Manual on Radiation Sterilization of Medical and Biological Materials
MANUAL ON
RADIATION STERILIZATION
OF MEDICAL AND
BIOLOGICAL MATERIALS
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The Agency's Statute was approved on 23 October 1956 by the Conference on the Statute of the IAEA held at United Nations Headquarters, New York; it entered into force on 29 July 1957. The Headquarters of the Agency are situated in Vienna. Its principal objective is "to accelerate and enlarge the contribution of atomic energy to peace, health and prosperity throughout the world".

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MANUAL ON RADIATION STERILIZATION OF MEDICAL AND BIOLOGICAL MATERIALS
As a result of extensive investigations into the bactericidal effects of radiation and of the continuing improvements in irradiation technology, ionizing radiation is increasingly being employed for the sterilization of medical products. It has already become a well-established industrial practice in a number of technologically advanced countries, and a growing number of developing countries are planning to install radiation sterilization plants in the near future. Since the early 1960s approximately fifty gamma irradiation facilities on a commercial scale have been commissioned to sterilize various medical products ranging from complicated medical instruments to sutures and from pharmaceutical starting materials to biological tissues. At the present time radiation sterilization of medical products constitutes the most important industrial application of large radiation sources. The rapid increase in this application of radiation can be attributed to its high efficiency, its unique suitability for the newly introduced and inexpensive plastic constituents of medical products for which heat, chemicals and other conventional methods of sterilization have proved inadequate, and to its hygienic advantages that permit sterilization of pre-packed items.

During the last ten years, the Agency has attached great importance to this development, aiming at early application in both the developing and the developed Member States. The Agency's active role in the accumulation and dissemination of the relevant technical information in the fields concerned has been through panels, symposia, research contracts, and technical advisory and expert services to the Member States.

This Manual on Radiation Sterilization of Medical and Biological Materials is planned to serve as a guide and valuable source book for those who want to establish this sterilization technique or use radiosterilized products for medical practices. Furthermore, the Manual will provide useful information in the effort to standardize the procedures for sterility testing and quality control of the radiosterilized materials in the light of the experience gained by several technologically advanced countries.

The authors of the various chapters, most of whom have been participants in relevant Agency meetings and research contracts, are recognized international experts. It is hoped that the Manual will prove useful to scientific and technical personnel engaged in the radiation sterilization of medical products, radiation biologists, microbiologists and pharmaceutical quality control experts of national pharmacopoeias and experts in radiation dosimetry.
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GENERAL INTRODUCTION

The application of ionizing radiation for the sterilization of medical and biological products has played an important role in the program of the International Atomic Energy Agency for more than ten years. The Agency has organized a large number of international symposia and other meetings within this particular field and through support to a number of national research programs it has also helped to co-ordinate the relevant research in Member States. In addition, the IAEA arranged Symposia on the application of large radioactive sources in Warsaw in 1959 and in Salzburg in 1963. Several problems related to the radiation sterilization of medical products were discussed at both these symposia.

The first international conference on the radiation sterilization of medical supplies was held at Risø in 1964 with participation from 14 countries and representatives of the Agency.

In 1966 the IAEA arranged a panel meeting in Vienna with the aim of establishing international rules for the application of ionizing radiation for sterilization of medical products and during the subsequent year a working party set up by IAEA for the purpose prepared and agreed upon a proposed Code of Practice for Radiation Sterilization of Medical Products. This proposal was presented in 1967 at an international symposium in Budapest on the Radiation Sterilization of Medical Products. The code, which was published by IAEA shortly after the Symposium, aimed primarily at radiation sterilization of medical supplies and devices as the practical application of the new sterilization method to these particular products had already been realized on a significant scale.

The scope was widened in 1969 when IAEA assembled an expert panel in Budapest to discuss the Sterilization and Preservation of Biological Tissues by Ionizing Radiation.

The present manual is a further attempt by the IAEA to facilitate the introduction of the new sterilization method in Member States. It has been prepared by experts from many countries and it makes their individual experience with the method available to users and potential users all over the world. The large number of authors is the reason why somewhat conflicting viewpoints may occasionally be found between one chapter and another.

Another effort in this field has been to hold regional training courses in the application of ionizing radiation for sterilization arranged by IAEA. One such course was held in Bombay in 1969 and another was arranged in Buenos Aires in 1971. The training courses are directly geared to helping developing countries to introduce the radiation sterilization of medical supplies as early as possible, with due regard to the necessity of providing adequate safety and quality.

