects of irradiation on dry materials versus solutions of these materials. The object was to establish methodology for the sterilization of other drugs.

To: D. M. POWER — U.K., by: T. TALLENTIRE — England

Q. Is there any synergistic microbicidal effect of the pharmaceuticals and radiation? Do the decomposition products of radiation possess therapeutic activity?

A. We have irradiated spores of a radiation sensitive organism, *E. coli B* and a radiation resistant organism, *B. pumilus* E 601, in the presence and absence of sulphacetamide (10% solution). With *E. coli B,* under anoxic conditions, survival curves were identical in the presence and absence of drug. However, the drug itself was lethal to the bacterium, and when this effect was taken into account it was evident that the presence of sulphacetamide protected the organism from the lethal effect of radiation. Spores of *B. pumilus* E601 were unaffected by sulphacetamide alone. However, following irradiation in argon saturated solution, D_{10}-values of 200 krad were obtained in the absence and presence of sulphacetamide (10% solution), respectively. The protection of both organisms from the lethal effects of ionizing radiation can be attributed to the scavenging of radicals by the sulphacetamide.

Sometimes the products of irradiation are themselves pharmaceuticals. Thus irradiation of sulphasuxidine in argon saturated solutions yields sulphathiazole (G = 0.7). Here, obviously, the products of radiation do possess therapeutic activity.

To: J. KOMENDER — Poland, by: G. H. LORD — U.S.A.

Q. Have you any experience with irradiated bone grafts in nonunions (pseudoarthroses)?

A. In the material presented in our analysis there were no cases of pseudoarthrosis. As I understand it, pseudoarthrosis is not an appropriate condition to be treated by bone transplantation. Most often surgeons use strong metal plates for stabilization of pseudoarthroses.

To: H. B. RAINEY — New Zealand, by: W. S. MILLER — U.S.A.

Q. The question of viral survival on medical devices sterilized by radiation has come up on several occasions during this meeting. Would you comment on the probability of viruses being recovered from devices exposed to doses discussed here, i.e. perhaps 1 to 3 megarads?

A. In the laboratory we have been interested in survival of viruses at low temperatures, important in determining shelf life and the life of reference samples.

In the temperature range of 0-10°C Grieff suggested that the "molecular half-life" of RNA viruses was of the order of 20 days. This was the time taken for the RNA molecule to degrade to half its molecular weight.

Most devices and materials, certainly those which have been molded at high temperatures, probably contain few particles. I have always believed viral contamination to be low and 2.5 Mrads to give a "sterile" article.

To: I. GALATZEANU — Rumania, by: Y. COHEN — France

Q. Do you observe a parallel degradation between S-methionine and Se-methionine, or was the latter more sensitive to irradiation?

A. It was observed that Se-methionine was less decomposed than S-methionine. This we explained by the lower ionization potential of the 4p elections of selenium than of sulphur, which leads to a better scavenger because the selenium atoms in such a compound can easily accept or release electrons and hydrogen atoms. This is also dependent upon the chemical concentration of each compound in the mixture.

To: I. GALATZEANU — Rumania, by: A. CHARLESBY — England

Q. The decomposition of methionine and cysteamine irradiated in the presence of water can be largely ascribed to the effect of OH radicals and electrons. In the solid state could not another reaction be due to reaction with H? There is evidence that this accounts for radiation protection in some systems irradiated in the solid state, in the presence of small amounts of cysteamine.

A. Depending on the absorbed dose there could also be other reactions than with H by irradiation of cysteamine in the solid state. In the range of doses 2-5 Mrad the mean process observed was the

substitution of H atoms from sulfhydryl groups and probably amino groups. The H radicals remain as interstitial atoms trapped in the crystalline lattice. By dissolution in an aqueous solution they recombine and the damaged molecules repair themselves. At higher absorbed doses a cleavage of S-C bonds (6.15 kcal/mol) is possible by formation of volatile products as mercaptans. On the other hand, the cysteamine introduced into a system may act as a scavenger for free radicals which emphasizes the role of radiation protector.

To: I. GALATZEANU — Rumania, by: H. ROUSHDY — Egypt

Q. Sulfhydryl bearing compounds as cysteamine and cysteine are known to give effective chemical radiation protection to biological tissues through scavenging oxidizing radicals formed by irradiation.

Since the compounds have been shown to be easily decomposed by radiation, does this contribute in liberation of more free SH-groups affecting more scavenging of free radicals? If so, does it show dose dependency?

A. Yes. It is dose dependent.

To: N. DIDING — Sweden, by: Z. P. ZAGORSKI — Poland

Q. What preparations, other than pilocarpine, are presently allowed to be sterilized by radiation in Sweden?

A. There is no general permission in Sweden to use radiation sterilization for drugs. There must be on each occasion an application to the health authorities. In the special case I referred to, it was a quantity of pilocarpine hydrochloride in bulk contaminated with a small number of microorganisms. Talc, however, which is sterilized by irradiation, can generally be used in tablet preparations.

To: N. DIDING — Sweden, by: V. G. JENSEN — Denmark

Q. Do you have any experience/knowledge of change of crystalline structure of polymorphic compounds (drugs) caused by radiation?

A. We have so far no experience of changes in the crystalline structure of substances exposed to radiation.

When subjected to thermal analysis, two of the substances studied, namely chloramphenicol and tetracycline gave thermograms indicating a possible presence of polymorphic forms. Regarding chloramphenicol, the polymorph is present also in the non-irradiated sample.

The DSC-curves of the tetracycline samples, however, showed differences correlated to the differences in the dose levels used.

General Discussion

Comment by

T. ALPER — England

Radiation does not normally act in an "all-or-none" fashion. Surely, it would be valuable to derive proper dose-effect relationships (e.g. for effects of radiation on properties of bone, on viability of mycoplasma, on effectiveness of drugs) rather than use only the "magic" dose 2.5 Mrad. It should then be possible in some circumstances to compromise between a substantial (e.g. 99.99%) reduction in bacterial contamination and a destruction of desirable properties.

Comment by

A. DZIEDZIC-GOCŁAWSKA — Poland

The doses cited in the range of 2.0-3.5 Mrad are used nowadays in some tissue banks. The so-called "cold shock" used by Dr. Klens in the Tissue Bank in Hradec Kralove can kill only vegetative forms of bacteria. As far as I know, the spores survive "cold shock."

Because, as a rule, the initial contamination of tissues taken from cadavers is unknown and cannot be routinely controlled, the dose for sterilization should have a "wide margin of safety". As has been stressed by Dr. Christensen, another problem is the existence and possibility of inducing radiation resistant bacterial mutants, if low doses of ionizing radiation are used.

Of course, from the medical point of view it would be desirable to lower the sterilizing dose. It would be profitable not only with regard to mechanical parameters, but also for the osteoinductive properties of bone grafts.

As has been mentioned by the bacteriologists, the survival of bacteria depends greatly on the irradiation conditions: dose rate, water content, presence of oxygen, etc. Maybe in the future, irradiation conditions will be established which will permit a lowering of the sterilization dose and ensure the required assurance of sterility.

Comment by Chairman

R. W. CAMPBELL — Canada

If we are going to make a change, we will have to be very sure that we have a logical basis for what we do. I think those of us in the regulatory agencies, on the other side of the Atlantic at least, would welcome any guidance that can be given and are interested in getting a little bit of common sense into a situation which is mathematically a bit out of hand. Does anyone wish to comment on the matter of

dosage of 2.5 Mrad?

Comment by

T. ALPER — England
I should like to know what evidence there is for radiation induction of resistance in microorganisms? I have never observed it.

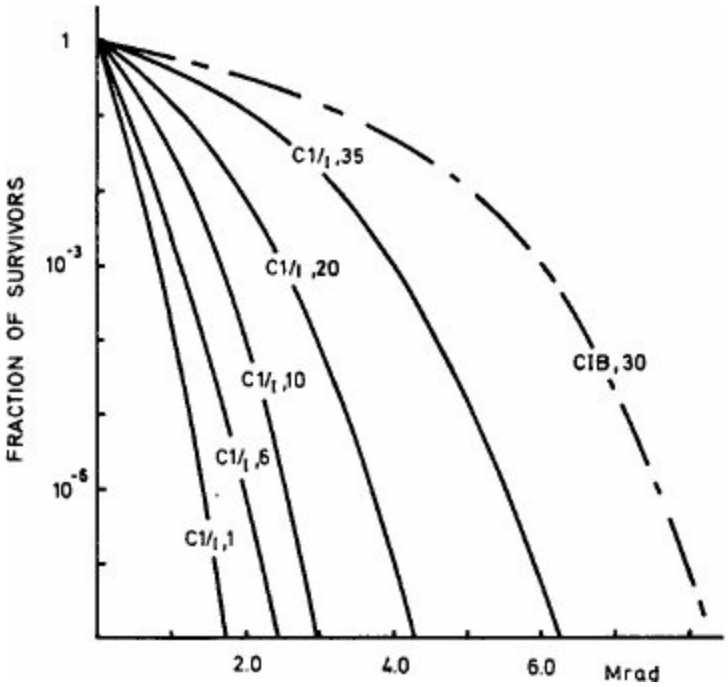
Comment by

E. A. CHRISTENSEN — Denmark

First, I should like to make reference to the Canadians. I think Dr. Thatcher was one of the first to demonstrate that if you irradiate cultures of microorganisms in several cycles, you can produce organisms with a significantly higher resistance. I also know that Dr. Tumanian in Moscow produced mutations by the same technique. Then, I can mention that we have done the same in several cases. It can be done with a single radiation and if you look for the colonies with changes in colony shape, you will have a good possibility of picking up organisms with changed radiation resistance. It is much easier if you irradiate several times. I have a slide giving an example.

The C 1/I inactivation curves demonstrate the radiation resistance of substrains of a strain of *B. cereus* after 1, 6, 10, 20 and 35 irradiations, respectively. The CID curve demonstrates the resistance of a selected mutant of a strain of *B. sphaericus* — a parallel to our test strain *B. sphaericus* CIA. (From: Christensen, E. A., Proceedings of U.S.P. Open Conference on Biological Indicators, Arlington, Virginia, 1970).

Repeated irradiations are, however, only a convenient tool for selection of resistant substrains. More than 10 years ago Dr. E. Kjems and I could demonstrate a change in radiation resistance after a single irradiation. We worked with enterococci, two strains of *Streptococcus faecium.* Colonies with changed appearance after one or a few irradiations were selected, and the radiation resistance of the substrains was determined. Among less than 20 substrains picked up after one irradiation, two with higher radiation resistance were detected (Christensen, E. A. and Kjems, E., Acta Pathol. Microbiol. Scand. 63: 281-290, 1965). Regardless of the explanation of the phenomenon the radiation resistant mutants should not be neglected in relation to the utilization of radiation for inactivation of bacteria.



Comment by

K. H. CHADWICK — The Netherlands

I would like to ask Dr. Christensen what the chance would be of finding these mutations, because mutation rates are notoriously very, very low in both higher cells and microorganisms. I think it is 10^{-6} or 10^{-5}, if you have high induction of mutation.

Comment by

E. A. CHRISTENSEN — Denmark

As far as I can see it varies very much from one strain to another. We have tried with some strains where it was impossible to change the resistance. With other strains it is very easy. But if you have information demonstrating that it is very rare in all cases, I should be pleased to learn of it.

Comment by Chairman

R. W. CAMPBELL — Canada

It seems to me irradiating a culture 35 times is a laboratory situation, rather than a real world situation.

Comment by

E. A. CHRISTENSEN — Denmark

As I mentioned, it happens also after one irradiation. How often it happens depends on the strain. It is not very common, but at least I have had the situation where we examined, as far as I remember, 20 colonies with small differences from the mother strain, picked up from a few hundred colonies. We were able to demonstrate that some strains had a higher resistance after a single irradiation.

Comment by Chairman

R. W. CAMPBELL — Canada

In 20 out of a few hundred?

Comment by

E. A. CHRISTENSEN — Denmark

Out of 20 examined. How many there were in total I cannot say because I took only changed colony morphology as an indication that at least there was some change. Therefore, I picked these 20 colonies and examined their resistance. Some of them had a higher resistance. It is, of course, a problem as to how much working time you can expend in such an examination.

Comment by

T. ALPER — England

Is there some danger of confusing the so-called induction of antibiotic resistance with an induction of radiation resistance? In either case, I think we are not dealing with a genuine induction. In other words, that the treatment itself is being mutagenic, is very difficult to establish. But it seems to me that it is philosophically too easy to confuse the situation that arises with antibiotic resistance, where you have plasmids which are able to convey resistance from one plasmid containing bacterium to another one which does not contain that particular plasmid, with killing by irradiation. It sounds analogous to say you can induce radiation resistance, but it is such a very different situation, as the Chairman said. You do not, in a situation where you are sterilizing, pick up a surviving bacterium, grow it up and irradiate it again. I think there is a great risk here of worrying about a phenomenon simply by analogy.

Comment by

T. OLEJNIK — U.S.A.

Is it possible with microorganisms that we are selecting out and growing up those cultures with a naturally higher resistance rather than inducing it by radiation? I find it difficult to believe that an organism, which is composed of chemicals which have bond energies of the order of kilovolts can develop a mechanism that will prevent these bonds from being attacked by radiation with energies in excess of many kilovolts. I do not think that the bacteria can make the chemical bonds so strong as to prevent them from being ruptured by an essentially random phenomenon.

Comment by

A. CHARLESBY — England

We are now getting right back into physics. The energy absorption by whatever you are irradiating is presumably random. However, we are not even sure of this. But the actual bond that breaks is highly specific. How this happens is a fundamental problem of radiation physics and chemistry. The strength

of a bond has remarkably little to do with the subject. You have bonds which are stronger and which break preferentially. So the question of the strength in the bond and the energy deposited in it have nothing to do with each other. If there is enough energy to do the trick, it will be selected. You can have a very big molecule and energy absorbed at random, and the break is at a very specific point. How the point of absorption of the energy is transferred to the bond that actually breaks is a very fundamental question. It has nothing to do with the present subject except as one of the applications. I would like to ask another question, if I may. We have heard all about the D_{10}'s and the effect of OH's on killing the bacteria and so on. We also know that the effect of the radiation absorbed in the water transferred to the spore by the OH's or the electrons depends on the concentration of water. In the spore, unless there is no water present at all, there should therefore be a D_{50} or D_{10} dependance on concentrations of water; I do not only mean high concentration of water; I mean very low concentrations, of the order of a fraction of one per cent. This is not because of the energy absorbed in this fractional per cent, which is very small and negligible, but in many of these materials which are crystalline or semi-crystalline or fairly rigid and amorphous, the presence of a minute trace of water changes the mobility of the system. The effect that you are going to get, the biological effect, the chemical effect, and the physical effect ultimately depends on the mobility of the material available. You can take a very simple polymer crystal or paraffin crystal and you will find that just by altering slightly the mobility of the system, the whole chemistry changes. Now this must also be true of our spore. In the present context, I would like to know how is it we get apparently consistent D_{50} or D_{10} results which should, in my opinion, depend on traces of water.

Comment by

D. M. POWER — England

I am unable to say whether direct or indirect effects of irradiation are more lethal. But I can offer some evidence from radical processes. Spores were irradiated in the presence of sulphacetamide. The D_{10}-value, I am told, was increased and it was predicted that this was because of radical scavenging by the presence of the drug. It, therefore, seems that there is some small evidence at least that in solution, indirect effects do help to kill spores. But I am sure this is not the whole story.

Anonymous comment

The whole question of induced resistance in microorganisms has exercised the minds of the radiation microbiologists on the food front. The works of Thatcher, that Dr. Christensen referred to, has been repeated in our country, particularly with the Salmonella. There was concern not only about increased resistance after a series of sub-lethal treatments with growth in between, but also about changing the pathogenecity of some of the strains of *Salmonella.* These things have been gone into in quite a lot of detail. However, the conclusion to it all was summarized very well by Dr. Alper. They decided that in practice it was quite unlikely that organisms would receive a sub-lethal treatment, be shipped away from the radiation facility, then grow and find their way back to the radiation facility as contaminants, be reirradiated and survive. The number of sub-lethal treatments was in the order of six or eight before these changes of significance were evident. So I think that the summary that Dr. Alper made of the significance of this has been accepted on the food front. There is no doubt, however that

these changes can occur and could be important.

Comment by Chairman

R. W. CAMPBELL — Canada

I think it is so easy for us to wrap ourselves in our own little cocoons. From my background in drugs, before I came to devices, we did not speak to the food people. They were out in another building and we never walked across the street and spoke to them. Perhaps we should do a lot more than that. We should be talking to the other people in our own organizations, discussing our problems with them. It seems wrong that I should have to come all the way from Canada to Vienna to find out from somebody from Scandinavia that somebody from my own town has the answer.

Comment by

V. G. JENSEN — Denmark

I should like to give a small example. I do not have the explanation for it but it is a suggestion for an explanation. From one of the factories manufacturing medical devices we picked up a gram negative rod in an unreasonably high number and with a quite unexpected high resistance. It was an *Achromobacter.* When we asked the Diagnostic Department at the Institute for a number of strains of the same species, none of them had the same higher order of resistance. Then we looked for an explanation and found that the cooling water was exposed to UV radiation most of the day. Of course, we do not know whether this is the reason for this apparent selection, but at least, large numbers of this organism were present in the environment where devices were manufactured and radiation sterilized.

Comment by

F. J. LEY — England

In this regard, we should mention that perhaps we should look also were X-ray machines are being used, or where cobalt therapy units are being used. Radiation, of course, is not only used for sterilizing medical supplies. This is the irony. We must look as well at areas where radiation is used for other purposes.

THIRD SESSION

Co-chairmen

A. Bishop

I. Galatzeanu

Introduction to Regulatory Session

A. Bishop

Department of Health and Social Security, London, England

This session is concerned with the control of radiation sterilization processes, both voluntary controls, which some of us try to exercise, and regulatory control, which is exercised in fact in very few countries at the moment. Before we start, could I remind you of the remark that Dr. Campbell made at the end of the discussion period yesterday evening, about the whole question of radiation dose and the various papers that have been presented here suggesting that the time has come for a change. When I was young, history books used to contain a quotation from Canning referring to emigration of European populations to America saying that the New World had redressed the balance of the Old Now the position of the Old World on radiation sterilizing is fairly clear. We have by dint of a lot of experience, a lot of discussion and a lot of conferences, arrived at a reasonably concensus view of the dose that we want, the dose that we think is reasonable and the dose that has served us well. We would ask that everybody thinks very carefully before they change it, particularly on the evidence that we have been given here. Sitting, listening to some of the papers, I was reminded of the Duke of Wellington. The Duke of Wellington, you will remember, was an Irish Commander in the British Army at the beginning of the last century. He was reviewing some of his troops who were pretty raw and rather poor and he turned to his aide-de-camp and said, "I don't know what effect they will have on the enemy, but by God they frighten me". This is very much the effect that some of these papers had and I would like, if I may, strictly without reference to anything that has been said here or anybody who has said it, to make the proposition to you, that to gain commercial advantage by lowering standards is not an activity that the medical supply industry should engage in. Having said that, I hope that when we get to the discussion on these papers, all the people who did not rise to Dr. Campbell's bait yesterday afternoon will do so and state loudly and clearly what their view on all this is.

There is a small change in the program. There will be a short presentation by Mr. Frohnsdorff in his capacity as Vice-Chairman of the U.K. Gamma Panel and Co-Chairman with Dr. Karl Peter of an EEC Industrial Committee to tell you about the present position of that Committee. I think perhaps I ought to say that the present position in Europe in this. Two committees were formed under the auspices of the Director General III in Brussels; this is the Directorate General that was concerned with energy and nuclear affairs. The purpose of the two committees was to try and get agreement in the EEC on radiation sterilizing. Some of us would like to see this activity extended to other forms of sterilization. One of these committees was composed of government officials and the other of manufacturers. The intention was to produce a code of practice embodying conditions of manufacture and methods of running sterilizing plants which would meet with universal approval, at least through the EEC and which would enable goods manufactured and sterilized in one country to be sold without argument and question in another. Unfortunately, both of these committees have gone into abeyance,

largely because of changes in the Brussels bureaucracy, but we live in hope of starting them up again. If any of you have any influence in this field with your governments, it would be a very good thing to use it to get this work re-started.

Pharmacopeial Viewpoints on Radiation Sterilization

A. S. Outschoorn

Drug Standards Division
United States Pharmacopeial Convention, Inc.
Rockville Maryland, U.S.A.

Abstract: *Recommendations on radiation sterilization developed internationally have been largely adopted in national regulations. The main interest is for medical devices and less for drugs, other than solid forms, which are identified in pharmacopeias. There has been some application for non-sterile pharmaceuticals to reduce the microbial content. There may be some licensed sterile drugs, which have been radiation sterilized, but this information is not publicly available on the label or insert. The specifications in pharmacopeias usually deal with final products. The radiation minimum dose is sometimes specified, but recent emphasis is on determining the dose from the microbial and other characteristics of the product before sterilization. Biological indicators are regarded as superfluous, sterility testing of a final product is of limited value; there is less emphasis on these topics in pharmacopeias for radiation sterilization. Specifications relating to toxicity resulting from such sterilization may receive more attention in the future. Further developments in pharmacopeial specifications are likely in future revisions.*

Background

The pharmacopeial coverage of sterilization by ionizing radiation and standards for compendial items are characterized by the fact that there is not much specified in any of the compendia which are current in various countries of the world. The situation, however, is not as bare as it may seem, for several reasons. One is that sources of radiation and the uses of ionizing radiation have been subject to control in most countries, separate from the national control of food, drugs or medical devices and equipment. Another is that to assure sterility of a product or item, there are certain principles which are common to all methods of sterilization which come to be automatically applied if a new or revised method is used. Owing to various circumstances, national activities for the control and testing of radiation sterilized articles developed together with efforts at international cooperation in this field, and the countries which first applied ionizing radiation to the sterilization of medical items were also those which contributed most to the formulation of international recommendations on methodology, codes of practice and criteria which should be fulfilled.

Foremost among the international recommendations are the series of publications by the International Atomic Energy Agency (IAEA), based on output from meetings, symposia, groups of experts and individual national agencies and persons knowledgeable and experienced in the field (1). Many IAEA publications cover a wide range of interests and topics, but there is a substantial proportion

which relates to items of medical and pharmaceutical interest. Details should, therefore, be sought in these publications, and in any which national regulatory authorities issue for the same purpose, rather than in the compendia. Nevertheless, pharmacopeias do provide material of interest, and also have the advantage of presenting material so as to indicate tendencies and the direction of progress. This could be very useful to persons engaged in the manufacture, control or use of pharmaceutical items.

The development of national and international recommendations dates from the 1950's (2). Control of pharmaceutical items during the early periods depended largely on what has been designated "voluntary discipline." Many countries, however, did follow the recommendations and adopt their provisions as national regulations. However, national efforts at control of radiation sterilized products have varied greatly. Several advanced countries have constantly tried to improve and develop their standards. But there are others, particularly some developing countries, where the only action they could adopt seems to have been to prohibit the use of irradiated drugs.

Field of Applicability of Radiation Sterilization

In considering the pharmacopeial aspects of radiation sterilization it would be as well to pick out the categories of items which are now subjected to this procedure. The major interest in such sterilization is for medical devices. This constitutes a very large field, ranging from equipment for diagnosis and therapy; dressings, bandages and garments; tubes, catheters and pessaries; syringes and needles; transfusion and infusion assemblies and components (all of which are minimally in contact with human tissues and body cavities) to such items as implants, prosthetic devices and other materials which may actually be in the human system for protracted periods. Sterility of the article would be applicable to both classes, but the development of toxicity may also be particularly relevant to the latter. In the compendia, there have been no indications of differing specifications for these two broad categories of radiation sterilized objects.