The IAEA intends to continue these efforts in coming years. The application of sterilized medical products is increasing rapidly all over the world and the speedy and free exchange of experience among Member States is becoming more and more important. A number of additional areas exist where future applications of ionizing radiation for sterilization, decontamination or preservation may be suggested. The rapid progress of medical and surgical techniques is accompanied by the introduction of a large number of
new types of medical devices, of which many may only be sterilized by radiation in order to obtain the same bacteriological quality as heat-sterilized equipment. The demands on the bacteriological control of pharmaceutical products are becoming more strict year by year and one of the means of improving the quality here may well be the radiation decontamination of the raw materials for the pharmaceutical industry. It can also be considered quite likely that it will be of advantage to sterilize some of the pharmaceutical products themselves in their final containers by means of ionizing radiation. Lastly, it should be mentioned that irradiation of vaccines and of some biological tissues has yielded very promising results that suggest possibilities for better products within many product areas in the future.

The IAEA will continue to co-ordinate and encourage research work in these fields. The IAEA's Recommended Code of Practice for Radiation Sterilization of Medical Products was published in 1967. In the meantime new experience has been obtained in radiation sterilization and several countries have applied the Recommended Code or parts of the Code in their preparation of national recommendations and regulations for radiation sterilization. It is the intention of the IAEA to have the Recommended Code of Practice revised in the light of experience from Member States since the need for common rules, standards and minimum requirements is very pronounced.
PART I
THEORETICAL BASIS
1. INTRODUCTION

An important step in developing the background for the radiation sterilization process is to review the physics and chemistry of radiation interactions in matter and the quantities that are used for monitoring radiation energy depositions. From this basis we can go on in subsequent chapters to the practical considerations of how efficiently the most radiation-resistant living organisms (fungi, bacteria, viruses, etc.) can be inactivated without causing excessive damage to the host material.

Radiation interaction processes are like thermal or chemical processes in the one respect that changes brought about in a material are caused by deposition of energy. In any thermal or chemical process the incremental energy transfer is relatively small, ranging from a tiny fraction of an electron volt (eV) to perhaps 10 eV (where 1 eV is equivalent to $1.6 \times 10^{-12}$ erg/molecule and, for radiant energy, corresponds to a wavelength of about 1240 nm). The binding energies of molecular bonds are generally below 12 eV. The combinations of energy deposition processes are then limited by the thermodynamic properties of the particular system. In the case of ionizing radiation energy is imparted to the system mainly in quanta of 10 eV or more, with the result that practically any chemical bond may be broken and any potential chemical reaction may take place.

Detailed accounts of the mechanisms by which ionizing radiations inactivate microorganisms are given in later chapters, but it should provide a useful background for the understanding of the role of the physical phenomena to summarize briefly at this stage the kill mechanisms. Inactivations of bacteria are brought about partly by direct collision action in a sensitive part of the cell and partly by indirect action via highly reactive chemical radicals produced in the cell liquid by the radiation. In the case of direct action we may assume that the incoming particle ionizes, say, one part of a DNA molecule, an enzyme or some other sensitive region resulting in destruction or significant change of character of the cell. It is conceivable that a sufficient amount of damage of this type may result in complete inactivation of a given viable organism. Another effect could be damage of the cell membrane by which its life function is profoundly changed or disturbed, or perhaps the cell respiratory function is affected, or mitosis is thwarted. In an aqueous system (and all microorganisms contain a certain amount of water) the release of radiation energy will also lead to chemical reactions by which free radicals such as OH and H and molecules such as $H_2O_2$ are formed. These species are highly reactive chemically with vital components in the organisms and can thereby indirectly cause lethal damage. All types of ionizing radiation interactions lead to such reactions and appreciable variations in their effect can be observed as a result of differences in energy transfer mechanisms, dose-rate differences, etc.

The following sections of this chapter will describe the basic physical processes by which ionizing radiations interact with matter, and discuss
some of the practical implications for radiation sterilization. The radiation user will not need to apply the complicated details of the mechanisms of particle collisions and cascades of secondary processes in the preparation of routine sterilization procedures. The radiation doses are very high and sensitive 'hit' processes are averaged under constant irradiation conditions, i.e. as many irradiations are repeated under identical conditions, the chance of a failure remains insignificantly small. For research purposes we should be aware of the intricacies of the radiation interactions in order to solve troublesome problems and ambiguities that may arise from changes in radiation spectra, dose rate, etc. The discussions that follow are a rather superficial treatment of the subject. Interested readers are urged to go to the short bibliography at the end of this chapter as a starting point for deeper inquiry.