The most recent information which we have, collected from some pharmacopeial authorities, shows that there is much less interest in radiation sterilization of conventional drugs and pharmaceuticals. A possible reason for this could be radiation-caused chemical changes likely to produce unacceptable effects. Generally, solids are less likely to show such adverse effects. The production of toxic by-products in liquids has also been an experience in food production. In fact ionizing radiation is perhaps the only sterilizing agent which is required to be demonstrated not to produce unacceptable toxicity in the product. This type of toxicity must be distinguished from toxicity due to residues of the agent itself, as with sterilizing gases. For pharmaceuticals the use of radiation sterilization tends to be limited to dry solids or powders and perhaps certain oils and oily bases. Sterile injectables may, however, be packaged in their containers in the dried form, with sterile reconstituting fluid supplied separately.

There is, however, an area of compendial interest in microbial contamination of non-sterile pharmaceutical products. The United States Pharmacopeia (USP) has a chapter on *Microbiological Aspects of Non-Sterile Pharmaceutical Products* and another which deals with *Microbial Limits;* methods for enumeration and tests for specific pathogens are described (3). The BP, however, gives only tests for specific microorganisms (4). Most compendia agree that even for non-sterile products it would be advisable to use materials and processing methods in which the microbial load is kept to a minimum. This is recognized good manufacturing practice. A procedure which has been used occasionally in manufacture, is for raw materials or components of pharmaceutical products to be subjected to a small

dose, say 1 Mrad, of radiation, particularly if highly contaminated originally. This has been termed "light sterilization" or "partial sterilization". The USP chapter on microbial limits was formulated on the basis of conventional hygienic processing, and the methods described are for those microorganisms which are usually found naturally in the type of raw material or component of the pharmaceutical item. There is no information on the adequacy of the microbial limit tests in the USP in a situation where the product has been subjected to a process which selectively kills some microorganisms, attenuates others and leaves resistant ones relatively unaffected. The same question could be raised in a parallel situation where raw materials are heated in an autoclave deliberately at a low temperature and pressure and for a comparatively short time, to minimise the bacterial content but not sterilize the material.

The use of partially irradiated components of non-sterile products raises the question of irradiated components of sterile products. It has so far been the practice for sterilization by ionizing radiation to be done to the final product, not to portions which are later mixed. It is also generally accepted that in the event of failure of radiation sterilization, for whatever reason, the radiation procedure not be repeated. These are theoretically good practices, and there may be evidence in support of them. But pharmacopeias have little or nothing to say on these points.

There was quite some enthusiasm in the use of ionizing radiation for some biological materials (5). These include biologics, used for active and passive immunization, and biological tissues, as well as some antibiotics. Any use of radiation in antibiotic production would be for the purpose of destroying microbial contamination. The effect of ionizing radiation which has been studied on a number of viruses and bacteria, is to reduce toxicity with retention of antigenicity. The process has been reported useful for snake venoms which are notoriously administered to animals at the limits of tolerance, for antivenom production, and could with advantage be given in larger dosage if the toxicity were reduced. Such radiation usage should, however, be regarded as detoxification or inactivation procedures for these biologics, and not as sterilization. In fact all substances of this kind are not capable of being terminally sterilized, whether by heat or by chemicals or gases. A possible advantage of radiation sterilization is that it may be applied in the cold, sometimes even in the frozen state. Such sterilization has been tried, but there is no information publicly available whether or not there are any products of this kind which have been consistently rendered sterile by ionizing radiation. There is no mention of particular products in the federal Food and Drug Administration regulations, and none in compendial monographs. It is desirable that for the drugs and other substances identified in the compendia the standards applicable should be publicly available. Some compendia do prescribe, for each product that must be sterile, the method of sterilization which may be used. If a comparatively innovative method of sterilization, by ionizing radiation, has been used for any product that must be sterile, that information should be given on the label or package insert. So far this has not been a compendial requirement, and if there are any biologics or antibiotics or substances of biological origin which have been so rendered sterile, that would be unknown to the prescriber or consumer.

It must not be imagined, however, that the fact of radiation sterilization would not be known in relation to certification or licensing of federally controlled drugs. As far back as December 1955 the Code of Federal Regulations referred to an old sterile drug; it would be considered as requiring approval as a new drug (or under a modification to existing manufacture) if it was sterilized by ionizing radiation (6). This is still a current regulation and applicable, but federal regulation will not necessarily mean that this information must also be conveyed to the user of the drug form.

Compendial Role

It would be best to consider the possible role of compendial standards for radiation sterilization in the phases through which a product or item may be subjected. The first may be termed the pre-sterilization phase. This aspect is hardly dealt with in the compendia and not only in relation to radiation sterilization. This is usual since it is compendial practice to deal only with properties and tests of the final item itself in pharmacopeial monographs and chapters. Governmental regulations, however, are wider, particularly for products such as biologics, where certain procedures and in-process tests are crucial to the efficacy and safety of the final dosage form. What is pointed out in the compendia, is that validation of the sterilizing process includes prior determination of the microbial bioburden of the load, as well as the determination of the effect of the dose evaluated for typical loads.

In the sterilization phase the effectiveness of the sterilizing process depends on the absorbed dose of radiation. This has to be evaluated in relation to time of exposure, distance from the source of radiation to the object, whether it is a fixed load or one in a continuous process, the distribution of the dose through the load, having attention particularly to the location of minimum exposure in the load, and finally the determination of the likelihood of there being any survivors. This last depends on the characteristics of the microorganisms initially present and those of the product and should be calculated by accepted methods. Once determined for typical loads of a particular product, the validated findings would be acceptable as assurance of sterility of the product, but they would not necessarily be applicable if changes are made which could affect the sterilization efficacy. In this phase too, the compendia would have little role, except for pointing out that each sterilization of a load must be monitored to verify that the predetermined conditions for sterilization have indeed been applied or administered. This involves quality control of the radiation source and equipment and the mechanisms for measuring. Criteria for all such equipment have to be applied according to relevant regulations for them, and the recommendations in the publications of the IAEA (7–9).

Control of radiation source and equipment must be distinguished from control of a radiation sterilized product. As far as the dose is concerned, a limit of radiation is mentioned in some pharmacopeias and national regulations. It is often said that a dose of not less than 2.5 Mrad is recommended by the World Health Organization. This loses sight of the fact that the WHO itself makes no such recommendations. What is published are the recommendations of experts consulted for the purpose. It follows that such a recommended dose would be one generally specified in national regulations. In fact a minimum dose of 2.5 Mrad is used in a number of countries; this dose was also mentioned in USP XVIII. In some cases, however, as in the Scandinavian regulations, the minimum dose allowed is 3.5 Mrad. This order of dosage would perhaps be acceptable if the initial concentration of microorganisms is low. It appears illogical that when a dose is specified that it is not related more specifically to the pre-sterilization bioburden, yet this is common. Experimental evidence indicates that 2.5 Mrad would be adequate with good absorption and a wide margin of safety if the microbial content is of the order of 50 viable units per article; 3.5 Mrad may be preferred if the content is 100 viable units per object or greater. These general statements must be related to a probability of one in one million or less that there remains a contaminated unit, and, since the data must be monitored with a test organism, using *Streptococcus faecium* (ATCC 19581) or an equivalent (10).

Unlike in other methods of sterilization, biological indicators are considered unnecessary or superfluous, since the dose of radiation, once determined can be precisely measured and adequately

administered. This is in contrast to the generally accepted view of the value of biological indicators held some time ago. Nevertheless there are still some national regulations which demand that biological indicators be used. As far as pharmacopeias are concerned the tendency seems to be to indicate that microbiological controls *may* be used. If they are, of course, the general provisions regarding biological indicators should be followed.

The post-sterilization phase is where, perhaps, the compendia have the most interest and where it is most likely that any developments in pharmacopeial standards will occur (11, 12). One of the most important advances in the philosophy and practice of sterilization is the realization of the limitations of sterility testing alone to assure sterility of a sterilized article. It is becoming increasingly evident that the role of a sterility test is in a referee situation or in good manufacturing practice as an adjunct for assessing the efficacy of the sterilization process. These limitations of sterility testing of a final product are more pronounced where sterilization by ionizing radiation is concerned in view of the superior controls achievable through measurement of radiation dose and consistent reproducibility. However, a possible use for a sterility test even in radiation sterilization may be to show that the particular load or object has in fact been through the sterilization procedure. This too seems superfluous in the light of other and effective means, such as by recordings or effects on quantitative indicators such as colored (or non-colored) polymethyl methacrylate.

There could also be some use for sterility testing procedures in another context; in the pre-sterilization phase sub-lethal doses of irradiation may be given in order to draw up destruction curves for determining the dosage necessary for adequate sterilization. It would be preferable to consider this as part of validation of the sterilization procedure, rather than as a sterility test.

The USP, as well as other pharmacopeias, are concerned with assessment of the quality of finished products. They are not concerned with release criteria for lots or batches of products even though they may provide much informational material. However, questions are often received by USP about the validity of release of radiation sterilized products on the basis of physicochemical dosimetry, rather than by sterility testing of the lot with recommended sampling procedures. Of course microbiological audit and validation of the sterilization actually used are an essential prerequisite. An item claimed to be sterile must be capable of meeting the *Sterility Test* given in the USP, no matter what sterilization process it has previously undergone. It must be considered a reasonable position that such sterility tests are not an indispensably essential adjunct to all manufacturers' sterilization processes and batch release specifications. If other program safeguards yield demonstrably higher orders of confidence than obtained with random sampled sterility tests that a batch load is indeed sterile, then obviously such alternative measures would be preferred for quality control purposes. The acceptability of such an alternative in governmental regulatory procedures is a matter for the agency concerned.

Compendial Developments

Very little information is available on specific studies or actions taken by pharmacopeial authorities about radiation sterilization. One has, therefore, to evaluate their interest by what is published in the compendia, or what these authorities are prepared to pass on as information in an informal way. Previous work for the USP must, however, be mentioned. There was a USP Conference on Radiation Sterilization held in October 1972, in Washington, D.C. The interest of the USP in standards and tests of the properties of drugs that are sterilized by ionizing radiation "led the Planning Committee to

invite scientists outside the pharmaceutical industry to give a perspective to the Committee of Revision in this technology." The proceedings were published in the International Journal of Radiation Sterilization (13). At about the same period the USP Advisory Panel on Radiation Sterilization Procedures carried out a Collaborative Study involving a dose of 2.5 Mrad for different times, using an indicator of *B. pumilus* (ATCC 14884) at two different concentrations. The findings were used to assist in the material generated for USP XIX.

An interesting overview of developments and tendencies can be obtained if one compares the section dealing with radiation sterilization in USP XVIII with that in USP XIX, 5 years later in 1975 (3). Both these Revisions of the USP give a very general description of sources of radiation and of dosimetric measurements. This does not reflect the fact that there has been an immense increase throughout the world in radiation sterilization, and in use particularly of cobalt-60 as a source. This is not likely to have occurred if not for the fact that this method of sterilization is both efficient and safe in a variety of circumstances.

Where the two revisions do differ is in specific areas such as those relating to dosage, biological indicators, and sterility testing. USP XVIII (published in 1970) mentions that the amount of energy generally accepted as required for the sterilization of packaged medicaments and disposable medical adjuncts is 2.5 Mrad, delivered as uniformly as possible to all parts of the product being sterilized. USP XIX is much less definitive and states that the choice of sterilization dose should be determined by the microbial flora (types and numbers) and the nature of the article to be sterilized. This difference clearly illustrates a tendency among pharmacopeias. As mentioned previously a dose of 2.5 Mrad would be effective with a certain (low) level of initial microbial contamination, but it also follows that even smaller doses may be suitable if the procedure is validated and the product is subjected to adequate controls.

USP XVIII has a section on *Biological Indicators,* in general, but it was pointed out that even if a particular microorganism may be highly resistant to heat, this does not necessarily mean that it is suitable for monitoring a sterilization process that depends on the use of a gas or of radiation. Similar statements are made in USP XIX, but in even more general terms, pointing out that for selecting a microbial type the resistance to the specific sterilization process employed must be considered. Both USP XVIII and USP XIX give certain recommendations for the use of the sterility test as an adjunct to assessing a sterilization procedure, but these are mainly in relation to other means of sterilization. The applicability to radiation sterilization of different sizes and numbers of samples and incubation of inoculated media for different periods with and without biological indicators is questionable in view of the tendency not to use biological indicators for this procedure.

Both revisions have sections dealing with biological tests for plastic containers. The tests include implantation and the injection of specified extracts into animals. They are intended to test the suitability of materials intended for use in fabricating containers or accessories thereto. Once performed they are not mandatory tests to be repeated for each lot of pharmaceutical product, because it is believed that if the plastic itself has been found satisfactory the results would be applicable unless the sterilization alters its characteristics. Radiation is known to affect plastics, and generally to increase toxicity. Special considerations, therefore, would appear to be involved for this sterilization procedure, but there have as yet been no specific recommendations or mandatory standards.

This overview of pharmacopeial viewpoints has necessarily dealt with several related activities. It is clear that if guidance is sought then one must turn to international recommendations which are

relevant to technology and processing of medical and pharmaceutical items. For regulations that must be observed one has to become familiar with the national laws and regulations which are applicable in the country concerned both for radiation sources and for pharmaceutical products and devices. Pharmacopeias and other compendia have been much less advanced. This may be due, in part, to the fact that most of the current volumes (other than for interim revisions and supplements) were released in the early 70's, and there was not the present experience and knowledge available to draw from. One may expect, therefore, that the revisions to be expected in the next few years will treat the subject more fully, based on actual experience in applying the recommendations of experts. In this, a sincere tribute is due to the International Atomic Energy Agency for the amount of material provided.

Acknowledgements

Grateful acknowledgement is made for personal information to the Advisory Panels, Subcommittees and Headquarters of the U.S. Pharmacopoeia, used for the preparation of this communication from.

The following: —

Carl W. Bruch, Edward B. Seligmann Jr., William W. Wright: Food and Drug Administration, Health Education and Welfare, U.S.A.

Ebbe A. Christensen: Staten Seruminstitut, Copenhagen, Denmark.

Miles Fors: Department of Drugs, National Board of Health and Welfare, Uppsala, Sweden.

H. S. Grainger: European Pharmacopoeia Commission, Strassbourg, France.

B. R. Matthews: British Pharmacopoeia Commission, London, England.

O. Wallen, W. Wieniawski: Pharmaceuticals, World Health Organisation, Geneva, Switzerland.

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Role of the International Atomic Energy Agency and Current Activities with Emphasis on Regulatory Aspects

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Abstract: *The Agency's current programme activities include a considerable emphasis on the promotion of the beneficial use of cobalt-60 gamma radiation for the sterilization of medical supplies in the Member States. Factors determining the feasibility of introduction of this technology in the developing Member States have been discussed.*

Introduction

Extensive investigations into the bactericidal effects of ionizng radiation and the continuing improvements in the irradiation technology have led to the applications of cobalt-60 gamma radiation sources for the sterilization of a wide variety of medical products. It has already become a well-established industrial practice in a number of technologically advanced countries. Since the early 1960's approximately fifty gamma irradiation facilities on a commercial scale have been commissioned to sterilize various medical products ranging from disposable syringes and sutures to complicated medical instruments and from pharmaceutical starting materials to biological tissues (1). At present radiation sterilization of medical products constitutes among the most important industrial application of large radiation sources. The rapid increase in this application of radiation can be attributed to its high efficiency, ease of control and reliability, its unique suitability for the newly introduced generation of plastic constituents of medical products and packaging materials for which heat, chemicals and other conventional methods of sterilization have proved inadequate, and to its hygienic advantages that permit sterilization of prepacked hermetically sealed items (1, 2).

The current geographical distribution of the radiation sterilization plants and the concomitant massive introduction of ready-to-use sterile medical products in the routine health care services are rather unbalanced in the sense that they are mainly located in the technologically advanced regions of the world including Europe, North America and Australia (1). A sharply contrasting reverse situation is, however, encountered in the technologically lesser advanced developing parts of the world inhabited by the two-thirds of the total population. The status of the health care services of those populations is still gravely inadequate (2, 3). Against the figure of one doctor per 500 people in the advanced countries the corresponding figure in some developing countries may stand at one doctor per 60,000 people or even more! Similar trends may also be noticeable in the availability of the auxiliary supporting facilities and supplies including the trained nursing and other hospital staff. Furthermore, the limited available hospitals in many such countries are frequently located in the urban areas, while the majority of the populations living in the rural areas are scarcely served through mobile dispensaries and camp health

centres lacking in the facilities to ensure safe sterile uses of the medical instruments on the patients. This obviously leads to further deterioration of the efficiency of the health care services and an increased incidence of nosocomial infections with even greater and wasteful demands on the scarce resources of antibiotics and the hospital beds, not to mention the economic losses of the man-hours (3).

Such pressing demands on the existing health care system have resulted in a growing awareness for its upgrading through the introduction of the radiation sterilized ready-to-use medical supplies in a large number of developing countries during the recent years (3). Some of these countries have even become interested in the introduction of industrial radiation sterilization technology as part of their national development programmes and have sought the necessary assistance from the IAEA and United Nations Development Programme (UNDP). Economic feasibility and the market survey carried out in some of those developing countries (3) have reached the conclusion in favour of an early introduction of this technology. Despite the risk of somewhat higher costs for the radiation sterilized ready-to-use medical supplies their introduction can still be justified, for which the underlying philosophy is well-expressed:

"… money spent to safeguard public health produces wealth — for it protects human capital"
— Albert Calmette (1863-1933)

Role of the IAEA

One of the statutory tasks of the IAEA is "to accelerate and enlarge the contribution of atomic energy to peace, health and prosperity throughout the world". In keeping with these objectives and the particular regard to the interests of the developing Member States the Agency's radiation biology programme has promoted research on the understanding of the microbicidal activity of ionizing radiation for the practices of sterilizing medical equipment and supplies, pharmaceutical starting materials and biological tissues (1). During the past ten years the IAEA programme activities have actively contributed to help develop the operation and control of the radiation sterilization practices and the related technology in the interested Member States, with particular emphasis on the developing countries (4).

For the ease of discussion such promotional efforts of IAEA may be grouped into the following categories: a) Support of research activities to accumulate relevant technical information of local and/or regional significance aiming at the development and monitoring of the practices; b) Scientific meetings to freely disseminate the available information and to foster the necessary exchange of technical and operational experiences between the scientists from the developed and developing countries; c) Provision of the required technical training and expertise through the training programmes implemented by the awarding of fellowships as well as by holding regional and international training courses; d) Provision of the assistance of technical experts to undertake the necessary planning, including the market survey, economic feasibility assessments, instrumentation, man-power evaluation and the setting up of the laboratories for process development and control; e) Scientific publications including the proceedings, reports and technical manuals; and f) Assistance in the formulation of an international "Code of Practice" for possible standardization of the good manufacturing practices and the products' quality and safety to fulfill the requirements of the growing international consumer markets. In the following paragraphs some of these items will be further elaborated.

Encouraging recent developments in the practices of radiation sterilization of medical supplies in some of the countries of Asia and the Far Eastern region (Tables I and III) are most remarkable. Following the successful commissioning of the cobalt-60 gamma radiation facilities in India and South Korea, both through the support and joint venture of UNDP, IAEA and the governments concerned, the spurt of interest in this field of beneficial nuclear applications has extended to the Philippines, Indonesia and Thailand. In addition some small scale applied research activities relevant to the practices and process control have also been triggered off in the other countries of the region, such as Pakistan, Bangladesh and Burma, all under the IAEA regional co-ordinated research programme.

The process control and safety monitoring associated with the practices of radiation sterilization of medical products are undoubtedly at a highly satisfactory level of development through the generation of extensive information as well as operational experiences by the pioneering advanced countries as available in the literature. While introducing similar practices in the new geographical areas, such as in the tropical countries of Asia, best possible advantages should thus be availed of those valuable resources of knowledge (4) (Table I). Nevertheless, the striking differences in the ecological and the environmental hygienic conditions of the tropics together with the types and priorities of the medical supplies concerned may, however, impose certain limitations on the feasibilities of direct extrapolation of those available technical information and criteria (2). The situation may, therefore, necessitate the generation of some relevant technical information under the local conditions in order to ensure the establishment of the effective and safe practices significant to their local medical manufacturing industries and the environmental parameters. The Agency's regional coordinated research programmes on this subject (Tables I and II) have been designed to meet those objective goals.

Table I. — IAEA supported activities on radiation sterilization practices in Asia and the Far East

Country	Large-scale irradiation facilities	Research support & coordination	Expert assistance on technical, economic & & marketing services	Training of personnel & fellowships
Bangladesh		+		+
Burma		+		+
India	+[a]	+	+	+
Indonesia		+	+	+
Korea (South)	+[b]	+	+	+
Pakistan		+		+
Philippines		+	+[d]	+
Thailand	+[c]	+	+	+

Notes [a], [b] Co-60 gamma irradiators for sterilization of medical products under UNDP/IAEA project

[c] Co-60 gamma irradiator used mainly for food-irradiation

[d] Detailed pre-investment market survey of potential medical products has been completed under

UNDP/IAEA project

Table II. — IAEA supported activities on radiation sterilization practices in Europe and the Middle East

Country	Large scale irradiation facilities	Research support & coordination	Expert assistance on technical, economic & marketing services	Training of personnel & fellowships
Bulgaria				+
Czechoslovakia		+	+	+
Denmark		+		
Egypt			+[a]	+
Greece		+	+	+
Hungary	+[b]	+	+	+
Israel			+[c]	+
Poland		+		+
Turkey				+
Yugoslavia	+[d]		+	+

Notes:[a] UNDP/IAEA supported expert assistance project

[b] Co-60 gamma irradiator and the associated services provided under UNDP/IAEA project

[c] UNDP/IAEA supported expert assistance project

[d] Co-60 gamma irradiator and the associated services provided under UNDP/IAEA project

Development of gamma irradiator plants and their process control

So far through the support and involvement of IAEA programme activities commercial-scale gamma irradiator facilities for the sterilization of medical supplies have been established in India, South Korea, Hungary and Yugoslavia (Table III). The setting up of the demonstration facilities in these countries is expected to enable them to further intensify their relevant research, training and the upgrading development of the health care services and thereby enlarge the scope of these beneficial nuclear applications (3).

Table III. — Commercial gamma-irradiators for sterilization of medical products established by the joint support of IAEA, UNDP and the Governments

Country	Operator	Designer	Capacity, initial	(MCi) maximal	Date of commissioning
INDIA	ISOMED, Bhabha Atomic Research Centre, Bombay	H.S. Marsh NE Ltd.	0.120	1.000	1974

KOREA (SOUTH)	KAERI, Korea Atomic Energy Research Institute, Seoul	A.E.C.L.	0.100	1.000	1976
HUNGARY	Debrecen	A.E.C.L.	0.250	1.000	1976
YUGOSLAVIA	Vinca	French CEA	0.200	1.000	1978

Through the services of IAEA experts, as well as the coordinated research programmes, steps have been taken to control the sterilizing efficiency of several irradiator facilities in the Member States (5). Such services of the expert included the development of the suitable physical-chemical dosimeters and their applications to calibrate the radiation source and the relevant mechanical devices needed for smooth operation of the facility. Training of the local technical staff in the routine dosimetry procedures are also undertaken through the expert services.

Expert assistance has also been provided to a number of requesting facilities in the Member States for the development of the microbiological control of the radiation sterilization (6), including the advice on monitoring of the hygiénic standards of the production sites. Assistance including training of the counterpart technical staff has been given on the microbiological assay (initial counts) of the presterile medical materials. The microbiological efficiency of the cobalt-60 sources was investigated and calibrated using standard preparations of *B. sphaericus* strain C_1A and *B. pumilus* strain E601 (6). Based upon the requests from the Member States, the technical justification and the availability of the funds, some equipment has been provided for the various service control laboratories including microbiology.

IAEA publications

An essential role of the IAEA towards the promotion of the radiation sterilization practices in the developing and the developed Member States is served through the publications of the proceedings of the meetings as well as the reports and technical manuals (Tables IVa and IVb). The meetings have encompassed the diverse facets of the problems and the practice controls, which serve as the essential reference source and guidance for the workers in the field and also for the advanced students in the universities and the training courses. The recommendations for the relevant manufacturing practices have been utilized by a number of Member States while formulating their own national regulations and the "Code of Practice" (7).

Role of IAEA in the regulatory aspects of the radiation sterilization of medical products

In the previous sections of this article the promotional roles of the IAEA have been briefly enumerated in the field of application concerned. The regulatory authority for safety of the radiation sterilized medical products in a country belongs to the National Health and the Control Departments of the country's Government. Accordingly, the manufacture of the products should follow the specifically stipulated processes in the country's national Pharmacopoiea (7).

Table IVa. — Scientific meetings and publications on radiation sterilization of medical products organized by the IAEA

Title of Meeting	Date and venue	Date of publication
*Application of Large Radiation Sources in Industry [C]	27-31 May, 1963 Salzburg	1963
Radiosterilization of Medical Products, Pharmaceuticals and Bioproducts [P]	17-19 January, 1966 Vienna	1967
Code of Practice for the Radiosterilization of Medical Products [P]	5-9 December, 1966 Vienna	1967
Radiosterilization of Medical Products [S]	5-9 June, 1967 Budapest	1967
Radiation Sterilization of Biological Tissues for Transplantation [P]	16-20 June, 1969 Budapest	1970
*Utilization of Large Radiation Sources and Accelerators in Industrial Processing [S]	18-22 August, 1969 Munich	1969

Notes: [C] = Conference; [P] = Panel meeting; [S] = Symposium.
*Meetings partly related to radiation sterilization.

Table IVb. — Scientific meetings and publications on radiation sterilization of medical products organized by the IAEA

Title of meeting	Date and venue	Date of publication
Manual on Radiation Sterilization of Medical and Biological Materials [M]		1973
Revision of the IAEA Recommended Code of Practice for the Radiation Sterilization of Medical Products [W]	5-9 June, 1972 Risö	1973
Radiation Sterilization of Medical Products, Pharmaceuticals and Biological Tissues [R]	22-23 November, 1971 Risö	
Radiation Sterilization of Medical Products, Pharmaceuticals and Biological Tissues [R]	15-16 February, 1973 Budapest	
Ionizing Radiation for Sterilization of Medical Products and Biological Tissues [S]	9-13 December, 1974 Bombay	1975
Effects of Sterilizing Radiation Dose upon the Antigenic Properties of Proteins and Biological Tissues [P]	27 Sept. — 1 Oct. 1976 Athens, Greece	

Notes: [C] = Conference; [M] = Manual; [P] = Panel meeting; [R] = Reseach coordination meeting; [S]

= Symposium; [W] = Working group meeting.

However, in order to facilitate the necessary upgrading of the existing specifications in the light of the current developments in the technology and the relevant experiences, the IAEA organized several working group meetings of experts. The recommendations of the experts have been published (Table IVa) for use by the workers in the Member States (7). Furthermore, in view of the expanding uses of the radiation sterilized medical products by the consumers in the countries beyond the national boundaries of their production, such periodic upgrading of the document might become necessary in the future. IAEA wishes to remain responsive to such needs of the Member States.

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Comments on the Situation Within the European Economic Community

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You will have noted that it had initially been planned that speakers should be invited from two international organisations, the Commission of the European Communities and the Council of Mutual Economic Assistance. The organisers have not been successful in obtaining official speakers from these two bodies.

In the area of radiation sterilization, the countries of Europe have always played a very important role and a number of people attending this meeting have asked that some time should be given to consideration of the overall European situation in addition to the contributions from individual countries which follow. I have been asked to introduce this subject, but have not had the opportunity to prepare a paper before coming to the conference. I will not give you a detailed review, but will pick out several relevant facts. Please also note that I am not able to speak for, but only about, the situation in Europe. This will deliberately be a brief presentation and I ask colleagues who have also served on Brussels committees to add comments during the discussion period if any matter of importance has been omitted. However, my task has been lightened considerably for, a few months ago, this general subject was reviewed when preparing a paper which deals in part with the work of a European technical committee on radiation sterilization. This paper has recently been published in the names of Dr. K. H. Peter of West Germany and myself (1) and is the basis for many of my comments.

In Europe there were originally three Communities, each set up by a separate treaty. These were the Coal and Steel Community, the Atomic Energy Community and the Economic Community. Formerly each was administered by its own Commission, but since 1967 there has been one administrative unit in Brussels with the title The Commission of the European Communities. Of the Communities by far the best known to the general public is the Economic Community, usually referred to by the initials EEC. The Commission itself is one of the four institutions through which the Community operates but I will not enlarge on the detailed organisation of these European bodies as this information is readily available and simply set out in other publications (2, 3).

Since the introduction of radiation for the sterilization of single use medical products in about 1960 there has been a rapid increase in the use of the process and considerable practical experience has accumulated. Much of this experience has been obtained in Europe where about half of the installations are located and, in several countries, plants have been operating for a decade.

In February 1974 the Eurisotop Office, Directorate General for Industrial and Technological Affairs (DG3), held a meeting to consider the "Radiation Sterilization of Industrial Products". At this meeting it was agreed that the main interest was in the manufacture of medical products sterilized by means of gamma and electron radiation. It was noted that there were different official requirements in

some member countries. There was clearly a strong desire that there should be a uniform set of requirements acceptable to all national health authorities with reciprocal inspection arrangements. To advance this objective, two Working Groups were formed with the titles:

(i) "Technological and Industrial Aspects of Radiation Sterilization", with Mr. R. S. M. Frohnsdorff and Dr. K. H. Peter appointed as co-chairmen, and,

(ii) "Public Health Aspects of the Radiation Sterilization of Industrial Products", with Mr. A. Bishop and Dr. G. Kistner appointed as co-chairmen.

Rapid progress was made by one Working Group which was assigned the task of recording the principles and the practical considerations derived from the operation of radiation facilities used to sterilize medical products. All operators with radiation facilities in the EEC had the opportunity to join this Group and the majority were, in fact, represented at all subsequent meetings. In addition, several officials of the Community were present and we would particularly like to acknowledge the continued active encouragement of Professor G. Propstl and the secretarial assistance provided by Dr. E. Hertel.

This Working Group "Technological and Industrial Aspects of Radiation Sterilization" agreed on a number of objectives and recommended to the Eurisotop office that for the protection of the users of sterile medical products:

(i) All products should receive a dose of 2.5 Mrad (min.).

(ii) There was a need for a detailed and clear set of instructions for the procedures to be adopted when operating each of the three types of radiation facility — continuous gamma plant, batch gamma plant and electron accelerators.

(iii) There should be a system within the Community to compare dosimetry standards at one or more nominated laboratories.

(iv) A suitable certificate that a product has received the specified radiation dose should be accepted as proof of correct treatment.

It was further suggested that representatives of the health authorities should consider means of harmonising laws and regulations which tend to hinder trade in sterile medical supplies between countries of the Community. It was also thought that a monograph on radiation sterilization would be a useful addition to the European Pharmacopoeia.

It was found that there was an almost complete identity of view amongst all those with experience of the operation of radiation plants within the Community. This is perhaps not surprising for radiation is essentially a simple process to understand and control. A draft document "Recommendations for the Control of Radiation Facilities for the Sterilization of Medical Products" was prepared by the Working Group. The recommendations were agreed in February 1975 and subsequently have been circulated widely for criticism and comment. Subsequently they have been accepted by both the UK Panel on Gamma and Electron Irradiation and the Association Internationale d'Irradiation Industrielle and have now been published as representing the present practice of all European operators of radiation facilities.

I do not wish to deal with the recommendations in detail but there are perhaps one or two points that should be stressed. It was thought desirable to draw a clear distinction between the Manufacturer

who bears total responsibility for the quality of the product, including packaging and sterility, and the Operator of the Radiation Facility whose responsibility is limited to delivering the required dose. We also consider it essential that products should be manufactured with a low level of microbiological contamination and for this reason attention has been drawn to a number of national and international codes of good manufacturing practice that were available when the recommendations were drafted. Others are now available.

Concerning the control of radiation sterilization, it is a firm opinion that this must be based on the measurement of physical parameters of the process which, in turn, will be monitored by recognised methods of dosimetry. Dosimetry is also used at the commissioning of the radiation facility and there is deliberately no mention of the use of microbiological test procedures or sterility testing.

Although there was identity of view within the Working Group that a sterilizing dose of 2.5 Mrad should be recommended, and attention was drawn to the fact that this dose is quoted in a number of pharmacopoeias and related documents, to assist in obtaining full agreement, without delay a compromise suggestion was made that within the Community the sterilizing dose may be recommended by the appropriate health authority.

There has been another useful outcome of our work on the Brussels Working Groups. Arising from the request for harmonisation of official requirements in the member countries, a review of existing legislation and regulations was required. This project was undertaken by Dr. Eliane Mesmaeker. She has visited all nine countries of the Community to collect information. It appears that this was not a simple project for, in some countries, the regulations are not readily available and information could only be obtained from a number of different government departments. Dr. Mesmaeker's report was published in December 1975 and forms a convenient reference to European legislation, although at this time it is only available in the original French text (4).

To complete the present phase of establishing a firm European position, it is considered that the "Recommendations for the Control of Radiation Facilities" should now be combined with a general review of requirements for the manufacture of medical products under conditions of good hygiene. Suggestions for such a document have been made, the most recent being by Professor V. G. Jensen of Denmark. When an acceptable text has been prepared this will first be considered by the Working Group under the Chairmanship of Mr. Bishop and Dr. Kistner before submission to the combined Groups for joint approval. When this stage is reached our technical recommendations will be returned to the Community with the request that they should be translated into appropriate legislative action. It should be mentioned that, to this time, the initiative has come from the Eurisotop Office, Directorate General for Industrial and Technical Affairs. There has been some reorganisation within DG3 and it is probable that responsibility may pass to another Directorate General which would have the task of harmonising laws and regulations and it has been suggested that DG11, which deals with the Internal Market, may be the appropriate office. It is, however, not my purpose to speculate on the final form that our work will take as the Community has a number of alternative courses of action.

I hope that these few comments will be considered a useful and sufficient review of the situation as it exists in Europe today, but again emphasise that I am not speaking for the Community but giving my understanding of the present situation as it affects radiation sterilization.

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A North American Viewpoint on Selection of Radiation Sterilization Dose

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Abstract: *In North America Cobalt-60 gamma radiation installed capacity has increased rapidly in the past five years in response to its becoming a preferred method for sterilizing disposable medical devices. Consequently, a North American Working Group has been formed to develop guidelines for establishing appropriate sterilizing doses.*

The current position of the group regarding development of guidelines is presented in this paper, and while no actual guidelines have yet been formulated, we have concluded that a proper sterilizing dose for radiation should be based on the numbers of microorganisms that constitute the bioburden on the article to be sterilized combined with the radiation resistance of that bioburden and the margin of safety required. The group believes that the margin of safety should not be identical for all products, but should bear a relationship to the intended use of any product.

Radiation resistance profiles (D_{10}-values) and distribution data are being determined for a variety of naturally occurring organisms on products derived from the manufacturing line, and some preliminary results have been presented in this paper. Tests for sterility based on growth/no growth have been conducted on product after incremental doses of radiation, and the results compared with published and unpublished work.

The group recognizes that sterility must be viewed as a probability function and has demonstrated that sterility must be viewed in a probabilistic way. Since existing mathematical models have limitations, a program is under way to develop new models which will relate the radiation resistance and distribution profile of heterogeneous microbial populations to dose and margin of safety, (inactivation factor).

A plan for continuing work, including the development of standardized tables to aid in dose

determination is proposed.

This paper reports on the approach being taken in the United States and Canada by a Working Group considering practical methods for the determination of radiation sterilization dose. In addition, comments will be made on some of the preliminary results of studies that are now in progress.

The use of gamma radiation for the sterilization of disposable medical products has expanded rapidly in North America in the past 5 years. Figure 1 illustrates the increase in the installed cobalt-60 irradiation capacity between 1973 and 1976 from 3 million to 13 million curies. The reasons prompting this increased capacity stem primarily from a broader understanding of the well-known inherent characteristics of the process which include:

1. The desired dose can be accurately predicted on the basis of the geometry of the source, the product configuration relative to the source, and the exposure time.
2. Delivery of dose can be readily and accurately confirmed by dosimetry.
3. Reliable penetration by the gamma rays assures delivery of a known dose to every part of any article being sterilized.
4. Package seals are not subjected to vacuum stresses nor is dose delivery affected by the nature of the packaging materials. This allows free choice of packaging including the option to use hermetically sealed pouches or cans. (This option may become increasingly important as regulatory authorities require assurance of the sterile shelf life of sterilized products.)
5. A wide range of medical grade materials, suitable for gamma sterilization, are now commercially available.
6. The process has become more cost competitive.

Additionally, the adoption of radiation sterilization has probably received further impetus as a result of recent criticisms in the U.S.A. of ethylene oxide sterilization.

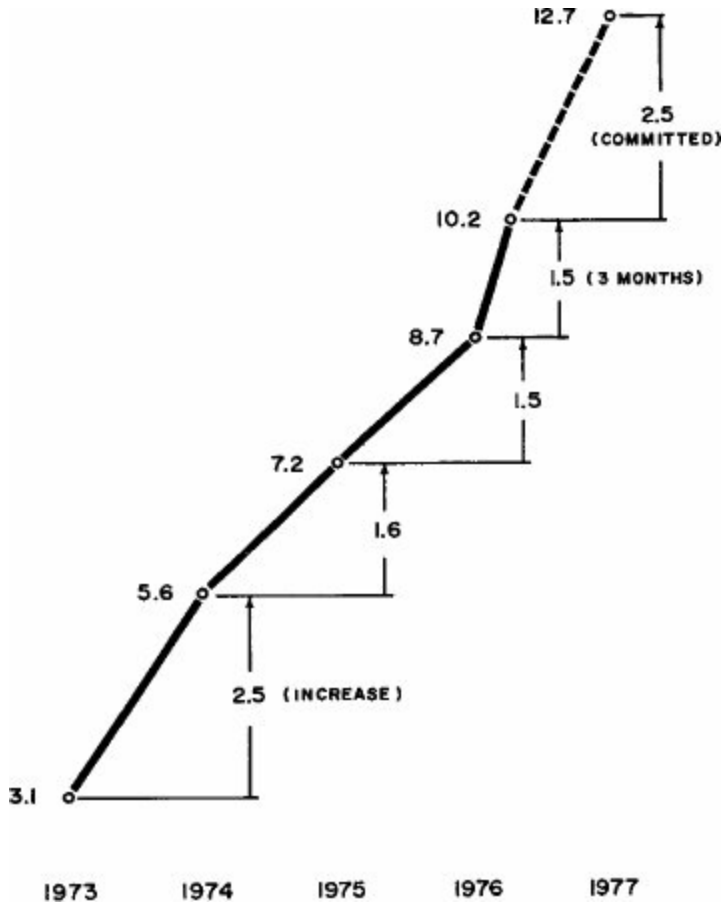

Figure *1.* United States and Canada Installed Co-60. (millions of curies)

Three classes of guidelines are currently recognized as they pertain to the selection of radiation sterilization dose.

Class 1 — may or may not specify a set dose but requires that sterility be certified by means of post-sterility testing.

Class 2 — specifies a minimum sterilizing dose, which is generally set at 2.5 Mrad, with additional limits usually based on the presterilization microbial challenge.

Class 3 — specifies no set dose but requires that the probability of sterility be defined as a function of the distribution and sterilization resistance of presterilization bacterial contamination (bioburden), and of the margin of safety sought, as determined by the intended use of the products.

The Class 1 guideline in specifying confirmation of sterility by post-sterility testing has serious statistical limitations. Reasonable sampling will not even detect gross contamination. For example, as illustrated in Table I, when post-sterilization U.S. Pharmacopoeia test criteria are applied, a manufacturer could accept a lot in which approximately 17% of the articles were unsterile 5% of the time. In fact, this sampling strategy is indifferent to 6% non-sterile material. No one needs this type of protection! Thus we conclude that the Class I guideline is not good enough even as a rough screening test for sterility.

Table I. — Probability of accepting non-sterile product.

Sample Size	Fraction Non-Sterile	Probability of Acceptance
20	.0016	.999
	.060	.50
	.168	.05
1000	.00003	.999
	.0012	.50
	.0037	.05
3,685,000	.000001	.05

The Class 2 guideline in which an arbitrary minimum sterilizing dose is specified, is unduly restrictive. Certain products are currently being sterilized to the satisfaction of industry in North America at doses lower than 2.5 Mrad with no evidence of hazard. Further, the reference to 2.5 Mrad as the "generally accepted sterilizing dose" has not been included in USP XIX. To reimpose the requirement for a 2.5 Mrad minimum dose might arbitrarily force the use of alternative, less reliable sterilizing processes. For instance, it has been shown that some products having physical properties which are impaired by a 2.5 Mrad dose (e.g., antistatic properties and odor generation) can be satisfactorily sterilized, without deleterious effects on these properties, at the lower doses currently being employed in North America.

The Class 3 guideline takes into account the actual product bioburden and its radiation resistance, and the margin of safety required as determined by the intended use of the products. This promises to provide the required sterility assurance without unnecessarily restricting the use of the process. This is also the strategy recommended in USP XIX and in the IAEA Code of Practice, and is the one favored by our Working Group.

There are still difficulties to be overcome before realistic guidelines can be provided for establishing the doses required to achieve selected levels of microbial inactivation. For instance, we have yet to agree on a final definition of an item for the purpose of quantifying bioburden. Although there is wide recognition of the idea that sterility should be classified in a probabilistic way, nonetheless it is still one thing to claim a probability of sterility, and quite another to show reasonable substantiation, or better yet, proof, of that claim. Reasonable proof requires extensive product bioburden knowledge which we believe must include details of the distribution and the radiation resistance of the bacterial species constituting that bioburden. Sound statistical methods must also be developed to reliably estimate the approximate dose and to provide proof that any statement of sterility confidence is reasonably correct.

Methods for determining the bioburden challenge and the statistical interpretation of that data require further development. These methods must be practicable so that they do not impose excessive costs on the manufacturer and therefore the consumer. A part of the justification for any new proposed methodology should be a comparison with existing practices.

We believe that the intended use of a product should be considered when determining the sterilizing dose. For example, if a product has little potential for causing patient harm when put to normal use then perhaps a smaller margin of safety would suffice. That is, a microbial inactivation level of one contaminated item in 1000, together with a less than 1 in 1000 chance of potential untoward effect in use, would yield a less than one in a million chance of a bacterial survivor causing harm. Not to

recognize this would result, in practice, in providing a greater margin of safety attending the use of a surgical drape than is demanded for an implanted heart valve.

When dealing with naturally (heterogeneously) contaminated product, we seriously question the practice of assuming a linear relationship between the log fraction of nonsterile items and dose levels, and the extrapolation of this relationship to predict a given sterilizing dose.

We have initiated a program to develop mathematical models, using minimal assumptions, for the inactivation of irradiated heterogeneous microbial populations subjected to varying doses of radiation. This is an essential first step in developing statistical models which will accurately estimate the radiation dose necessary to provide an assured level of sterility.

Thus far we have found that, in fraction negative studies, the Schmidt-Nank procedure consistently underestimates the true D_{10}-value. Hence, we have developed an *ad hoc* alternative D_{10} estimation procedure which corrects this problem of underestimation bias. To establish confidence limits for estimates of sterilizing dose, further statistical work, including a significant amount of computer simulation, remains to be done.

We have started preliminary investigations to generate data identifying the radiation resistance and distribution of organisms as they naturally occur on a wide range of products manufactured in North America.

Because the natural bioburden on product is large and diverse and is hard to quantitate precisely, and since it is impracticable to examine every bacterial cell present on the product before radiation treatment, we have attempted to define the bioburden of interest by the use of incremental radiation doses. Hence, we have used incremental doses of 0.1 Mrad in the 0.1 to 1.0 Mrad range. applied to conveniently sized samples of product. We have cultured the samples and scored them on the basis of growth or no growth, and the radiation resistance of the surviving microbes. If less than 20% of the samples show growth when tested by immersion, the positives will have resulted, by and large, from the outgrowth of a single bacterial survivor. For our purposes, the critical radiation screening dose will be that achieving sterility of 80% of the irradiated samples. A large number of samples are then exposed to the selected screening dose, and the bacterial survivors isolated. From these survivors, D_{10}-values are determined and used to assemble a profile of radiation resistance of the bacterial species remaining on the articles. We are confident that by working within the 50-90% sterile dose, the outgrowth obtained will be largely from bacterial survivors of the process and not from chance contamination.

We are not yet certain how large a bacterial profile will have to be collected, but we can already say with some degree of confidence that bacterial species of high radiation resistance are uncommon on the natural products we have examined. In fact, it has been possible to obtain a 100% sterile fraction well before the upper limit (1 Mrad) of the radiation dose series is reached. We examined isolates obtained from 0.2 g samples of stockinet cuff from surgical gowns carrying an initial bioburden of approximately 18,000 organisms per gram. A dose of 0.5 Mrad yielded 47 nonsterile samples out of 110 samples irradiated. From the radiation resistance profile of this study, (Table II), it can be seen that while two isolates were obtained with radiation resistance slightly higher than that of *B. pumilus,* (D_{10} = 0.17 Mrad), no isolates had a resistance greater than 0.25 Mrad. Work is continuing and so far, the D_{10}-values for 180 isolates from survivors of incremental doses have been determined. We did not discover a single organism with a D_{10}-value greater than 0.25 Mrad. This indicates with 95% confidence that after 0.5 Mrad exposure, organisms with a radiation resistance of more than 0.25 Mrad

do not occur with a frequency of more than 1.7% in this population. Statistical claims such as this will be further refined by the planned pooling of D_{10} data by a number of North American manufacturers and universities.

Table II. — Radiation resistance profile and distribution.

	D_{10}-Value					
	0.075	0.10	0.15	0.20	0.25	>0.25
Isolates	19	15	11	0	2	0

We are all well aware of the data of Czerniawski and Storlarczyk (1) who found a very large number of radiation resistance isolates in the environment of an injection needle factory, and that they then used their data to calculate the radiation sterilization dose. This seems unsound as it is the type and distribution of organisms on the product before sterilization which should determine sterilization dose.

The conditions of our experiments are different in that we are irradiating natural product and examining survivors, and this perhaps explains the disparity in our findings.

An independent study by White (2) further supports our initial finding as to the infrequency of highly radiation resistant organisms (on actual product). He found that after delivering a dose of 0.5 Mrad to a large number of 0.25 g cotton rolls with an average contamination of 1884 organisms, only 1.39% were nonsterile. Had the bacterial population been minimally similar to that described by Czerniawski and Stolarczyk, we calculate that a dose of 0.5 Mrad would have yielded more than 95% nonsterile items. This significant difference is borne out in other studies also, and provides additional support for our proposed approach to establishing dose.