2. TYPES OF RADIATION AND THEIR BASIC PROCESSES

Ionizing radiation includes many types of incoming particles, those that are directly ionizing (e.g. heavy and light charged particles) and those that are indirectly ionizing (uncharged particles such as neutrons, X- and gamma-ray photons, etc.). Non-ionizing electromagnetic radiation, such as ultra-violet light, is sometimes used in radiation sterilization but for the purposes of this manual the discussion will be restricted to the radiation types used in process sterilization. These comprise intense ionizing charged particles (electron beams) and electromagnetic radiation (X- and γ-rays) having primary energies ranging from 10 keV to 10 MeV. The lower limit is set because of the limited penetrating power of lower energy radiation, and the upper limit because of the increasing probability of causing induced radioactivity as the energies extend much above 10 MeV. The electron beams are usually provided by particle accelerators, and photon fields are produced either as X-rays resulting from intense electron beams striking high atomic number metallic targets or as γ-rays from powerful radionuclide sources, such as cobalt-60 and caesium-137.

Although we limit our considerations to primary radiation energies above 10 keV, degradation and complex interactions with matter produce a cascade of low-energy secondaries over a broad spectrum. Most of the first interactions are forms of ionization in that they make secondary charged particles. Eventually, as very slow non-ionizing photons and electrons are formed as a result of energy degradation, atomic and molecular excitations, displacements, chemical reaction transfer and thermal processes occur in great abundance. Whereas the quantum yield of ionization events caused by very low-energy radiations (e.g. the far ultra-violet) is around unity or less, more than 1000 atoms can be ionized by the absorption of one γ-ray photon and many thousands of molecules can be affected chemically, some for only short times and some more or less permanently. Figure 1 is a semiquantitative diagram showing the main photon and electron interaction processes as a function of the primary (and secondary) photon energy and electron energy from 10 MeV downward.

For photons pair production takes place at incident energies above 1.02 MeV (twice the rest mass of the two produced particles, electron and positron) and is caused primarily by the interaction of a photon with the electromagnetic field of an atomic nucleus. The resulting recoil energy of
FIG. 1. Interaction processes of ionizing photon and electron energy (< 10 MeV) deposited in matter and including important degraded secondary particles and events.

The two particles is shared equally. If the positron in its flight remains in the medium, it will combine quickly with an electron to create by annihilation two gamma-ray photons of 0.51 MeV, which in turn can cause other secondary particles by Compton processes. The probability of pair production increases with energy above 1.02 MeV and as the square of the atomic number of the absorbing material.

Compton scattering results from elastic collision of an incoming photon (mainly 0.1 to 2 MeV) primarily with the electronic orbital system of an
atom. The incident photon loses energy and changes direction as it ejects an orbital electron, which also has some lower energy. However, because of its higher momentum, this Compton electron carries with it most of the kinetic energy and goes on to produce further secondary radiations. The scattered Compton radiations occur at various preferred angles and kinetic energies as a function of the incident photon energy. But according to the more or less random processes, the energy imparted per event to the absorbing material is essentially independent of its atomic number. The Compton photons can go on to produce further scatter and photoelectric absorption.

Photoelectric absorption of photons occurs mainly below 0.1 MeV in the case of low atomic number absorbers. It causes the ejection of a bound orbital electron with a kinetic energy corresponding to the difference between the energy of the incoming photon and the binding energy of the electron. This total absorption process is followed immediately by the emission of characteristic fluorescent photons, which is called atomic fluorescence. The ejection of orbital electrons as Auger electrons also occurs in competition with fluorescence emission as a result of orbital readjustments. The latter process is apt to predominate with low atomic number media. The photoelectric effect is to some extent a discrete process determined by the various electron orbital binding energies of the K, L, M etc. atomic shells. The efficiency is proportional approximately to the fourth power of the atomic number of the target atom and inversely proportional to the third power of the incident photon energy.

Another type of secondary photon is bremsstrahlung, caused by streaming high-energy electrons being slowed as they pass through the charge fields of absorbing atoms. The ratio of this type of energy loss $(dE/dx)_{\text{radiation}}$ to the energy loss due to collisional ionizations by electrons $(dE/dx)_{\text{collision}}$ is approximately proportional to the increase in incident electron energy and the atomic number of the absorber. Bremsstrahlung production is appreciable for low atomic number systems only for primary electron energies above 1 MeV.