Here, in outline then, is the plan we are working with to establish the appropriate radiation sterilizing dose for a product. An incremental dose series will be set up to determine the dose that yields approximately 80-90% sterile samples, by a pilot determination. This dose will then be applied to a sufficient number of samples, and surviving bacteria will be recovered and isolated from the nonsterile fraction. The isolates will be qualitatively and quantitatively characterized and their D_{10} radiation dose determined. Then, statistical analyses will be conducted to ascertain the dose necessary to provide sterilization to less than one in a million or whatever microbial inactivation level is considered appropriate. In addition, computer simulations will be run to quantify the quality of our findings.

Another key part of our efforts will be directed to an attempt to provide standardized tables which will allow a reasonably easy determination of required dose in specific applications without the need for running complicated studies and computer programs for each individual product. Until the data being generated from our program is more complete, it may be necessary to continue the traditional practice of assigning a single arbitrarily high D_{10}-value to all bacteria in the bioburdens. This results in an unnecessarily high dose being specified, and we are anxious to discontinue this approach.

We are hoping, therefore, that some of you will collaborate with us in this microbiological/statistical effort by providing criticism and input data which will lead to greatly increased knowledge and improved standard operating procedures for the radiation sterilization of medical products.

We believe our study will eventually provide:

- A means by which dose can be related to desired sterility levels for most products and a more

accurate method of substantiating probabilistic levels of sterility which will stand the test of time.

- An extensive body of data characterizing the current pre-sterilization microbial bioburden of most key products.
- The elimination of post-sterility testing for radiation sterilized products, which has already been agreed to in Europe and Canada for a dose of 2.5 Mrad.
- Theoretical and empirical support for whatever radiation sterilizing dose is employed rather than merely the traditional 2.5 Mrad dose.
- A set of standard operating procedures including sets of statistical tables by product class which will provide dose requirements to achieve different probabilistic sterilization levels for given heterogeneous bioburden challenges to the sterilization process.
- The development of guidelines for statistically valid microbiological auditing of bioburden so as to validate the continued use of the initially established dose.

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Present State of Legislation in the Field of Radiation Sterilization of Drugs and Medical Products — West Germany

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A rational legislation should be based on the following principal ideas:

1. Sterility is an ideal state of a drug or medical product, which never can be attained actually or not even be proved with absolute certainty.

2. The actual attainable state of microbiological purity is a limited number of certain microorganisms, given either as a concentration (e.g. microorganisms per ml) or as the probability of finding one microorganism in a certain volume, which is essentially the same.

3. In principle, the number of microorganisms contaminating a drug or medical product can be reduced to any level except zero. There are several methods available for this purpose. Not one is universally applicable, or is without influence on the quality of the treated products. The duration and severity of the antimicrobial treatment are of importance. The antimicrobial treatment finally chosen and the severity of the conditions are always a compromise between the acceptable maximum number of surviving organisms and the range of reduction of quality by the treatment of the product in question.

4. One of the available large scale methods is the treatment with ionizing radiation, for convenience called "radiation sterilization." This method is not inferior to others with regard to its efficacy. The irradiated products do not show, if at all, special risks, which could not be detected by the usual analytical methods. Therefore, there is no reason to hinder this method by restrictive legislation or to restrict its applications, compared with other methods. The general legal regulations for safeguarding efficacy, safety, and quality of drugs or medical products and the rules of "Good Manufacturing Practices," as well as of the pharmacopeias, should be sufficient, as they are in the case of other antimicrobial treatments. On the other hand, a legal regulation of how to run a radiation plant is necessary for reliably controlling the general hazards arising from operating with radioactive materials.

Legislation in the Federal Republic of Germany and the regulations ensuing from it meet only partly the considerations mentioned earlier. The Drug Act from 1961 generally prohibits the use of ionizing radiation on drugs in par. 7 and makes exceptions, depending on respective regulations. Likewise, the new Drug Act, coming in force in 1978, sticks to this fundamental prohibition, and moreover, covers further "fictitious" drugs, e.g. *in vitro* diagnostics. Regrettably above all is the amateurish equalization of "drugs treated with ionizing radiation" with radiopharmaceuticals, e.g. radiation emitting materials. This has resulted in the completely unnecessary unwieldiness of the pertinent text of the Act.

Characteristics of the Drug Acts of '61 and '76 are the extensive interpretation of the term "drug." The Acts cover and subjects to the pertinent regulations concerning radiation sterilization, besides dressings and surgical sutures, also a number of other medical products, e.g. prosthetic materials. On the other hand, other medical products, which do not count as drugs, such as medical instruments, are not subjected to any legal restrictions. A special law concerned with medical devices, comparable with the "Medical Device Amendments of 1976" of the Federal Food, Drug and Cosmetics Act of the U.S.A. is still lacking in the Federal Republic of Germany. This has caused an uncertainty for the producers relative to the quality standards to be met, especially with respect to the competition with importers from low cost countries.

Already in 1967 it proved necessary to license the radiation sterilization of resorbable sutures and several similar products by a regulation. In 1971 an extension followed, and since 1975 a further bill has been under consideration, which generally admits radiation sterilization of drugs and limits the radiation energy limit to 8 MeV and the absorbed dose limit to 5 Mrad.

In spite of the generally acceptable formulation, the "Bundesverband der Pharmazeutischen Industrie" already pointed in 1975 some details which were to be disadvantageous. Thus the order of antimicrobial treatments in the bill allows the application of ionizing radiation merely in the case that other treatments fail. Inconsistent and, in addition, not clearly defined is the demand that by radiation treatment, "sterility" should be guaranteed, while the antimicrobial treatment of "non-sterile drugs" gains increasing interest.

For the rest, it seems essential that no minimum dose is prescribed. It enables us to reach an optimal antimicrobial effect at a minimum radiation damage to the products treated by choosing an individual dose dependent on the microbiological precontamination level.

The German Pharmacopeia DAB 7, and the European Pharmacopeia do not refer as yet to radiation sterilization, which is in contrast to the Pharmacopeias of Switzerland and the German Democratic Republic, which are, however, also very reserved.

As a consequence this has slowed down the development of a promising technology for two decades and is understandable only in light of the still deep rooted distrust of nuclear energy, which becomes especially clear in the opposition to the construction of nuclear power plants.

Safety and Regulatory Aspects of Radiation Sterilization in France

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The practice of radiation sterilization in France falls under several types of regulations. They deal on the one hand with the safety of the industrial facilities and with the protection against ionizing radiations. On the other hand, the French Pharmacopeia has set forth detailed rules ensuring that the various articles sterilized by irradiation are effectively safe when they are utilized.

The study of these legal dispositions show that the irradiation facilities are not always treated in the same way depending whether they use electron beam generators or radioisotopes as radiation sources. It also demonstrates that the legal and administrative framework at least in part is of recent origin. Further developments which are being prepared by scientific and technical researches might be expected.

Safety of Irradiation Facilities

New legislation has been recently established in France as regards the safety of nuclear facilities: it includes all the dispositions to be taken in order to enable the normal functioning of each nuclear facility, to prevent accidents or felonious actions and to limit their consequences.

(Decrees of December 1963, March 1973, and August 1975).

The conditions to be met for irradiation facilities under this legislation are quite different whether the radiation source used is an electron beam generator or a radioisotope.

Electron beam generators:

Electron beam generators are not considered as a potential hazard for the neighbourhood of the facility in which they are operated, provided the energy of the accelerated electrons is less than a legal threshold value of 300 MeV. This is always the case in radiation processing and no authorization is needed to build such a facility.

(Arret of September 1965).

Radioisotopes:

On the contrary, the facilities using radioactive sources fall under the nuclear safety regulations, but only if the total activity of the radiation source is higher than a threshold value: for Co-60 or Cs-137, which are the radioisotopes most likely encountered in radiation sterilization, this value was fixed at

100,000 Ci (Arret of January 1967). Since almost every irradiation facility used in radiation sterilization has activities well above 100,000 Ci of Co-60, in most cases, an authorization will be necessary to establish such a facility.

The procedure includes the following steps:

a) Submit a preliminary "safety report" to the Ministry of Industry and apply for an authorization to create the facility.

b) The preliminary safety report must include the description of the main features of the projected facility and a map showing the location and the boundaries of the irradiation plant.

c) A public inquiry is opened.

d) The Ministry of Industry examines the preliminary safety report. The degree to which the safety of the facility is achieved is evaluated by a specialized Department of the French Atomic Energy Commissariat (C.E.A.).

e) The Ministry of Industry requests the advice of the Ministry of Health and of the other ministries concerned.

f) An interministerial Committee is consulted for decision.

g) The Ministry of Industry eventually delivers the authorization to build the projected facility.

When the facility is commissioned a definitive safety report corresponding to the actual conditions of exploitation will replace the preliminary one.

It is to be noted that a surveillance of the facility will be organized with Inspectors of the Ministry of Industry, in addition to other inspections by Inspectors of the Health and of the Labor Administrations, respectively.

Table I summarizes the status of irradiation facilities of this category.

Table I. — Nuclear safety status of irradiation facilities

ELECTRON BEAM GENERATORS — (E ≥ 300 MeV) RADIOACTIVE SOURCES (Co-60 or Cs-137 ACTIVITIES ≥ 100,000Ci)
PRELIMINARY AUTHORIZATION (PRIOR TO BUILDING) (Decrees of 1963, 1973 and 1975) Given by the Minister of Industry, with advice of the Health Minister
SURVEILLANCE: Nuclear Safety Inspectors (Ministry of Industry) Health Inspectors Labor Inspectors

Protection Against Ionizing Radiations

The legislation dealing with radiation protection concerns any radiation source. However, in this presentation, we shall restrict ourselves to individualized sources only and to those which are not to be used directly on man for diagnostic or therapeutic purposes.

In this legislation, both the Health and the Labor administrations impose their own regulations:

1. *Health legislation.*

a) Electrical radiation generators.
No authorization is requested.

b) Radioactive sources.

A preliminary authorization is requested to use radioactive sources. This authorization is delivered by the President of an interministerial body in charge of artificial radioisotopes (CIREA), after obtaining favorable advice from the Health Minister.

A report must be submitted by the user of the source in which his ability to utilize the source and the security of the facility are demonstrated.

2. *Industry legislation.*

The facility must be eventually classified to the extent that it might represent a potential danger for the vicinity. (Law of 1917).

3. *Labor legislation.*

The labor legislation does not make any difference between electrical radiation generators and radioactive sources.

According to this legislation the employer of workers using irradiation equipments has to *declare* his establishment to the Minister of Labor and to the Minister of Health.

(Decree of 1967 and Arret of 1968).

In particular, a specialized service of the Ministry of Health (SCPRI) is responsible for keeping a national compilation of the radiation sources used in France.

In this declaration, the employer has to designate a person having the required knowledge and training to use the irradiation equipment. He has to keep an inventory of his radiation sources. Other obligations are:

a) to provide suitable information to the workers,
b) to delimit an area with a controlled access defined as having an irradiation risk for the personnel above 1.5 rem/year,
c) to signal this controlled area with proper symbols,
d) to admit in this controlled area only those workers who are medically recognized as fit for working there (to be renewed every other six months). These workers will have their individual medical file in which the results of their personal dosimetry will be recorded.

A surveillance of the establishment is organized by periodical inspections (SCPRI or related organisation).

French Pharmacopeia Regulations

The French Pharmacopeia is continuously up-dated in order to keep up with the developments of science and techniques.

A new chapter dealing with radiation sterilization was introduced in 1974 (Arret of the Health

Minister of May 1974). It was modified in October 1976 in particular to extend the legal storage period to a maximum of two years.

The prescriptions of the Pharmacopeia deal with the various aspects of the radiation sterilization process:

a) Radiation sources to be used and articles which can be sterilized,
b) Rules for the preparation of the articles,
c) Irradiation,
d) Material,
e) Post irradiation microbiological control,
f) Storage prescriptions,
g) Labeling.

1. *Radiation sources and articles to be radiation sterilized.*

The radiation sources which are utilized are gamma radiations or accelerated electrons.

The articles which are sterilized by irradiation are disposable medical and surgical articles such as syringes, needles, perfusion assemblies, catheters, probes, blood dialysis apparatus, as well as dressings or sutures. They must be conditioned in leak-proof wrappings.

2. *Rules for the preparation of the articles.*

It is stated that the efficiency of radiation sterilization depends on the initial number of contaminants. The production process of these articles must therefore be carried out in such a way that the contamination is reduced as much as possible.

In particular the air contamination should be checked regularly and working rules should be followed to reduce the probability of contamination.

The contamination should be checked at the end of the manufacturing process and prior to irradiation. The total number of the contaminants in every conditioned unit should be less than a maximum value specified in monographs for each identified type of microorganism.

This determination will be periodically carried out at a frequence depending on the nature of the article.

Finally, the wrapping of the articles must permit them to remain sterile. It should be possible to remove each article out of its wrapping in an aseptic way.

3. *Irradiation.*

The prescriptions concerning the irradiation process deal with:
a) a preliminary dosimetric study of the process;
b) the dosimetric control during the process;
c) the definition of the doses to be used.

a) *Preliminary dosimetric study.*

A preliminary dosimetric study must be carried out before a radiation sterilization process is started.

This study involves the irradiation of several “packages” designed for the process, each one

containing several *dosimeters.* These dosimeters will allow the measurement of an absorbed dose by physico-chemical changes and will be distributed at the surface and inside the package.

This procedure will allow the determination of the irradiation conditions ensuring that the minimum sterilization dose is effectively obtained throughout the package.

It is to be noted that the reference dosimeters to be used in this study must have been calibrated by the specialized services of the Ministry of Health (SCPRI).

The results of the study will be noted in a report with the corresponding physical characteristics of the facility together with the dose distribution obtained within the article. The proper irradiation conditions will thus be defined.

b) ***In line dosimetric control.***

During the irradiation process each sterilization "batch" will be controlled by means of dosimeters, in order to check that at some well defined places in the packages, the absorbed doses are in conformity with the doses obtained during the preliminary dosimetric study.

A "batch" is defined as a number of packages having a similar content, irradiated in sequence without interruption, in the same facility, during a well defined period of time not exceeding 24 hours.

The dosimetric control takes into account the nature of the radiation source.

Accelerated electrons

In the case of irradiation facilities using electron accelerators the dosimetric control is carried out by means of a dosimeter properly placed on each package and by recording the main physical parameters of the irradiation facility in order to check that these parameters are permanently set at the values they had during the preliminary study.

In addition the accelerated electrons should not be energetic enough to induce any radioactivity in the materials of the articles.

Radioactive sources

In the case of irradiation facilities equipped with gamma ray sources, a dosimeter will be placed on each of the first five packages, then on every tenth one, and on the last five packages of every batch.

The duration of the irradiation period will be accurately measured.

In any case, an "irradiation indicator" (go-no-go) directly readable, will be placed on each package in order to distinguish between irradiated and non-irradiated packages.

c) ***Dose definition***

The definition of the dose depends on several factors:

— the extent of the initial contamination,

— the radiation sensitivity of the microorganisms,

— the security degree wished for the sterility.

The Pharmacopeia states however that for articles manufactured following the working procedure and the rules of hygiene as indicated above, provided that the number of contaminants should be less

than the maximum permissible value taking into account the radiation sensitivity of the contaminating microorganisms, a minimum dose of 2.5 Mrad uniformly distributed, generally gives a sufficient degree of security.

4. *Materials*

It is stated that the articles treated with the above mentioned dose should not undergo any modification of physical or chemical nature which would make them incompatible for their future utilization.

5. *Post irradiation microbiological control*

A microbiological control is carried out for each batch by means of "biological indicators" placed within or at the surface of the articles to be sterilized.

These indicators may be the articles themselves, voluntarily contaminated, or they may be constituted by a piece of material as close as possible in nature to the constituent of the article to be sterilized or of its wrapping, contaminated with a given number of desiccated spores of known radiation sensitivity *(Bacillus pumilus* E 601 or *Bacillus sphaericus* C_1A).

It is to be noted that such biological indicators prepared with 10^8 spores of either type are available at the "National Laboratory for Microbiological Reference".

A detailed procedure should be followed based on the fact that for a minimum dose of 2.5 Mrads all *Bacillus pumilus* E601 are destroyed. It is also possible to determine the inactivation factor of *Bacillus sphaericus* C_1A which should be approximately 10^3 in that case.

For minimum sterilization doses higher than 2.5 Mrad the inactivation factor obtained should be checked against a reference inactivation curve and should correspond to the required dose.

6. *Storage*

During storage the sterilized items should be protected from the effects of humidity and heat and from the risks of tearing or puncturing the wrapping.

The integrity of the wrapping should be checked before utilization.

The maximum duration of the storage period is two years running from the sterilization date.

7. *Labeling*

The labeling must be in conformity with the general international and national prescriptions.

In addition each package and each article must carry a label with the name and address of the irradiation facility, the minimum absorbed dose of ionizing radiation, the sterilization date (month, year), a number identifying the corresponding batch and the date before which the article must be utilized.

Conclusions

An important effort has been made in France to master all the potential hazards due to the use of

ionizing radiations. In particular it may be said that the practice of radiation sterilization is well controlled.

However this effort is continuing from the standpoints of both the nuclear safety and the quality of the products.

Extensive researches are undertaken for this purpose in C.E.A. laboratories.

As regards nuclear safety an institute specialized in nuclear protection and safety was created within C.E.A. at the end of 1976 (I.P.S.N.) in order to systematically study all the factors, technical and human, which are involved in potential failures.

On the other hand a better knowledge of the behaviour of materials under irradiation is necessary if one wishes to be sure that the irradiated materials will remain compatible with their medical or surgical utilization. Researches in this direction are under way.

Regulatory View Point — Egypt

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Introduction of radiosterilization technique in developing countries is stimulating the industrial development of the manufacture of single use disposable medical products with the potential for marketing of sterilized medical items at a reasonably competitive price.

Economic feasibility studies on transfer of the technology of radiation sterilization of medical products and biological tissues to developing countries should take into consideration the measurable anticipated saving in view of the considerable reduction in cross bacterial and viral infections particularly encountered during bulk vaccination requiring a long-period of hospitalization and excessive efforts and expenditure on treatment.

However, for successful transfer of such a technology to developing countries, efforts should be made to solve the following problems:

1. Production of medical items under poor hygienic factory conditions:

   It is known that equipment containing a higher number of microorganisms offers problems with regard to sterilization, pyrogenicity and toxicity. Although special products might, in some cases, justify special approval in spite of high contamination levels prior to irradiation, yet increasing the radiation dose should not, in ordinary cases, be taken as a measure of compensation for poor hygienic conditions at the production premises (1).

   However, production of medical devices of fairly low pre-irradiation basic contamination requires excessive efforts and basic modification on the part of developing countries. Under the present production conditions prevailing in many of the developing countries, and in order to avoid significant increasing of the required sterilizing radiation dose which would possibly affect the material itself, efforts should be made to investigate possibilities of radiosensitization of contaminating microbial organisms in order to attain perfect sterility using a reasonable radiation dose level.

2. Shortage in skilled personnel:

   Developing countries have to set up the principles for creating infrastructure for the transfer and adaptation of irradiation technology to local conditions of developing countries in the area of sterilization of medical products and biological tissues. This requires competent specialists and skilled technicians in the fields of irradiation processing, sterility and quality control, operation and maintenance of irradiation facilities, safety measures and radiation protection.

3. The socio-economical aspects of developing countries and the prevailing merits of their

societies.

Problems of lower standard of living and cost of medical disposables create problems in view of the utilization of disposable items for more than one time. Moreover, rough handling of packed medical products may affect their safety. Furthermore, the customers are obliged to deliver and collect their products at the exact time fixed by the irradiation facility in order not to disturb the working schedule of the plant or to block the available storage areas of the facility by unnecessary accumulation of finished products.

As recent microbiological investigations proved that protein nutritional status of the radiation exposed host would significantly influence the magnitude of organs damage and rate of their recovery (2), and in accordance with the trends of radiation technology transfer to developing countries suffering protein-calorie malnutrition and endemic parasitic infestations encountering haematological disorders, it seems of great importance to reconsider the maximum radiation dose levels permissible for occupational exposure for workers from developing countries in order to ensure adequate protection measures for them. It is well known that such permissible exposure levels have been drawn up based mainly on biological standard parameters common to developed communities enjoying good health and high nutritional status. National health authorities in developing countries, in collaboration with WHO, IAEA and interested developed countries, are invited to undertake an active role along this line.

For successful adaptation of such a technology to conditions of developing countries, the following parameters should be taken into consideration:

A. In the field of Irradiation Processing:

1. Official regulations for the manufacture of medical disposables should be formulated to ensure that medical appliances are produced under good hygienic conditions and subject to the survey and responsibility of competent specialists.
2. Microbiological sterility testing and quality control should be carried out continuously on a routine basis during the first 5 years of operation using well equipped microbiological laboratories belonging to the radiation facility. Selective confirmatory tests should be independently carried out by other specialized laboratories belonging to the universities and ministry of public health.
3. The efficiency of radiation dosimeters should be periodically evaluated. Effects of hot, dry or humid storage conditions should be investigated and taken into consideration for adequate calibration.
4. Attention should be made to deliver as much as possible the required sterilizing level as an integral dose, since fractionated doses with long time intervals could encounter possibilities of microbial survivals which could induce radiation resistant mutants of microbial strains.
5. Radiation source activity should be adjusted according to the actual need in view of the anticipated volume and density of medical products to be sterilized.
6. Radiation dose rate should permit the delivery of the required sterilizing level in a few hours time. Utilizing of decaying radiation sources delivering low dose rates would be less economic

and could be inadequate.

7. Critical evaluation of the proper sealing of packages should be made and subject to inspection and testing. Packaging material should be able to tolerate and able to withstand inadequate handling or long-term storage under adverse environmental conditions.
8. In view of the increasing interest on the part of developing countries to install multi-disciplinary irradiators, strict precautions should be made to eliminate any possibility for mixing of medical products with any other industrial or agricultural items to be irradiated. This would ensure elimination of any possible biological cross contamination in the irradiation chamber or in storage areas.
9. Atmosphere of irradiation premises should be kept clean especially against dust and sand particles which formulate a common problem to many areas of developing countries.
10. Optimal sterilizing dose level should be adjusted in view of the pre-irradiation microbial load of the medical products and the number of contaminating radiation resistant micro-organisms at a minimum dose level of 2.5 Mrad.
11. Complete filing of the medical product batch numbers should be made including detailed information on the measured absorbed radiation dose, dose rate, irradiation time, microbiological testing and date of receipt and despatch.
12. Supervision by the production manager of the running sterilization process utilizing visual aids as closed circuit television system and lead glass window fitted in a biological shield.
13. Concerned personnel of irradiation facility are fully responsible for the irradiation processing, including the production sites, for the quality of production and handling; the end users are responsible for the proper utilization of such radiation sterilized products.
14. Availability of necessary spare parts for the irradiation facility in stock in order to be able to meet on the spot with any accidental failure; by a competent maintenance group belonging to the facility personnel.
15. Restriction on commercialization of certain non-sterile medical products as glass syringes, catheters, surgical gloves, adhesives … etc.
16. Statistical evaluation of the magnitude of cross bacterial and viral infections through the introduction of radiation sterilized medical products to the country.
17. Critical supervision should be made on the disposal of radiation sterilized disposables after a single use. This should be of extreme importance in bulk vaccination carried out in public health hospitals and clinics. Mechanical damage of such disposables after being used could be the solution.

B. In the field of Radiation Safety:

1. Evaluation of the cumulative radiation levels to which the radiation workers have been exposed.
2. Periodic clinical examination of the radiation workers in collaboration with public health

authorities, for the assessment of any symptoms of radiation hazards.

3. Periodic calibration of radiation monitors and testing of the efficiency of biological shield, interlocks and other safety devices.
4. Strict supervision of the personnel to ensure that they always carry their radiation monitoring devices, such as film badges and pocket dosimeters, during the whole working hours.
5. Getting radiation workers aware about biological effects of radiation developed due to inadequate safety parameters.
6. Maintenance of enough ventilation inside the irradiation chamber to ensure a minimal concentration of ozone and its proper dilution before being despatched to the outside environment.

C. In the field of Manpower Development:

1. Getting the personnel from the production areas and irradiation facility as well as the end users aware of the technological requirements for successful application of radiation sterilization of medical products.
2. Maintenance of progressive educational development and training of personnel in different disciplines of irradiation technology.

Adoption of R & D Studies:

Developing countries would take part in the international R & D efforts made in the field of radiation sterilization of medical products, particularly along the following lines:

1. Radiation sensitization of contaminating microbial organisms and evaluation of the synergistic effect of radiation with other factors, e.g. heat.
2. Evaluation of radiation resistant bacterial strains in local environment and study of their dose-response curves.
3. Reconsideration of the maximum permissible level for occupational radiation exposure under local conditions of developing countries.
4. Evaluation of the possibility of irradiating certain pharmaceutical preparations and the radiation induced effects on their pharmacological response and chemical constitution under local storage conditions.

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Regulatory View Point — Australia

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Australia's attitude to radiation sterilization can be described in terms of the reactions of commerce, customers and Commonwealth authorities to this technology. Some of these reactions, which are based on up to 18 years' experience, are discussed.

Commerce

Industry has provided the capital to build three commercial radiation plants in Australia.

In 1959 the world's first commercial gamma irradiation plant was constructed at Dandenong, Victoria, for Gamma Sterilization Pty Ltd. This plant has had a chequered history. It was designed as a means of quarantine control for goat hair bales imported by the parent company to manufacture carpets. However, the carpet industry developed new technology — namely, the use of synthetics — which made the importation, and hence, sterilization, of goat hair redundant. The radiation plant continued operating as a general purpose irradiator, sterilizing a wide variety of medical goods and biological tissues on a contract basis until 1976. In that year, it was declared obsolete and dismantled, the cobalt-60 being sold and transferred to the Australian Atomic Energy Commission.

The shutdown of Gamma Sterilization Pty Ltd.'s plant was probably precipitated by competition. In 1971, an Atomic Energy of Canada Limited (AECL) JS6500 radiation plant was installed — also at Dandenong — for Tasman Vaccine Laboratory (Australia) Pty Ltd., a subsidiary of Tasman Vaccine Laboratory (New Zealand) Pty Ltd. These companies were acquired in 1976 by I.C.I. (New Zealand). Source strength is currently 510,000 curies of cobalt-60. Contract irradiations, chiefly medical goods, account for 90% of the throughput, with the company's own products, mainly dressings, making up the remaining volume.

In 1972 a third industrial radiation plant, also an AECL JS6500, was built. It is owned by Johnson & Johnson (Australia) Pty Ltd, Botany (New South Wales) and serves to sterilize only their own products or those of their subsidiaries. The construction of this plant culminated Johnson & Johnson's 10 years of experience with contract radiation sterilization.

Customers

The wide range of products now being gamma sterilized in Australia provides an index of the acceptance of this sterilization technique by both manufacturers and consumers.

Medical goods, with high volumes of syringes and needles, dressing packs, sutures, and rubber

gloves, supply 90 per cent of the throughput of the radiation plants. Other disposables being radiation sterilized include catheters, tubes, blood transfusion sets, bags, vials, petri dishes, swabs, etc. Some of these items are exported. A few topical pharmaceutical products containing radiation sterilized antibiotics are marketed, as well as some radiation sterilized ophthalmic and veterinary preparations, and lyophilized reagents manufactured by the Australian Atomic Energy Commission for complexing with the radionuclide technetium-99m, which is widely used in scintigraphy. These products have been described in more detail elsewhere (1). As well as sutures, some biological tissues such as fascia lata, veins, and arteries, are sterilized by radiation.

Interpreting "medical goods" broadly to cover cosmetics and toiletries, radiation is also used in Australia to sterilize a few raw materials used in manufacturing cosmetics, and to sterilize imported brushes, for example, shaving and cosmetic brushes.

Commonwealth

The official attitude toward radiation sterilization was set in Australia in 1959 with the acceptance of its suitability for quarantine control. It is still used for this purpose. To prevent the entry of anthrax, the Australian Department of Health recently stipulated that imported goods containing animal hair or bristles must be treated by an acceptable sterilization method. Unless the brushes are accompanied by a certificate to this effect, they are bonded until they have been gamma sterilized.

The Australian Department of Health has also been responsible for drawing up an Australian Code of Good Manufacturing Practice for Therapeutic Goods which was first published in 1971. The Code contains an appendix on radiation sterilization. This section remained unchanged in a recent revision of the Code. Briefly, it specifies that the minimum dose for radiation sterilization shall be 2.5 megarads. However, after application to the appropriate authority, permission may be granted to use higher or lower minimum doses if circumstances warrant it. As yet, companies have not availed themselves of this provision in the Code.

Development of Australia's multi-million dollar radiation sterilization industry has been actively promoted by the Atomic Energy Commission. Technical advice is freely available to potential customers who are encouraged to use the Commission's radiation facilities (spent fuel elements, 300,000 curies of cobalt-60, electron accelerator) to develop and assess the technological requirements for their particular products before implementing the process on a commercial scale. Specialized hospital equipment is sterilized for a nominal charge. Research contracts, on a fee-for-service basis, enable industrial organizations to employ Commission personnel to investigate specific problems. Training courses for radiation plant operators, with particular emphasis on health and safety aspects have been organized. A fortnightly calibration dosimetry service for users of commercial gamma irradiators is provided on a contract basis. A direct reading megarad meter (for the cericcerous dosimeter) was developed for calibration and routine dosimetry of radiation plants. The meter is being marketed under license by AECL. Research programs have studied various physical, chemical, microbiological and dosimetric aspects of radiation sterilization.

Summary

Australia has 18 years of experience with radiation sterilization. Currently, radiation from

1,000,000 curies of cobalt-60 and spent fuel elements is utilized by two industrial plants and the Australian Atomic Energy Commission to sterilize a wide range of materials for local use and export. Adherence to the provisions for radiation sterilization detailed in the Australian Code of Good Manufacturing Practice for Therapeutic Goods ensures that both the manufacturers of medical goods and their customers can be assured of receiving products that have been sterilized to a standard recognized by the regulatory authorities.

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United Kingdom Approach to Radiation Sterilization

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Sterilization by exposure to ionising radiation has been a recognised method in the UK since 1960 when the then Ministry of Health agreed to accept materials (absorbable sutures, etc., subject to Therapeutics Substances Act) which had been exposed to a radiation dose of 2.5 Mrad. The plants then in existence in the UK used cobalt-60 as a source and it was not until a few years later and after considerable investigation that the use of high energy electron sterilization was also accepted.

The basic principles for acceptance of this method laid down by an expert Committee in those early years have remained largely unchanged, but additional requirements have been made as experience dictated.

The minimum dose of 2.5 Mrad is accepted as sterilizing materials which have a minimum level of microbial contamination prior to processing. No figures have been attached to this qualitative statement as it is the firmly held view that the effort to reduce the presterilization contamination level should be a continuing process. This view is based on the fact that the majority of items sterilized by irradiation are produced using a high level of manual assembly. It is well known that the major risk of microbial contamination in any controlled environment is from people and that the incidence of risk is not predictable. Discussion of average "bioburden" figures must be based on sampling techniques which cannot take account of occasional high level contamination resulting from inevitable lapses in hygiene. The risk from variable contamination load on the raw materials of biological origin are equally unpredictable, e.g. unbleached cotton, wool and starch.

The major emphasis in the UK has thus been on the development of the highest possible standards for manufacturing conditions involving clean rooms, filtered air, protective clothing for operators and, above all, discipline on all hygiene matters and for the routine control of raw materials known to be subject to contamination.

Ensuring that the products which are to be irradiated have a low level of contamination is the responsibility of the manufacturer. The operator of the irradiation plant is responsible for ensuring that all the products are subjected to a minimum dose of 2.5 megarads and he is required to certify this to the manufacturer. The manufacturer knowing the presterilization contamination level or "bioburden" and the irradiation dose given can then claim that this product is sterile, providing of course that the packaging is satisfactory. Nowadays, apart from presterilization counts, no form of microbiological testing is normally required or considered necessary. The sterilization factor achieved when subjecting microbiological tests pieces to 2.5 Mrad doses has been proved repeatedly and these are not used except occasionally in the initial approval of exceptional or unusual products.

Proof that a dose of 2.5 Mrad has been applied to all items processed is obtained from the instruments which record the process and from the dosimeter readings. One dosimeter must be going

through the plant at any one time. It is vital that control over the accuracy of both instrumentation and dosimetry is maintained and this forms part of the quality assurance procedures of the plant. All plants regularly cross check their dosimeter readings with an independent laboratory or against a primary dosimetry system.

Positioning of the dosimeters is also important and it is usual to place them at the point of lowest dose found by experimentation using typical loads. If new material or loads of different densities are to be processed re-checking is of course necessary. It is usual also to record the overdose ratio in order that the maximum dose any material may receive is known. This may be of particular importance when new products or the use of new packaging materials is under assessment. Although irradiation is one of the most versatile of sterilizing methods in allowing the use of a wide range of material it is also one of the most time consuming from a product assessment point of view. Experience has led my Department to insist that certain materials, e.g. polypropylene, which are to be irradiated must be shelf life tested for a minimum of two years with no evidence of significant physical or chemical change before we can accept them. Materials which will not withstand a 2.5 Mrad dose can be very adequately and successfully sterilized by ethylene oxide.

Finally I must add that my comments are concerned with the irradiation of nonmedicinal products. Medicines controlled under the Medicines Act 1968 are subjected to individual assessment by the Committee on Safety of Medicines and they of course not only require proof of sterility assurance but also that the potency of the drug is unaffected by the process and that any degradation products are not harmful.

Regulatory Aspects of the Radiation Sterilization of Medical Products in Scandinavia

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In Sweden, an act governing the control of industrially sterilized articles for public health and medical purposes came into force on January 1, 1976. The statute applies to products intended for single-use in public health and medical care which are sterilized in conjunction with manufacture. It covers products used in direct treatment of patients as well as products used for other purposes in public health and medical care, e.g. certain laboratory supplies. Products regarded as drugs under the Drug Decree (1962:701) are excepted.

The provisions state that, "The manufacturer shall take those precautionary and other measures which are necessary in order that the products shall be sterile when used and which can be considered reasonably called for in order to prevent or counteract their causing injury when used." This includes all properties such as sterility, mechanical and other properties of the material, freedom from biological risks (toxic compounds), package and fitness of the product for its purpose.

According to the principles of the statute:

1. There is no system of approval of a product or of a manufacturer. There are no fees to be paid by the manufacturer.
2. The manufacturer is responsible for his products. He or the importer must inform the National Board of Health and Welfare about the products marketed and, on request, furnish the Board with documentation.
3. The National Board of Health and Welfare

   a) exercises the supervision of the law,

   b) may issue standards and make inspections,

   c) may issue injunctions and prohibitions, and

d) may institute public prosecution for offenses of the law.

Thus, the manufacturer carries the entire responsibility, but is required to notify the authorities of the product marketed. The products are not registered as approved or not approved. The idea of the regulations is simplicity; no time delay in registration, no extra costs on the products to pay for the controls. The control system is diagrammed in Figure 1.

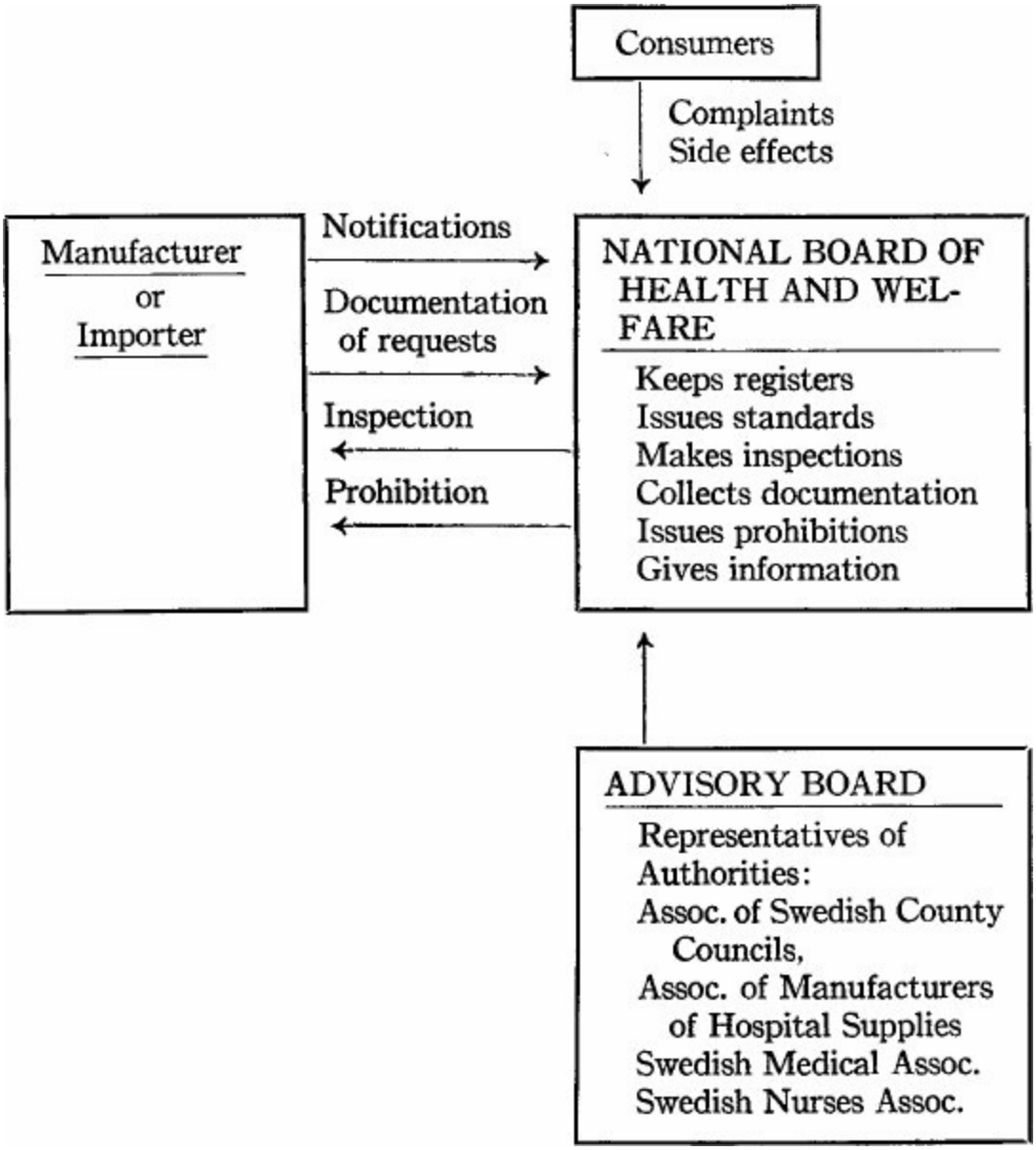

Figure *1.* The Swedish control system of industrially sterilized articles for public health and medical purposes.

The following list may serve as an example of the notifications that must be made by the manufacturer or importer, as far as the sterilization procedure is concerned:

Presterilization counts
Place of sterilization
Method of sterilization
Method of control of the sterilization process
Sterility tests of final product (according to the standards issued, sterility tests are not requested for radiation sterilization)

Testing laboratory.

The drug legislation in Sweden is different from that of sterile disposables. Drugs must be approved and registered. Certain industrially sterilized single-use articles are considered as drugs and must be registered. These include single dose syringes used as containers for drugs and any article which is packaged or delivered with the drug, e.g., infusion sets, disposable syringes, eye drop applicators.

The quality of drugs is regulated by the Nordic Pharmacopeia. The Nordic Pharmacopeia recognizes the following group of pharmaceuticals: sterile, aseptically produced and others (non-sterile).

I would like to strongly underline that drugs can only be labelled as sterile if they are subjected to an active sterilization process in their final containers, e.g. by saturated steam, ionizing radiation or ethylene oxide. Filtration, for example, is *not* considered as a sterilization method.

For Sterile Preparations the following are requested:

1. Manufacturing procedures aiming at no more than one non-sterile unit per million.
2. Test for microbial contamination before sterilization.
3. Microbial test of sterilization procedure.
4. Should conform with the sterility test.

For Aseptically Produced Preparations the following are requested:

1. Sterilized raw materials.
2. If (1) is not possible, sterility test of raw material.
3. If (1) or (2) is not possible, 100 microorganisms/gram in acceptable if the final treatment kills or removes the microorganisms.
4. Aseptic working facilities (laminar air flow, etc.).
5. Manufacturing and filling procedures should be regularly checked microbiologically.
6. The most effective method for removing and/or killing microorganisms should be used.
7. Sterility test should be carried out on each batch.

I would like to draw your attention to the strict definition of sterile preparation. This, I think has bearings on the discussion here; whether one could accept different degrees of sterility. For drugs labelled as aseptically produced, the level of microbial cleanliness corresponds to about 1:100 — 1:1000 nonsterile units versus 1:1,000,000 for drugs labelled as sterile.

Denmark

In Denmark, there is a new Drug Decree in force since January 1, 1976. A paragraph on medical utensils is included in the Decree. The paragraph gives the right to the Minister of Interior to issue statutory provisions that the law or part of it shall apply to products intended for use in the prevention, diagnosis or treatment of disease. An Advisory Board has been appointed, but the government has not yet issued the statutes governing the control. It is reasonable to believe that the present voluntary registration of approval will be succeeded by a compulsory registration. This compulsory registration for approval may not necessarily cover all but the most important medical utensils from the sterility point of view — at least in the beginning.

Norway

In Norway, since 1960, approval and control of special medical utensils have been required, i.e. for blood donor, transfusion and infusion sets. Provisions governing the control of other medical utensils are in preparation.

The control of these other products will not be as stringent as for the afore-mentioned groups of products, but the quality will be requested to be on the same level as for Denmark and Sweden.

As the capacity for official control is limited, priority will be given to new groups of medical devices and to those products which by reports from the consumers or for other reasons, are suspected to be below standard.

Common trends in Scandinavia

It should be obvious from this presentation that important differences exist between the regulations for medical devices in the three Scandinavian countries. For pharmaceuticals, on the other hand, the regulations are almost identical.

In Sweden, the authorities will be engaged only if there are specific reasons, e.g. through the system of reporting side effects and complaints from the consumers or if the information from the producer according to the compulsory notifications give rise to suspicion that the product does not comply with the standards issued.

In Denmark, the producer may request official approval of medical devices on a voluntary basis. The Drug Decree gives the possibility, however, to apply compulsory registration.

In Norway, there is compulsory registration for some critical devices. Regulations concerning other devices will come.

It should be emphasized, however, that although the official regulations are different, the quality levels applied by the authorities are very similar in the three countries. The standards for production hygiene, production control and sterilization control are identical or almost identical. Sterile medical devices are defined in the same terms, i.e. devices prepared and sterilized under conditions which are designed to ensure that the final product does not contain more than one viable microorganism per one million units.

The Nordic Pharmacopeia is common to all three countries until January 1978 when all these will change to the European Pharmacopeia.

Official biological indicators are used in Scandanavia according to the Nordic Pharmacopeia. Denmark, Norway and Sweden utilize the same preparations of biological indicators. The indicators are distributed by the National Medical Microbiological Institutes.

I would like to particularly emphasize that the indicators are based on the principle that sterilization methods, whether it be saturated steam, ethylene oxide or radiation, shall produce the same safety as to freedom from viable microorganism on the products (that is, not more than one viable microorganism in one million units of the product).

The official biological indicators are intended for control of hospital sterilization as well as for industrial sterilization. We have the ideal position of being able to influence hospital sterilization as well as industrial sterilization. The situation in our countries is that hospital sterilization is generally not inferior to industrial sterilization. For industry the official indicators are intended to be reference

preparations, thus not for routine use. It seems more rational for industry to design indicators that are more suitable for routine checking purposes.

The Scandinavian official biological indicator for saturated steam conforms with the USP performance characteristics. The ethylene oxide indicator complies with the USP as well, provided that the sterilization process includes a humidification procedure that rehydrates even very dehydrated microorganisms which are likely to occur in the dust of production premises.

In radiation sterilization, we do not recommend the routine use of biological indicators. The physical or chemical dosimeters are more accurate and less expensive. We recommend that the minimum dose be related to the requested safety margin (e.g. one in one million), and to the number and resistance of the naturally contaminating microorganisms as based on examination of the products in question. In addition, we request that the antimicrobial efficiency of the dose used be compared with the efficiency of a reference plant. Therefore, we actually supply microbiological reference preparations consisting of highly radiation resistant microorganisms. These preparations can be used as biological monitors or dosimeters by assaying surviving microorganisms after exposure to different doses and by plotting the values in dose-response curves.

Concerning the choice of radiation dose, with low initial counts, a minimum dose of 3.5 Mrad (3.2 Mrad for Co-60 irradiators) generally gives a satisfactory safety margin. If, however, continuous determinations of the radiation resistance of the initial microbial contamination shows that a minimum dose of 2.5 Mrad is sufficient to attain a satisfactory margin and the bioburden is proved to be consistently very low, this dose may be used.

Panel
Questions and Answers

To: J. R. PUIG — France, by: J. MASEFIELD — U.S.A.

Q. How do the French define a batch in a continuous irradiation process?

A. As I said in my paper, a batch is considered as a series of individual packages irradiated continuously, one after the other without any interruptions and, of course, in the same facility, for a continuous period of time, not exceeding 24 hours.

To: J. R. PUIG — France, by: J. MASEFIELD — U.S.A.

Q. What is the shelf life for ethylene oxide sterilized products?

A. I am sorry I cannot answer that. The shelf life of radiation sterilized products is now limited to two years in the French Pharmacopeia because the responsible authorities do not have enough evidence that it can safely be extended beyond two years. The shelf life was previously limited to eight months in the requirements in force before October 1976. This means that the regulation on this subject is amended whenever it is necessary. I think that the limit of the shelf life will be extended as soon as full knowledge of the long term storage is available.

Comment by Chairman,
A. BISHOP — England

Could I exercise a chairman's privilege and say to Mr. Masefield this is a very odd question. If the thing is sterile and if it is properly packaged, it should maintain its sterility indefinitely.

Comment by
J. MASEFIELD — U.S.A.

That is really why I asked the question. I was puzzled as to how shelf-life can be assigned when it surely would be a function of the constitutents and their age, whether they were going to suffer from long range degradation as a result of aging and the particular type of packaging being used. The question was to provoke, to express my puzzlement at any statements that said that all things irradiated should have a specific shelf-life. Since we are speaking of sterility at this Conference, it seemed relevant to see if this was a requirement purely for radiation sterilization or whether this statement applied to all other types of sterilization and to see what sort of correlation there might be. On the first part of the question, since the radiation sterilization parameters do not change over a 24, 36 or a 48 hour period of

continuous operation, is the 24 hours an arbitrary definition of a lot?

A. Yes, all these figures are based on the available experience which was gained so far. And I should emphasise that all these regulations are still in the stage of transition. They may be amended and modified. As one example, I mentioned at the beginning that, at first, since the people did not know exactly how long these articles could be stored initially, a limit of eight months storage was set. Now it is two years and presumably, if one gets sufficient experience that these articles can be stored for a longer period of time, this storage time might be extended.

Comment by Chairman,
A. BISHOP — England

I think that one could add that long experience of failures of single use plastics in hospitals suggests that they ought to be date-stamped for reasons not particularly connected with sterilization, but degradation.

Comment by
J. R. PUIG — France

There are two components. The limitation is not directed particularly at the sterilization itself, but rather, the behaviour of the material. Some additional knowledge is required on the long term behaviour of the irradiated materials.

To J. R. PUIG — France, by: K. H. PETER, — Germany

Q. Why does the French Pharmacopeia prescribe that the irradiation of a given product with a given dose must be uninterrupted? Publications by Ley show clearly that interruption of dose delivery, with storage intervals between fractions up to one week, has no influence on the sterilization effect. It is practically impossible to run a gamma irradiation plant continuously.

Comment by
J. R. PUIG — France

Is that a comment or a question?

Comment by Chairman,
A. BISHOP — England

Why does the French Pharmacopeia prescribe that the irradiation of a given product with a given dose must be uninterrupted?

A. This does not mean that a given package must be continuously irradiated. Of course, this is generally so. We are not contemplating to irradiate the package with first a given dose and then re-irradiate the same package. I think this regulation originates just from technological consideration. You can consider that the parameters of the facility are set and can be controlled for such a given period of

time.

To: J. R. PUIG — France, by: K. H. PETER — Germany

Q. The number of dosimeters prescribed by the French Pharmacopeia seems to be exceedingly high. International practice is to have at all times a maximum of two dosimeters inside the irradiation cell in plants of the continuous type.

A. I cannot answer this. This is just the regulation which was derived from the experience of the people who carry out this kind of processing in France.

To: F. J. LEY — England, by: C. ARTANDI — U.S.A.

Q. Why must one dosimeter go through the plant at any time?

A. Because we have in mind, if an inspector visits the plant he can actually see a dosimeter which is being irradiated in the course of the day. A very simple arrangement and it covers all exigencies.

Comment by
C. ARTANDI — U. S. A.

I asked for a logical reason and I would like to see how the inspector can inspect the dosimeter while it is being irradiated. My concern is that we are creating the myth that if you have one dosimeter at any given time in your cell you are safe. You know what goes on. I am saying that you check your process daily or every shift or whatever frequency satisfies you that the conditions are right. This fable of having one dosimeter at any given time in the irradiator does not make any sense because if you assume everything that we say about radiation, that there is constant emission of irradiation, the speed is controlled, the geometry is controlled, the product is controlled, then the results should be the same. If you test it once a day, that should be enough.

But the point really is that there is some basic work, which you have to do with your products. If they have a variety of densities, you have to test out what is the maximum density product and what is the effect of your process on the maximum density product so that your range of maximum and minimum is in the right place. That you can do even with infrequent dosimeters and on a control chart. But to talk about statistical sampling of dosimeters, is gilding the lily and is contrary to twenty years experience.

Comment by
F. J. LEY — England

I did take it for granted that everybody accepted that dosimeters are used in large numbers and that each batch has got dosimeters at the beginning, the middle and at the end. There is a large number of readings done. The comment about the one dosimeter is only a part of our procedure. Irrespective of the wholesale dosimetry that we do, there is always at least one dosimeter in the plant at any time.

Comment by Chairman,

A. BISHOP — England

I think this is absolutely right. We would not wish to make a mountain out of this and, in fact, when we are looking at the dosimetry in a new plant. we are obviously looking at it in total.

To: M. DUNCAN — England, by: J. MASEFIELD — U.S.A.

Q. Do you feel you have adequate statistical proof that, for example, complex hospital packs, large in size, with many folds, can be sterilized with ethylene oxide gas to the same probability levels as those obtained by doses even lower than 2.5 megarads?

A. Yes.

To: L. O. KALLINGS — Sweden, by: K. H. PETER — Germany

Q. The Swedish Act applies only to single use devices. How do you treat:

a) devices intended for *repeated* use, but delivered sterile to the user?

b) devices intended for *repeated* use, but delivered unsterile to the user?

A. The legislation is new. We have worked with that for a little more than one year and we are working with industry. There is our Advisory Board to the National Board of Health and Welfare and there is also an association for industry, so we have endeavored to find out some of these more tricky and practical questions. I think that one should not put too much emphasis on the single use, but rather if the device can be used repeatedly. But I am not sure what you are referring to. Could you give me one example?

Comment by
K. H. PETER — Germany

For example, endotracheal tubes made from red rubber which the hospitals in recent times are more and more interested to have delivered to them sterile. They say the sterilization process done in the factory does less damage to the instruments than the steam cycle used in the hospital.

Comment by
L. O. KALLINGS — Sweden

Then I understand. In such a case I think the law would be applicable. It would be applied to the first time it is sterilized. If it were to be sterilized in the hospital, then it comes under the other regulations concerning the sterilization in hospitals which we have not discussed here. I think it is applicable as a single use item.

Comment by Chairman,
A. BISHOP — England

Could I ask at this point, what is the point of supplying sterile an article which is going to be reused?

Comment by
K. H. PETER — Germany

We believe that the process we apply in the factory is far more controlled than the process which is applied in the hospitals. They have to perform certain cleaning operations which also cause greater cost to the hospitals than do items which are delivered sterile. You said all these instruments which are delivered into your country as single use devices have to be reported. What if endotracheal tubes were imported unsterile into Sweden?

A. In such case they would not come under the law. If they are not labelled as sterile, they will not be considered under this regulation.

Comment by
K. H. PETER — Germany

But your rules also apply not only to the sterility, but also they must function. You have apparently a loophole in your regulations.

Comment by
L. O. KALLINGS — Sweden

Yes, we know that. There are other products used in hospital which should be checked for their toxicity and functional properties. There is no rule for that for the time being, but we are attempting to cover that loophole also.

To: A. S. OUTSHOORN — U.S.A., by: F. J. LEY — England

Q. You refer to treatment of products with an average dose of radiation at the approved level. It is more usual to apply the approved dose as a minimum dose and to accept overdosing according to the density of the product. I must say that we do not accept overdosing.

A. I really do not have a comment. This was really a way of putting it because, if the dose is applied unevenly then one would have to make some kind of investigation beforehand to make sure that the adequate amount was delivered to low dose points. This is really a round about way of saying that you distribute the objects to be sterilized as evenly as possible, apply the dose as evenly as you can and this is really all I can say. It is not supported by a great many experimental results.

To: J. R. PUIG — France, by: K. H. MORGANSTERN — U.S.A.

Q. With respect to the production dosimeter requirement, would "go-no-go" dose indicators, such as Avery® labels, be acceptable under present French regulations?

A. No, I cited two points in the regulation. First, is the obligation to use these so-called "go-no-go" dosimeters so that you can tell if a package was irradiated or not, second is the need for carrying out the on-line dosimetric control which was described before.

Anonymous Comment

There are situations in fact, one in particular, that we are aware of where individual products will be sterilized in quantities upwards of 10,000 per hour. It is going to be patently impractical to use dosimeters in that quantity.

Comment by
J. R. PUIG — France

When this situation occurs, the regulation will be modified and improved. As I said, this is a recent regulation we have in France and it is constantly being up-dated. But I would like to refer to the case of electron beam irradiation. In that case it is required that every package has its own dosimeter. They are producing very large quantities of irradiated products and still they found out a way to carry out this dosimetry. I think that the trend is to make the dosimetry more and more automatic.

To: PANEL, by: A. CHARLESBY — England

Q. Is there any information available on the occasional failure of the packaging material?

Comment by Chairman,
A. BISHOP — England

The answer to this of course is that there is a great deal of evidence that half of the packages you find in hospitals are torn, or folded, or something. I would think that the answer to that question is that if the packaging is done right and is treated with respect that a sterile product has a right to be treated with, we will not have any trouble.

Comment by
M. DUNCAN — England

Could I make one comment that is equally applicable to irradiation or ethylene oxide gas sterilized products. Practically all of the failures that we have had with packaging have been seal failures which had nothing to do with the method of sterilization. They have had to do with the sealing equipment.

Comment by Chairman,
A. BISHOP — England

One of the most recent failure of seals is the result of a thing called vacuum stress. This is the cycling of first vacuum and then pressure when you use the ethylene oxide. You have all heard of the expression "blown packages." We find them very frequently in the the hospitals. That is caused by the sterilization process.

Comment by
M. DUNCAN — England

I think this is a fair comment that we had equally large number of seal failures with irradiated packages. What I am saying is that the seal is the thing that fails and perhaps the sterilization method has something to do with it, but you cannot say irradiation is a great deal better than ethylene oxide. The fact is that seals fail equally.

Comment by
A. CHARLESBY — England

I am not sure whether I am answering the question or raising a supplementary one. I ought to explain why I have asked this question. It is simply that, in practice every time I buy anything in shops, quite often the package is open or contaminated. I am talking about sterilized material. Packages are very sensitive to handling. If this is true for the type of medical devices we are talking about, sometimes this will happen. If there is any leakage of bacteria into the system after the seal is broken, this represents the maximum sterility we can get. There is no point in arguing whether it can be 10^{-6}, or 10^{-9} or 10^{-12}, if one in a thousand packages is torn, what we should discuss is the overoll sterility in use and not the theoretical sterility.

Comment by
E. A. CHRISTENSEN — Denmark

I may be able to give you a more reasonable answer to the question. We can demonstrate one leakage per hundred items in some packages. This is not good. But of course, we have the possibility of using several layers of packaging material for the equipment in the hospitals in Scandinavia, for example, 3 or even 5 layers of packaging material. In such cases, we can have any degree of security we wish.

Comment by
O. HEISENBERGER — Austria.

I missed a vital point in this discussion, that is independent from sealing defects. If the user can check whether the package is defective or not, we have a high security. With irradiation sterilization you can use air-tight packaging. If you have flexible packaging materials and you apply vacuum to the package, the user can easily see when there is a hole, a pinhole, or a defect in the seal because the package is not growing thick. This is high security in my opinion. This is the advantage of irradiation sterilization because you cannot use air tight packaging in ethylene oxide or steam sterilization.

To: M. DUNCAN — England, by: M. STEPHENSON — Canada

Q. Ms. Duncan said that the determination of average bioburden relies on sampling and cannot take into account occasional high levels. Surely, if the bioburden program takes into account frequency of distribution of counts on items, occasional high levels will be taken into account in calculating the required dose?

A. First, I should say I am no statistician but, as I understand the argument against sterility testing, it is

not possible to pick up the very low frequency of contamination if you are doing sterility testing because of the pure statistics of the sampling. You cannot talk about frequency of distribution, if you have an infrequent and unpredictable number of contamination occurrences. It is equally impossible to predict what these high levels of contamination will be and, therefore, you cannot really say when you are going to get the very occasional, very high level of contamination.

The Future of Radiation Sterilization

K. H. Morganstern

Radiation Dynamics, Inc., Melville, New York, U.S.A.

Abstract: *The applications for ionizing radiation, including sterilization, are entering their third decade. Although substantial progress has been made on a number of fronts, it is expected that rapid acceleration will take place from this point on. This is due in part to the energy shortage and the recognition that radiation processing is less energy consumptive, provides lower costs, and a better understanding of the interaction of radiation on various meterials. In addition, and with respect to the sterilization of medical disposables, it is expected that radiation will take over a number of the sterilization applications presently using ethylene oxide gas because of radiation's many advantages; i.e., better sterility assurance, better control, no residuals, and less environmental impact.*

This paper reviews the past and present situation in the radiation sterilization field and projects those areas of future growth.

An appraisal is made of the advantages and disadvantages of gamma and electron beam radiation with the conclusion that both have an important role to play in proving the efficacy of this technique.

Introduction

In trying to organize my thoughts on the subject of the future of radiation sterilization, I found myself faced with a real dilemma. Since radiation sterilization is now entering its third decade, it certainly is not new and over the years many experts had already predicted its "rosy" future; in fact, just three years ago, at Johnson & Johnson's First International Conference on Sterilization by Ionizing Radiation (1), many of the speakers concluded, rightly so, that the growth of the radiation sterilization industry was practically assured.

So, consequently, what is new? What can be said now that is different?

For starters, since the last Johnson & Johnson meeting:

- The world's population has increased by a few hundred million.
- The world suffered a severe economic recession — exacerbated by an oil embargo.
- The cost of crude oil has practically quadrupled.
- We are now aware that energy is no longer an infinite commodity.
- Our environment has continued to deteriorate.
- And, there is now a greater awareness of our delicate ecological system.

And, all of these factors impact radiation sterilization and its future.

However, before trying to assess these affects on the future of radiation sterilization, let's step back and try to view this aspect of radiation as it relates to the whole fabric of the radiation processing industry.

Radiation Processing

After the tremendous investment made in atomic weaponry during World War II, it was quite natural to seek humane and peaceful benefits that would be derived from the same basic technology. Nuclear power was obviously one; the use of ionizing radiation, although a poor second cousin, was another. In this latter case, radiation was viewed as a tool, both medically and industrially, and in both small and large quantities. (See Table I.)

Table I. — Radiation as a tool indicating both medical and industrial applications with isotopes and machine-produced radiation.

	Application	
Quantity	Medical (Biological)	Industrial
Micro-curies	Isotope Tracers (Thyroid Uptake, Tumor Localization, Etc.)	Tracer Techniques (Wear Studies, Flow Measurements Etc.)
Curies	Therapy	Radiography
Megacuries	Sterilization	Sterilization

In those early days ("the tantalizing stage" as Niels Holm aptly put it), great predictions were made. For example, Sterling Cole, Director General of the International Atomic Energy Agency, at the beginning of the IAEA 1st Conference in 1959 on "Large Radiation Sources in Industry", stated "Uses of radiation in industry are among the most effective ways in which atomic energy can help economic development." (2) Paper after paper pin-pointed practical advantages of radiation in rubber vulcanization (3), cross-linking of plastics (4), polymerization of chemical systems (5), synthesis of graft copolymers (6), radiation thermal cracking of normal hydrocarbons (7); at the same time, and related to this meeting, papers were published on the inactivation of virus, sterilization of pharmaceutical and hospital supplies, sterilization of insects, and "the old standby", preservation of food (8–11).

In spite of great expectations, industry was slow to embrace radiation processing. In fact, as an aside, the medical profession and the kindred health care industry became the radiation champions. The use of isotopes both as a diagnostic tool and for therapy has continued to grow at a respectable rate since the early 1950's. And the health care industry, as evidenced by this meeting and the countless others before, has certainly made its mark in the radiation field.

Although Table I indicates in a simplistic fashion isotope applications, one can overlay it with machine-produced radiation in the shaded area.

However, stimulated by increasing energy costs and lower radiation costs, other industries have slowly started to fulfill those earlier predictions. Today the list of practical applications is sizable and growing as evidenced in Table II. It is interesting to note that in all but three applications (sterilization, food, and plastic composites) particle radiation is the technique of choice. The reasons are primarily economic (12).

Table II. — Practical radiation processing applications.

- Upgrading Of Polymeric Insulation On Wire And Cable.
- Crosslinking Of Polyethylene Foam For Better Foam Control.
- Crosslinking Of Polyethylene For Improved Stress-Crack Resistance.
- Vulcanization Of Sheet Rubber.
- Curing Of Organic Coatings On Plastic, Metal And Wood Substrates.
- Curing Of Glass Reinforced Polyester Sheets.
- In Situ Polymerization Of Plastic In Wood And Concrete.
- Curing Of Adhesives.
- Irradiation Of Wood Pulp Chips To Increase Pulping Yield.
- Improving Switching Speeds Of Semi-Conductor Diodes.
- Sterilization Of Medical Disposables.
- Ion Implantation In Semi-Conductor Materials.
- Extension Of Shelf Life For Food Stuffs.

As for the future of radiation processing in general, I believe time is in our corner. The factors cited above, namely, increasing energy costs and lower radiation costs, should continue to weigh in favor of the radiation method. In addition, as radiation continues to be "accepted" — other psychological factors diminish.

With this as background, let's examine radiation sterilization, past, present and future.

Radiation Sterilization — Past

As mentioned earlier, the application of radiation for sterilization was one of the early "radiation winners". And in point of fact, the application had its beginning prior to the advent of atomic energy. Table III enumerates some of the milestones in the growth of this application. Others include:

- the first commercial radiation sterilization activity on the Continent in 1958 by the Danish AEC using Cobalt-60 (Co-60) and transferred to a linear accelerator in 1961.
- initial commercialization of radiation sterilization in the USSR with Co-60 in the early 1960's.
- in 1964, the first commercial plant in Czechoslovakia using a DC accelerator.
- full scale plant operation in the Federal Republic of Germany in 1967 with Co-60.

What is evident from this historical overview is that both Co-60 and machine radiation played a role in the development of this field. However, as the process became more widely used, it is fair to state that Co-60 has dominated the scene.

Table III. — Radiation sterilization milestones.

1895 — X-Rays Discovered.
1896 — X-Rays Shown To Kill Microorganisms.
1930 — Exponential Relationship Demonstrated Between Dose And Kill.

1956 — Ethicon Radiation Sterilizing Sutures Commercially With EB.

1960 — UKAEA Demonstration Gamma Facility Operational At Wantage.

1960 — First Commercial Gamma Plant Built In Australia For Sterilizing Goat Hair.

1964 — Ethicon Establishes Commercial Gamma Sterilization Facility In Edinburgh.

1975 — RDI Opens World's Largest EB Service Facility (7 Million Curie Co^{60} Equivalent) For Medical Product Sterilization On Long Island.

Radiation Sterilization — Present

The growth of radiation sterilization up to the present is presented in Figure 1. It shows the growth of both gamma and electron beam techniques. It should be noted that the graph is a plot of the nominal plant capacity for both Co-60 and machine facilities. If installed capacities were used, the Co-60 curve would be considerably lower. Figure 2 shows the geographical distribution of facilities as per cent (%). It is evident that the major portion of gamma sterilization is in Europe, whereas the U.S.A. presently occupies that position for electron beam sterilization.

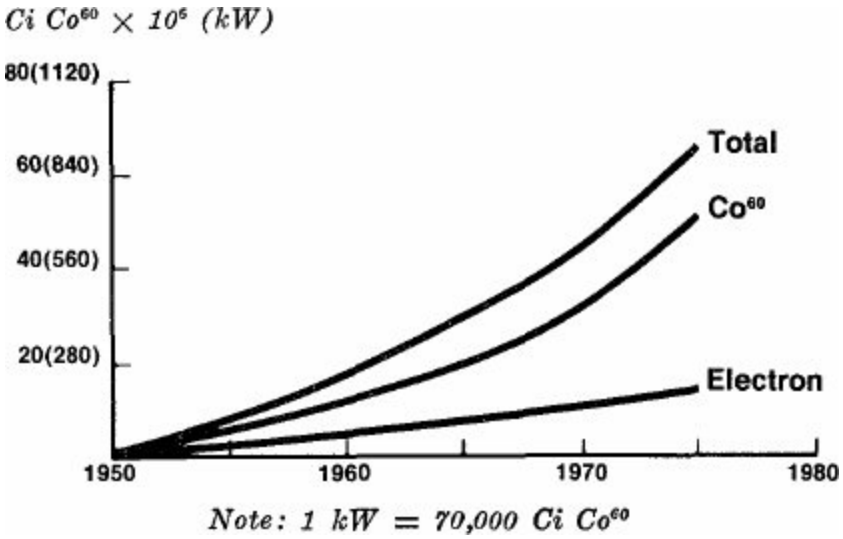

Figure *1.* Growth of radiation sterilization.

It is not necessary to reiterate the number of medical products presently being radiation sterilized for this has already been described in numbers of papers (13). Needless to say, the list is long and growing.

What is perhaps more meaningful for gaining a perspective on the future is to identify the industrial areas where radiation sterilization is or can be employed, to make judgments on the likelihood of radiation being used, and to attempt to predict the growth patterns if and when they develop.

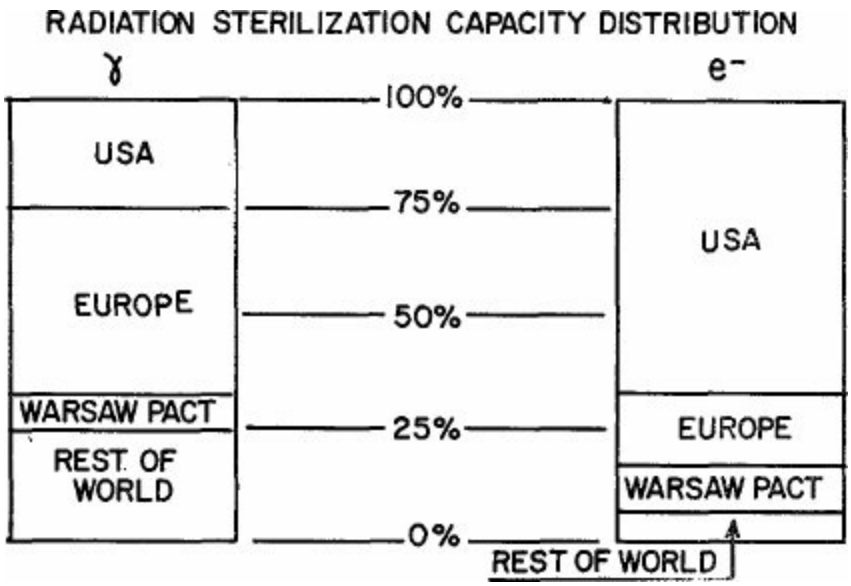

Figure *2.* Worldwide geographic distribution. In absolute quantities, the gamma capacity is approximately three times that of the electron capacity.

At Johnson & Johnson's first conference, Mr. Frohnsdorff conveniently indicated the main sterilizing areas as (14):

- Medical Devices
- Pharmaceuticals
- Vaccines and Sera
- Agriculture
- Biological Tissues and Related Materials
- Hospital Sterilizers
- Toiletry and Cosmetic Products
- Food
- Waste and Effluent Treatment

Using this as our roadmap, let's examine the future.

Radiation Sterilization — Future

Earlier it was mentioned that the changes that have occurred in the world since 1974 have had and will continue to have an impact on the radiation sterilization field. Let's examine this statement in a little more detail.

Obviously, the growth of the world's population means increasing demands on the health care industry. This growth is further accelerated by the evidence that the developing nations are following the direction of the more developed countries and turning increasingly to single-use products.

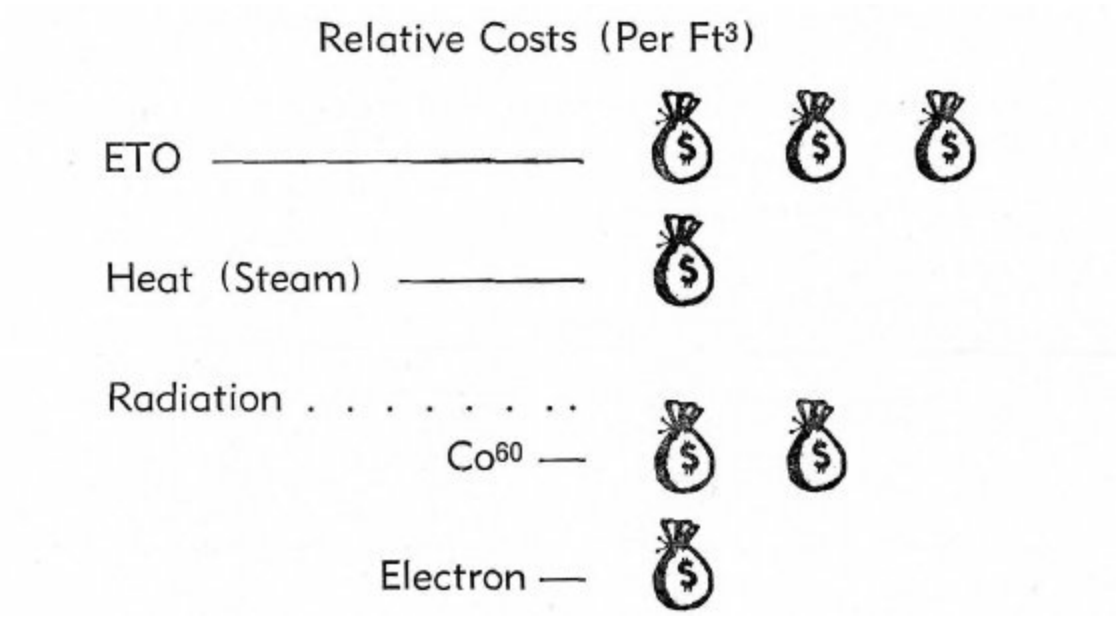

Figure *3.* Relative costs of various sterilization techniques.

The economic recession, as is the case in all recessions, put demands on industry to "shave costs" and try to improve profit margins. This, coupled with the higher cost of ethylene oxide (ETO) due to crude oil's astronomical rise in price, has led device manufacturers to look for less expensive alternatives to gas sterilization. (Figure 3)

Not only are today's enlightened manufacturers interested in cost effectiveness but also in energy conservation — recognizing the finite limitations of energy resources. And here, too, radiation looks good. (Figure 4)

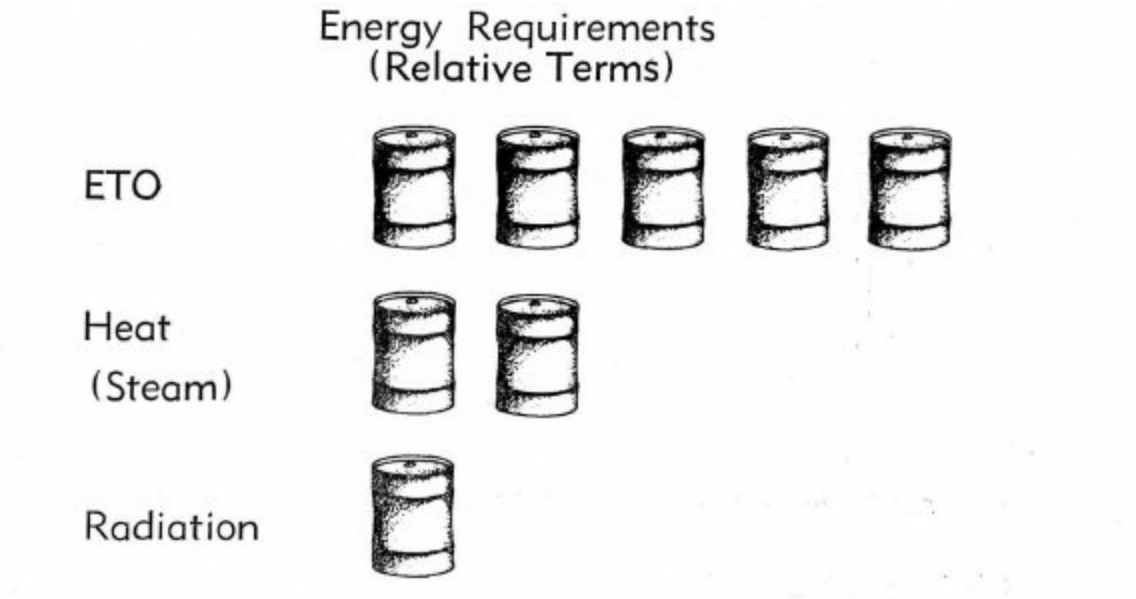

Figure *4.* Energy input for sterilization techniques.

In addition, the environmental concern, coupled with toxic residuals as related to ETO sterilization, furthered the advantages of radiation versus ETO.

And finally, with the point repeatedly made at this and earlier meetings, and confirmed with the passage of time, that one has better sterility assurance with radiation, we have the "capstone".

Now coming back to Mr. Frohnsdorff's list —

Medical Disposables

Certainly, the largest single application area now and in the immediate future for radiation sterilization relates to disposable medical devices (including laboratory wares) — products used once and then discarded.

In 1976, the market for disposables in the U.S.A. was estimated to be approximately $1,000,000,000 with a projected growth to $2,300,000,000 by 1985 (15) — yielding an annual growth rate of approximately 9.5%. The size of the market for the rest of the world is estimated to be currently $2,000,000,000; at the same growth rate, the world's total market by 1985 would be approximately $7,000,000,000. Needless to say, that would represent a sizeable radiation requirement if all such products were to be radiation sterilized.

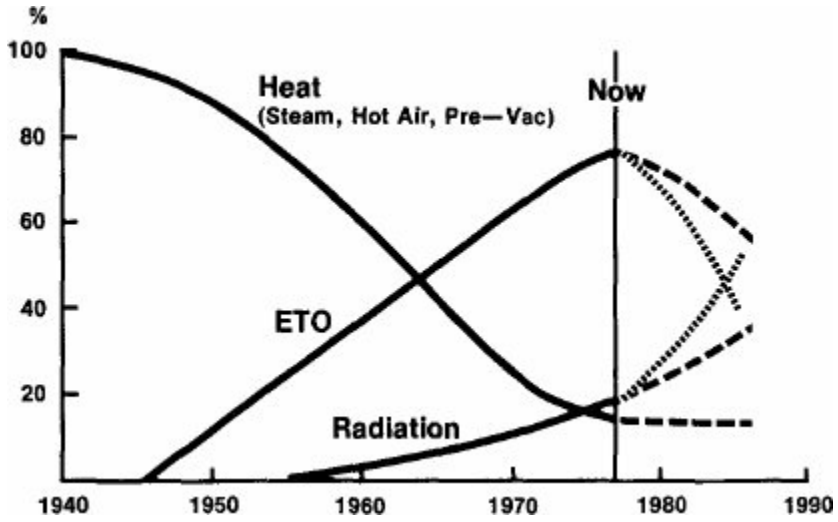

Figure 5. The percentage of sterilization techniques as a function of time.

Obviously, that will not be the case. Figure 5 shows what I believe is the present situation between the various sterilization techniques in the U.S.A. The big unknown is not whether ETO will diminish as a per cent but only how fast. Its decline could be heavily influenced by regulatory restrictions — based on toxicity as well as environmental considerations.

It now appears evident that the decline of ETO sterilization will be balanced by the growth of radiation sterilization as the viable alternative. It is interesting to "crystal-ball" gaze and consider what the consequences of ETO's demise would be on the radiation industry.

An educated guess puts ETO sterilization volume today in the U.S.A. at 500,000 to 1,000,000 cubic feet/day. If we choose the mid-point of 750,000 cubic feet/day and use 300 production days/year, there is a requirement to sterilize 225,000,000 cubic feet/year of products. At 1 cubic foot/curie, this means a requirement for 225,000,000 curies of Co-60 — considerably above the annual production rate for Co-60 projected by the present producers (16). This requirement would equally tax the production capacity of the world's accelerator manufacturers — assuming a substantial portion would go the electron beam route. In fact, just the 9.5% growth rate of the medical disposable industry turning 100% to radiation sterilization from this point on would represent a sizable increase in both the cobalt and accelerator manufacter's level of U.S.A.'s activity. (A recent meeting in Belgium on the "European Market for Hospital Disposables" indicates volume requirements similar to those in the U.S.A.) (17).

Pharmaceuticals

Although, as evidenced by papers at this meeting, major activities continue to be aimed at radiation sterilization of pharmaceuticals, the problem remains a complex one.

Recently, Fleurette, Madier, and Tronsy reported on 33 antibacterial and antifungal antibiotics (18). Irradiated in the dry state, 31 maintained normal activity and two showed only a slight loss of activity at 2.5 Mrad. In an aqueous solution, there was a complete reversal with all but two showing a complete loss of activity.

Although this is encouraging, it would be suspected that there will be only slow growth in this area. One major reason is the requirement that each irradiated drug be considered as a "new drug" with the concomitant time and expense in proving its necessary efficacy and safety.

Even if the New Drug Application (NDA) problem is overcome, from a radiation producer's standpoint, the volume requirements would be low.

Vaccines and Sera, Biological Tissues and Related Materials

As with pharmaceuticals, investigations in these areas continue and the results look encouraging as was reported at the last IAEA meeting in Bombay (19). However, again the volume, in contrast to medical disposables, would be relatively low. Consequently, they represent excellent product, though low production, potential.

Toiletry and Cosmetic Products

In today's world, "cleanliness" seems to come before "godliness" — and what could be cleaner than sterile face powder. Although there is no requirement for sterility with respect to this class of products, there appears to be increasing interest, fostered in part by governmental agencies, to limit contamination. Recently, an examination was made of the irradiation of talc prior to its formulation to reduce microbial levels, and the procedure looks satisfactory. Performing the irradiation prior to the addition of perfumes avoids the problem of radiation sensitive aromatics changing characteristics after irradiation.

Waste and Effluent Treatment

Since Mr. Frohnsdorff's paper of three years ago, there has been considerable activity in this sector. Not only has the Co-60 plant outside Munich continued to function effectively in treating sludge, but the Deer Island plant in Boston using electron beam equipment has come on-stream. In addition, the City of Albuquerque, New Mexico is planning a new facility for sludge irradiation in the U.S.A. and a similar plan is underway for the City of Bern in Switzerland.

Although these are not sterilization requirements in the strictest sense of the word, they do represent practical applications using radiation, rather than heat, as the kill mechanism for sanitizing this material. It is believed that the potential here is extremely large since the radiation treatment offers salient advantages over heat, such as:

- lower cost

- less energy consumed
- less noxious odors
- better dewatering
- better nutrient value when the sludge is used as fertilizer.

Whereas sludge irradiation today looks attractive, the radiation treatment of the total effluent from municipal waste plants does not. Unless there are stringent restrictions put upon chlorination, the economics will dictate against radiation (20).

Food

This potential sterilization/pasteurization application for radiation, although at the end of the list, is the one with by far the greatest potential — but the one that perennially is always "just around the corner".

Table IV. — Present status of foodstuffs having government approval.

Foodstuffs	No. of Countries
Fruits & Vegetables	
Potatoes	15
Onions	9
Garlic	2
Dried Fruits	2
Fresh Fruits & Vegetables	1
Mushrooms	1
Asparagus	1
Coco Beans	1
Spices & Condiments	2
Grain & Grain Products	
Grain	2
Wheat & Wheat Flour	2
Meat & Fish	
Semi-prepared Raw Beef	1
Poultry	3
Prepared Meat	1
Shrimp	1
Cod and Haddock Fillets	1
Other	

Deep-Frozen Meals	2
Fresh, Tinned and Liquid Foodstuffs	1
Dry Food Concentrates	2

In spite of great effort and the expenditure of large sums of research funds, the real potential is still unrealized.

Table IV indicates the present status of foodstuffs that have governmental approval (21); Table V shows those countries with two or more food items approved (21). Both tables are impressive in numbers. Yet there still appears to be little real commercial utilization of this powerful technique for preserving food.

Table V. — Countries with two or more food items approved.

Approval By Countries	Numbers
(With Two Or More)	
U.S.S.R.	8
U.S.A.	2
Canada	4
Israel	2
Spain	2
Hungary	3
Netherlands	10
Bulgaria	7
Italy	3
FRG	2

This should be most distressing to everyone. For here is a method that could alleviate some of the worldwide starvation that presently exists. However, what is more discouraging is the future. Our world's population will be double today's 4,000,000,000 people by the year 2010. Unless substantial progress is made on many fronts, the food shortages will be even more severe and the attendant potential political problems could become explosive.

Certainly food irradiation is not the complete solution; but isn't it criminal not to make use of all existing technology — including food irradiation — if this will assist in providing additional food for the world's population?

Because the food irradiation effort has been languishing so long, predictions on its future could be difficult. However, Dr. Ed Josephson, formerly head of the U.S. Army's Natick Laboratory's food irradiation program, was willing to predict that "food irradiation will make the transition from adolescence to maturity … before the last decade of the 20th century." (21) It suffices to state that if and when it goes commercial, it could dwarf any other radiation sterilization activity.

Radiation Sources

In spite of the obstacles evident in some sterilization areas, the overall future for radiation is seen as most promising.

If one accepts that premise, then the remaining question is "What kind of radiation?"

Here one will find valid differences of opinion. However, a "middle of the road" statement that both gamma rays and electrons will share in the growth of radiation seems reasonable — for each has its particular advantages.

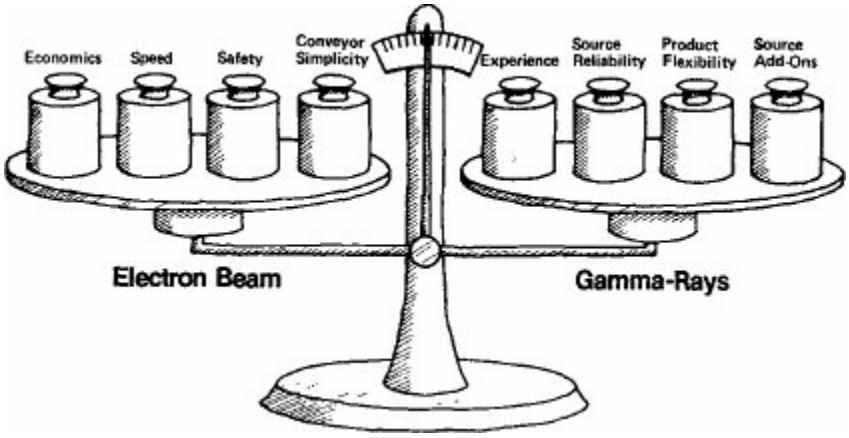

Figure *6.* A comparison of the relative advantages of electron beam and gamma ray sterilization.

In a paper presented in 1975 at the International Atomic Energy-Agency Meeting on Radiation Sterilization in India, an attempt was made to objectively appraise the relative position of gamma rays and electron beams (22). The balance is as shown in Figure 6. Now, let's examine those weighting factors for each.

Gamma Rays

a) Broad experience — As indicated earlier in this paper, although both electron beams and X-rays played a role in the initial stages of radiation sterilization, Cobalt-60 has been the dominant factor. The experience gained and continuing to develop in sixty plus Cobalt-60 facilities worldwide is obviously most helpful to others contemplating the use of radiation. However, it is well to note at the same time that electron beam facilities do exist and are growing as well, so that the experience factor today is only of secondary importance.

b) Source reliability — One of the major reasons for Ethicon's shift from early generation electron beam devices to Cobalt-60 was the assurance with Cobalt-60 that radiation would be available at all times. And there is no disputing this irrevocable law of nature. However, as source size has grown, the natural tendency has been to attempt to make maximum use of the source, i.e. better source efficiency and loading factors. Unfortunately, this has implied more sophisticated product conveyance systems — systems which have their own reliability factors to contend with. The net result has been to bring the total system reliability, both source and conveyor, to a level comparable to those experienced with electron beam systems.

c) Incremental changes in source strength—Since most Cobalt-60 facilities are designed for nominal loading in excess of the initial, the facility's capability can be matched to increasing production

requirements by the addition of more Cobalt-60. Although theoretically this could be accomplished in small incremental changes, from a practical standpoint one would be expected to make relatively large step changes in source strength — to avoid "downtime" during changeover and the subsequent new dosimetry.

d) Flexibility in product selection—This is, in all likelihood, the major advantage with Cobalt-60. Because of the penetration ability of the Cobalt-60 photons, one can often continue to handle products in the same physical configurations — package shapes, etc. — as was the case with heat or ETO. At first glance, this simplifies considerably the substitution of radiation for gas or heat. However, if one is looking to maximize the loading factors and minimize the minimum/maximum ratio — change is often necessary.

Electron Beams

a) Safety (on-off operation) — Since the electrons employed in radiation sterilization are machine produced, one has the ability to turn the device on or off. This not only allows product flow and radiation to be tied together, but inherently adds to the basic safety and simplicity of an electron beam facility. A typical electron beam shield and facility as shown in Figure 7A and 7B reflects this fact. The simplicity of an electron beam facility results in substantially lower costs as compared with a Cobalt-60 facility.

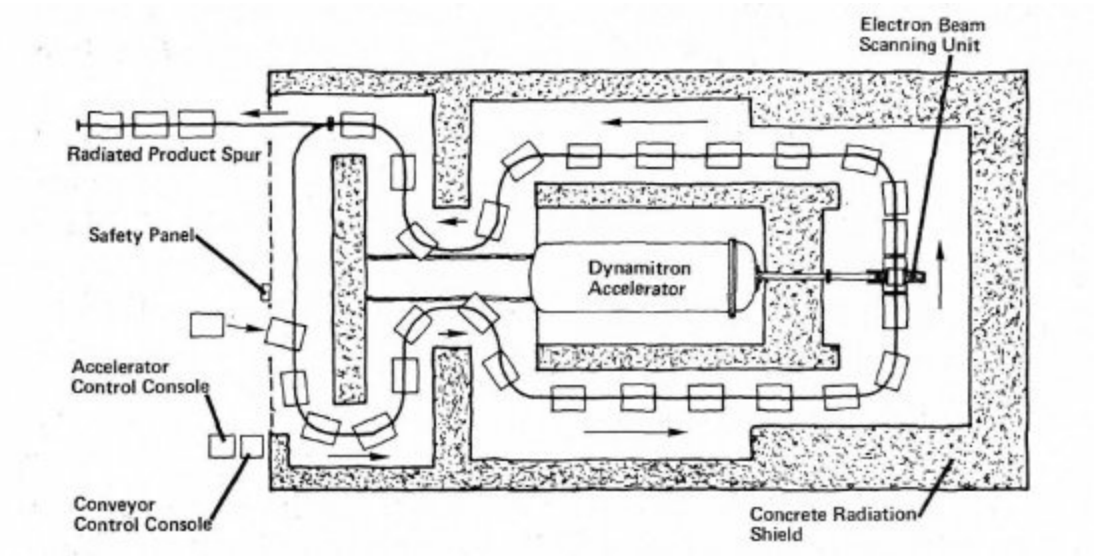

Figure *7A.* Plan view of a typical electron beam facility.

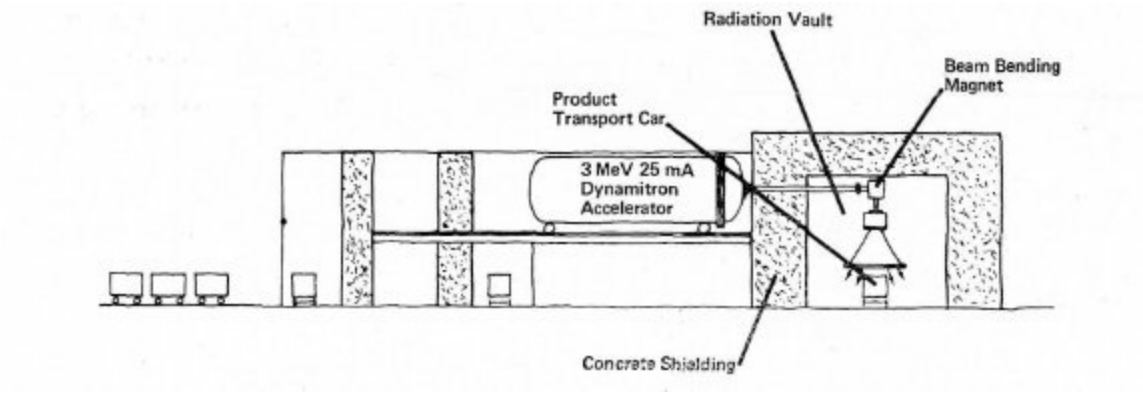

Figure *7B.* Elevation view of a typical electron beam facility.

b) Speed — With an electron beam device, the time to sterilize is of the order of a second. This fact, together with the ability to start and stop, allows one, as mentioned above, to tie together product flow and sterilization. Although most electron beam devices are still off-line, the ability to process product "on-line" can have distinct advantages for disposable items produced in large quantities. Figure 8 shows an artist's sketch of a facility just now being established by a major U.S. manufacturer. Their product, a urine analysis indicator, will be fabricated, individually packaged, sterilized and boxed all on-line. Since the product will be made in the thousands of units per hour, this type of automated production was essential for the product's economic viability.

In the system in Figure 8, multiple sensors and sophisticated read-outs and controls will be employed to assure product integrity. If a fault should occur at any point in the system, the system shuts down automatically, the fault is indicated visually on the controls, and any products whose sterility might be questionable because of the stoppage are eliminated.

c) Conveyor simplicity — This factor is evident in the example discussed above. The conveyor system employs a stainless steel mesh belt whose speed is "slaved" to the accelerator's output. This type of conveyor system is common to many industrial operations and has a high reliability factor.

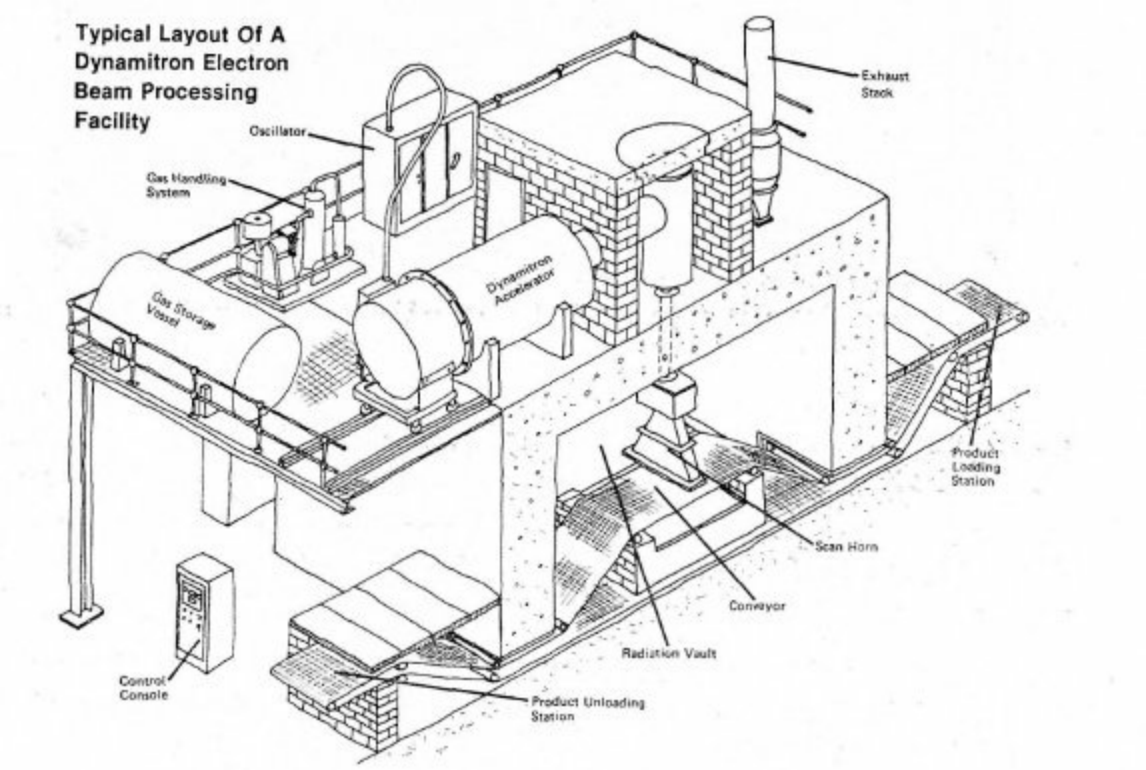

Figure *8.* A schematic representation of the electron beam accelerator installed on a medical device line by a major U.S. manufacturer.

d) Economics — This last element is the most important one for electron beam sterilization and may well tip the scale toward electron beams if two criteria are met:

- the product is produced in large volumes
- the product has low density and is relatively uniformly distributed in a configuration matched to the beam.

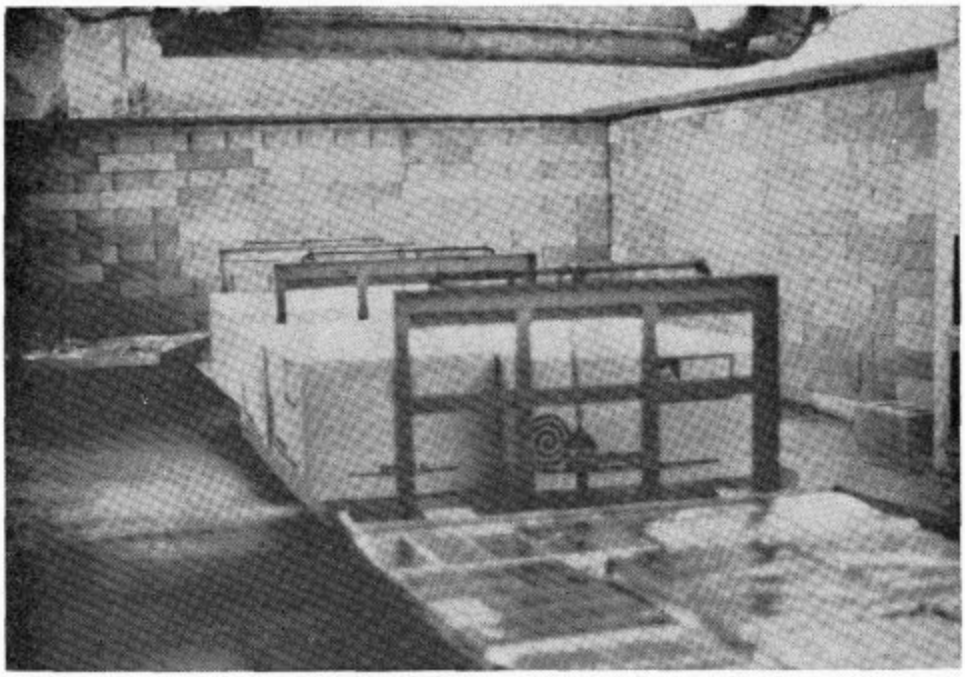

Figure *9.* Two different type medical disposables being irradiated under a 3 MeV beam.

In Figure 9 two very different type products are shown being irradiated under a 3 MeV beam. One is a single use plastic thermometer; the other cotton sponges. These products are produced in large volumes; and both, as packaged, provide a product configuration which, when irradiated from both sides, can be electron beam radiation sterilized with acceptable minimum/maximum ratios.

Since the statement has been made that economics is the most important factor for electron beam sterilization, let's examine this aspect in more detail.

Figure 10 shows the change in the capital cost for radiation over the last two decades for both electron beams and Cobalt-60. What is very evident is that electron beam costs are not only lower but have continued to stay down — in particular as the equipment's power levels have increased.

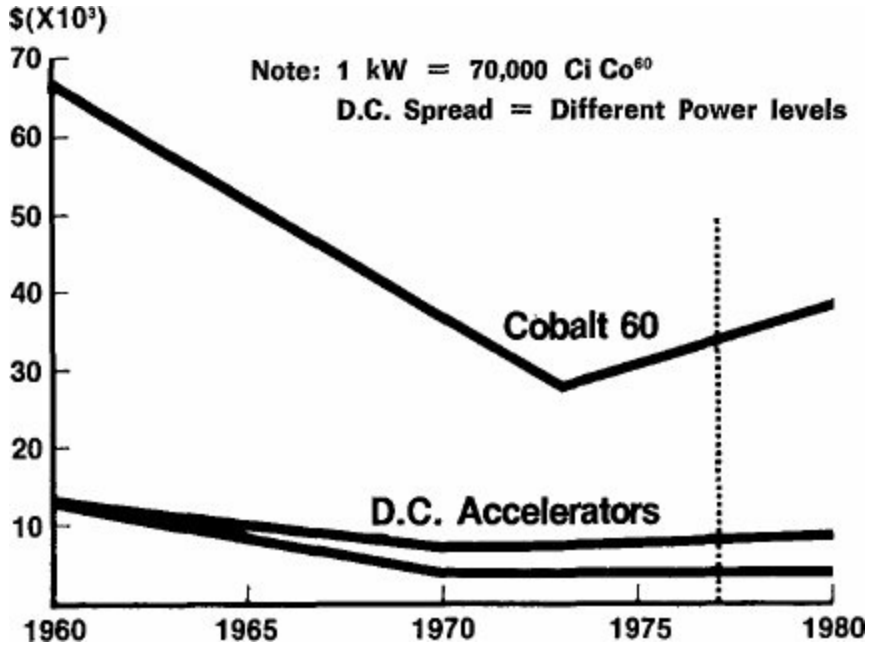

Figure *10.* Capital cost for cobalt and D.C. accelerators as a function of time.

A more meaningful way to look at the cost differences is to take a specific case. (Figures 11–14.) In this example, a yearly production requirement of 800,000 cubic feet (22,671 cubic meters) per year has been used. For the sake of completeness, the costs for steam and ETO are included as well.

Parameters = 800,000 Cu Ft./Yr.
Product Density: 0.2 Aver.
Autoclave Capacity: 250 Cu Ft.
Average Load: 200 Cu Ft.
Cycle Time: 2 Hrs.
Pressure: 15 Lbs

Capital Costs: $100,000

Annual Operating Cost: $75,000

Cost Per Cu. Ft. = 10¢

Sterilization Rate = 100 Cu Ft./Hr.

Figure *11.* The economics for sterilization with steam.

Parameters: 800,000 Cu. Ft./Yr.
Product Density: 0.2 Aver.
Autoclave Capacity: 1000 Cu Ft.
Average Load: 800 Cu Ft.
Cycle Time: 8 Hr.
Gas Mixture: 88/12

Capital Cost: $290,000

Annual Operating Cost: $275,000

Cost Per Cu. Ft. 34¢

Sterilization Rate: 100 Cu Ft./Hr.

Figure *12.* The economics for sterilization with ETO.

As evidenced in the summary, shown in Figure 15, although electron beam devices provide lower costs per cubic foot than Cobalt-60 for 800,000 cubic feet/year, their costs improve still further as one more fully utilizes their production capacity.

Certainly it is obvious that in radiation sterilization the single most important factor has to be assurance of sterility. However, recognizing that both electron beams and gamma rays can provide such assurance, then the economics should begin to have increasing importance — in particular as the volume of sterilized products increases.

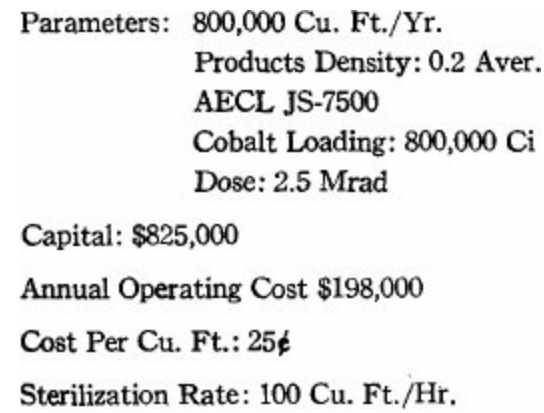

Figure *13.* The economics for sterilization with cobalt.

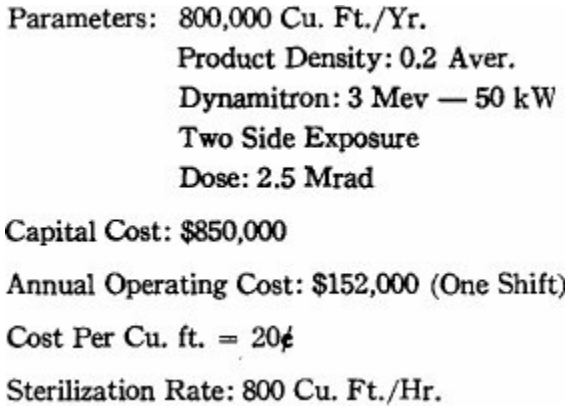

Figure *14.* The economics for sterilization with a D.C. electron accelerator.

Summary

In summary, I should like to state again that both gamma rays and electron beams will play increasingly important roles in the sterilization of medical products and pharmaceuticals. Equally evident is the fact that product release based on dosimetery and process control monitoring will become the accepted norm.

As radiation sterilization gains wider acceptance, it is my belief that we will find more complete integration of radiation sterilization with the product's manufacture. In addition, one will find increasing willingness to consider material modifications, package changes, and production flow alterations in order to avail oneself more fully of this better sterilization technique.

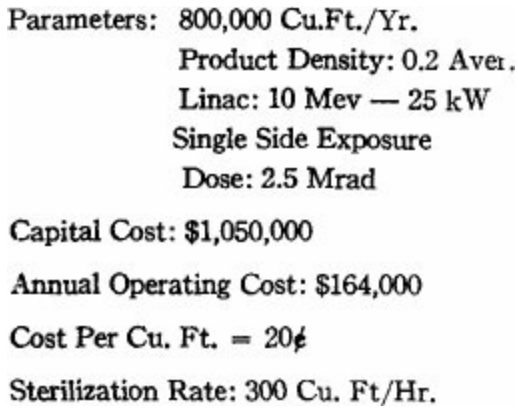

Figure *15.* The economics for sterilization with an electron beam linac accelerator.

	For 800,000 Cu. Ft./Yr.		For Maximum Yearly Production	
	Cost/Ft³	Rate/Hr.	Cost/Ft³	Quantity/Yr.
ETO —	34¢	100 Ft³	34¢	800,000 Ft³
Heat (Steam) —	10¢	100 Ft³	10¢	800,000 Ft³
Radiation				
Co^{60} —	25¢	100 Ft³	25¢	800,000 Ft³
Linac —	20¢	300 Ft³	13¢	1,000,000 Ft³
Dynamitron —	20¢	800 Ft³	6¢	4,800,000 Ft³

Figure *16.* The summary of sterilization costs — steam, ETO, cobalt and electrons.

The future is bright indeed for the health care industry and the radiation industry servicing its needs. The future will be brighter yet if through broad based acceptance of radiation sterilization we lead the way to using radiation to assist in those critical areas facing manking — food, environment and energy. This group, as leaders in the radiation sterilization field, can be instrumental in bringing this about.

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General Discussion

Comment by Moderator

R. W. CAMPBELL — Canada

I think the reason I have been put up here at this stage of the game is to try and keep me quiet, but I do not guarantee that it is going to work. I suppose what one should do is to try to stimulate a discussion which will sum up all we have been talking about this week. Probably one way of approaching the matter would be to tell you why we are interested in taking a second look at 2.5 megarads. The first reason is that we have a problem in North America that you do not have in Europe, and that is the almost universal use of ethylene oxide. In North America gas sterilization means ethylene oxide and as most of you know, there are many problems involved in ethylene oxide sterilization. One is that there are so many parameters which must be accurately controlled to achieve effective sterilization. The other is the induced toxicity in the product. We do not even take into account all the environmental problems of ethylene oxide. Second, is that North America is probably more sensitive to the influence of the work that was done by NASA. When we are sending a station to outer space, it is important that you have a degree of assurance of the absence of organisms in this case, which does not apply in any other circumstances. To find out whether that thing is sterile before it leaves you have to evolve new techniques because I have yet to see a sterilizer to put a space ship into. So NASA evolved a new set of techniques for assuring sterility of objects without doing end product testing on them. When we started setting up organizations to control medical devices, both in the U.S.A. and in Canada, we started looking at the sterilization of devices and the first place we looked was in the USP, which told us if we had a first-class lab, very highly trained operators, extremely good technicians, and lots and lots of luck, we might achieve an assurance of 10^{-3}. Whereas the NASA people were saying "What do you want? We will give it to you—10^{-12}? Fine we can do that." And so we thought there was something wrong with the existing methods as applied to drugs. We were not content with 10^{-3} for devices, particularly implantable ones. We were looking for 10^{-6} for implants. We had to evolve new techniques to achieve that assurance because it cannot be done by end-product testing. Having decided it could be done, we then started looking at the economics of it, not only in money, but in resources required in hospitals to assure that sort of level for everything and decided that it was not a practical proposition. So now we are working on the concept of different levels of assurance of the absence of microorganisms, according to the end use to which the product is to be put. This concept has not reached the point where we are prepared to publish it as a new charter for mankind, but we have it in the stage of a discussion paper. I have several copies with me, if anybody wants to look at it. At the moment, it is under discussion in North America, because we found that in our sterilization committees, we have become a tight little in-group, which talks to teach other in a language that the strangers do not know. We got so far ahead of our definitions that we are not leading any more, we are out of sight of our horizon. We have gone back now to the Royal College of

Physicians and Surgeons, in Canada, and we started talking to them about what it is we are trying to do and asking what kind of levels they would be happy with in various circumstances, and for various groups of devices. Then, there is the background against which we came to the Conference and why we came here with the object of arguing about 2.5 megarads. We do not think any figure, whether it is 2.5, 3.5 or 4.5, is the answer to our problem. We think the answer to our problem is an approach which will let us decide in a logical fashion what the appropriate dosage is in a given set of circumstances.

I hope that somebody is going to start off the discussion by telling me why, if a drug is sterilized by any means other than radiation, the Pharmacopeias of the world would be very happy if it reaches 10^{-3}, will accept then 10^{-2}, or even 10^{-1}. But if it is going to be radiation sterilized it is expected to meet 10^{-6}. If you have 1 microorganism and give it 2.5 megarads you are asking for 10^{-6}.

Comment by

E. A. CHRISTENSEN — Denmark

I think all of the Scandinavians could give you an answer to that because you have a misunderstanding. Sterilization of drugs follows the same rules as for devices. We gave an example about aseptically prepared products. There we have a lower standard, but the Nordic Pharmacopeia uses the standard of 10^{-6} also for sterilized pharmaceuticals.

Comment by Moderator,

R. W. CAMPBELL — Canada

Let me get that absolutely clear. The Nordic Pharmacopeia calls for 10^{-6} for pharmaceuticals?

Comment by

E. A. CHRISTENSEN — Denmark

All sterilized medical products.

Comment by Moderator,

R. W. CAMPBELL — Canada

What is the methodology recommended by your Pharmacopeia?

Comment by

E. A. CHRISTENSEN — Denmark

It is the indirect method by the use of biological indicators and the contamination level before sterilization.

Comment by

L. O. KALLINGS — Sweden

I think we had the opportunity to discuss with you at the Stockholm Conference, whether one could accept different degrees of sterility. As usual in these discussions, we end up with a lot of practical complications if we have different degrees of sterility or if we have different degrees for pharmaceuticals and for devices. The way that we cope with this in the Nordic Pharmacopeia for drugs, is that sterilized means one in a million, but when you cannot sterilize in the final container, then an aseptically filled product is no longer labelled as "sterile". They are labelled "aseptically produced." There is no reason to go into it now, but I could see that for medical devices, for instance, for drapes especially in surgery, sterility actually may not be needed. One should have a clearer definition for the cleanliness of that type of medical device, and I think that your approach to go back to the surgeons is quite the right way to do it. Personally, I would feel happy if these products were not called sterile, but another designation. It is quite clear that they are not sterile in the sense we usually use the word, but that they are safe for the surgical procedure. If anybody could invent a new designation for that, I think we would all agree to it. A lot of products currently used in the medical sphere are sterilized, when actually there is no need for sterility. I heard for instance, during lunch, about oral thermometers which are sterile. I cannot understand why. It would be all right if they were clean. We can give many other examples of unnecessarily sterile medical products.

Comment by Moderator,

R. W. CAMPBELL — Canada

The question that Dr. Kallings raises is an extremely difficult one. What do you call such a thing? The way we propose at the moment to get around it in U.S.A. and Canada, where our legislation differs from the United States in that it is much more flexible. In Canada our basic Act is very short indeed; it only has three short paragraphs that refer to medical devices. You must not sell one that is hazardous and if there is a standard, you must comply with it. All the rest is in the regulations that we write ourselves and which we can amend and change quite quickly. They are very flexible. What we propose to do at the moment is that as each section of the regulations is written covering another class of devices, we will specify in the definitions of that section of the regulations that for the purposes of that section, the word sterile shall mean that the manufacturer has established that the probability of survival of an organism is not greater than 10^{-6} or 10^{-3}, or whatever the limit is. With that kind of wording in the regulations, it is possible to call all of these sterile, but the word sterile has different meanings for different classes of devices. There could be a problem and I agree that it would be very nice if somebody could come up with an agreed set of terms. The only thing is that if we do this, we are discussing the possibility of having a third category like the Swedish one, which would replace what in the Western World used to be called "surgically clean." The advantage of doing this is that if we say that you must achieve a particular level, it can probably be achieved without going through a sterilization process. However, it does mean that you have to have a microbiologist in the plant, which we do not have at the moment. The majority of our plants do not have a microbiologist on the production line. The microbiologist they have is sitting in the laboratory somewhere doing end product testing according to USP or whatever.

Anonymous Comment

This is astonishing for me to hear that you do not have a microbiologist at the radiation plants and facilities.

Comment by Moderator,

R. W. CAMPBELL — Canada

Oh, no. I mean in manufacturing plants in general.

Anonymous Comment

It was more than ten years ago that we started the discussion about an international code for radiation sterilization and one of the conditions generally agreed upon was that there should be access to at least one microbiologist. Therefore, I can see this kind of difficulty is relevant.

Comment by Moderator,

R. W. CAMPBELL — Canada

No, we are at cross purposes there. The irradiation sterilization is based on some microbiology. I was talking about in the plants which are manufacturing medical devices.

Comment by

A. CHARLESBY — England

I just want to raise a rather different point, if I may, which is directly related to what you have been saying. This is based on the radiation chemical aspect of the problem. If you irradiate materials with 2.5 megarads, you expect to find one chemical change in something of the order of one molecule out of 300,000. This means that a fair number of your molecules have been modified; some will become ineffective, others may be less effective, or may even be harmful, or there may be a great improvement. Is there any instance that not only do you sterilize your drugs, but you modify the reaction of the drug as a result of sterilizing it, because you have some new ingredient in it now?

Comment by Moderator,

R. W. CAMPBELL — Canada

Do we have a pharmacologist in the house?

Anonymous Comment

I am not a pharmacologist, but it may be relevant to comment that in radiation of some drugs you do produce other molecules which have pharmacological activity. They also are equally reactive towards the initial products of irradiated water as are the parent compounds. So, if you choose any dose,

whatever it may be, you are continuously breaking down the parent molecule, thereby producing a second molecule which is also breaking down into something else. It is very complex.

Anonymous Comment

Along similar lines, one of the problems of the radiation sterilization of foods has been, particularly with certain products that we worked on in the United States, that not only are some of the pharmacological molecules modified, but certain other molecules are modified to produce carcinogens. This is the real problem with radiation sterilization of certain molecules.

Comment by Moderator,

R. W. CAMPBELL — Canada

There is another point that was left hanging the other day, the question of free radicals and the possibility of their being mutagenic. Is there anyone in the audience that can contribute anything to that point?

Comment by

K. H. CHADWICK — The Netherlands

We are busy working on food irradiation and have been for a long time. Two points: one is that, personally, I would warn against deriving a set of new words to describe parts of the sterilization process. This was an exercise which was carried out in 1966 in food irradiation. We derived, what I find quite distasteful, terms for various levels of pasteurization, disinfection and sterilization. This led to words like radappertization, radurization and radicidation. Personally, I do not know which is which anymore, but I find this is not a good system to go into. The second thing is we have also been concerned for a long time with the sword of Damocles which hangs over everybody who irradiates food, namely, that you are making carcinogenic chemicals when you irradiate food. In fact every claim in this regard has been unsubstantiated. To my knowledge, the last one was that if we irradiated spinach we would make nitrates or nitrites which would become nitrosoamines in the digestive system. Test show that there were so many nitrites in the spinach before it was irradiated that the effect of irradiation was actually to diminish, rather than increase, the amount of nitrites.

What has surprised me about this meeting is that no one has mentioned the Ames Test. We are now starting with the Ames Test on irradiated foods and concentrates of irradiated food in an attempt to check for mutagenic activity. I would think, although this might be more difficult than pharmaceutical testing with the Ames Test, this might be one way to check for the creation of carcinogens or mutagens.

Comment by

T. ALPER — England

I think Dr. Chadwick's remarks are very helpful in this respect. I would like to say that it is extremely difficult for a radio-biologist who works on carcinogenesis by direct irradiation of animals to find suitable systems for quantifying the carcinogenic effects of irradiation. For example, there is a great

deal of work done on animals that are going to develop certain cancers whether they are irradiated or not, and the test of the carcinogenic action of radiation is just that you induce the cancers earlier.

Comment by Moderator,

R. W. CAMPBELL — Canada

Could I ask you one question? Do you think that we can take mutagenicity as an index of carcinogenicity?

Comment by

T. ALPER — England

A lot of people do I suppose *faute de mieux.* I think it was during conversation with you that we were talking about someone who was working on mutation induction and you were equating it with chromosome breakage, which I think is absolutely not really equivalent. I think Dr. Chadwick would agree.

There is some doubt about whether the induction of cancer is really a somatic mutation. So, as I say, *faute de mieux,* these things are taken as synonymous, but I do not think the case is proven.

Comment by

K. H. CHADWICK — The Netherlands

Dr. Alper is an extremely difficult person to disagree with, but I do have a slight difference of opinion on this matter. Our personal opinion, that is my colleagues and myself, of the effects of radiation on biological cells, which is highly disputed, I should say, has led us to believe that the induction of DNA double strand breaks by irradiation may lead to cell killing, it may lead to the induction of mutation and it may lead to the induction of chromosome mutation. So we have an idea that these things are all intercorrelated with each other. In extending the idea to the induction of cancer by irradiation, which, I think, is of great interest for the group of people here, there are two important pieces of scientific works which have supported our ideas. One is that the work of Ames with his bacterial test systems has given an extremely good correlation, I think it is about a 90% correlation, between chemicals which are known carcinogens with mutagenic activity and chemicals which are non-carcinogens with non-mutagenic activity. Ames has concluded that one of the initial steps in the induction of cancer arises in the form of a somatic mutation and represents damage to the DNA. I think this is published, almost in those words, in The Proceedings of National Academy of Sciences in 1976. The other piece of work is the work of Henry Harris at Oxford, who has fused malignant and non-malignant cells and injected these into host animals to study the growth of the cancer. What he found was that the new cells, the hybrid cells, were mainly non-malignant unless there was loss of chromosomes. He came to the conclusion that the factor which governed malignancy behaved as a recessive genetic character. In other words a chromosome in the malignant cell is carrying the genetic phenotype for the malignancy. But it is dominated by the chromosome in the non-malignant cells and this gives a basis for chromosome mutation, giving rise to malignancy.

I think this is superficial. I do believe there is a relationship between somatic mutation and carcinogens. I do believe in the Ames test for this effect.

Comment by Moderator,

R. W. CAMPBELL — Canada

Well, I gather we have to stop our discussion at this stage. Mr. Bishop opened the day with two quotations: one from the 18th century and one from the 19th century. I would like to close with a quotation from the 20th century, a quotation from the late Mr. Churchill, who speaking of another situation in which the United Kingdom found itself in some distress, said:

> "And not by eastern windows only
> When daylight comes, come in the light;
> In front the sun climbs slow, how slowly
> But westward, look, the land is bright."

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