It is the collisional energy losses due to fast electron inelastic collisions with atoms that are most important in the radiation absorption effects discussed here. Since the electrons undergo Coulombic scattering with atomic nuclei and electron orbital fields, the electron stopping power of a material $(dE/dx)$ for non-relativistic electrons (< 0.5 MeV) increases approximately with the reciprocal of the square of the velocity of the electron (except for very low energies) and depends on the electron density of the interfering medium. Each interaction as electrons slow down in their erratic path through the medium results in part of the energy being absorbed to form other secondaries, both electrons and photons. The main-line charged particle track consists of a random bundle of tiny tracks (or spurs) of varying density. This density increases with the value of the linear energy transfer (LET), which is defined as the average increment of energy $(dE_1)$ deposited locally to a medium by a charged particle having a specific kinetic energy, divided by the increment of distance transversed in the medium $(dl)$. This quantity $dE_1/dl$ is different from stopping power $dE/dx$, which, instead of referring only to local energy deposition, includes the energy deposited at all distances from the site of the particle's trajectory $dx$. In the vicinity of intensely ionizing slow electron tracks many very low-energy electron cascades (delta rays) are produced (primarily in the energy range 10-1000 eV),
which can cause much local damage. Average spur separation for delta rays is generally less than 100 Å, but for a 1-MeV electron track the spurs are about 3000 Å apart.

As the various photons and electrons decrease in energy the ratio of ionization to excitation of atoms and molecules decreases, until eventually in the region of 12 down to approximately 3 eV, ionization is completely replaced by the low-energy processes of excitation, fluorescence from singlet and triplet excited states, non-radiative chemical reactions, atomic and molecular displacements, bond polarizations and heat dissipation. As was indicated in the introduction, these events are especially important in radiation biology. Low-energy events (< 100 eV) must be weighted heavily in attempting to project from physical interactions to chemical and biological effects. Although in practical irradiation situations one deals with unchanging primary spectrum, target geometries and environmental conditions, where the results (survival fraction versus dose) are averaged macroscopically, it is advisable for researchers not to ignore the complexities outlined above in a microscopic sense. One cannot deal adequately with average LET in a critical small volume irradiated with different primary radiation spectra at widely varying dose rates and environmental conditions. In such situations the biological effects of changing ratios of secondary radiation components, say, < 100 eV to >100 eV, are apt to vary considerably.

3. PRACTICAL CONSEQUENCES

In practical applications of ionizing radiation much effort has gone into dosimetry, which involves the measurement of absorbed dose and associated quantities, according to recommendations of the International Commission of Radiation Units and Measurements (ICRU). Absorbed dose is a useful quantity for describing the integral amount of energy deposited by ionizing radiation in a small volume of a given medium. It is defined as the incremental energy, ΔE, deposited in an element of volume of material divided by the mass, Δm, of that element. The unit of absorbed dose is the rad (1 rad = 100 erg/g = (1/100) J/kg). It is usually measurable with good reproducibility and relatively small error by means of calorimetry as a macroscopic average in a relatively large absorber, or is determinable by relating ionization or chemical measurements to known energy deposition effects.

Recently, considerable effort has gone into microdosimetry in order to understand the kinetics of energy depositions and dose distributions in very small volumes of materials simulating biological substances. The measurement techniques use knowledge of LET spectra (or the random valued 'lineal energy density' for single events) along segments of charged particle tracks to determine the distribution of absorbed dose as a random valued quantity ('specific energy' for single and multiple events) in microvolumes according to statistical extrapolation from experiment.¹

1 The specific energy, z (rads), is related to the lineal energy, y (keV/μm), according to

\[ z \approx 16 \frac{1}{V} (y) \]

where I is the mean path length (μm) and V is the size of the microvolume (μm)³.
experiment uses a proportional counter of biological tissue-like wall materials (gridded 'wall-less' counters are also used) and very low pressures of gas with atomic constituent ratios matching those of tissue so that the effective gas volume corresponds to the tissue volume with a diameter of the order of 1 μm.

This approach has not been entirely satisfactory because the minimum size for measurable energy deposition in a single gas ionization is for, say, $^{60}\text{Co} \gamma$-rays not much less than 0.5 μm in diameter. Besides, what is wanted by radiobiological researchers is detailed knowledge of the kinetics of reactions occurring in tracks, and especially those close to sensitive microvolumes and interfaces of cells (local sites in polynucleotide strands, critical membrane sites, etc.). In addition, we need to know the influence of radiation dose rate, recombination, phase differences, environmental parameters, chemical side effects, etc. Unfortunately the statistics of space-time relationships of a biological inactivation process in the vicinity of radiation particle tracks is not sufficiently known at present. Considering a microscopic scale of less than 100 Å, this is simply too small for measurement by current methods. Moreover, microbiological radiation cross-sections are too complex to be predicted accurately by theory.

There are macroscopic weighting factors by which one can correct the absorbed dose reading according to the effects of differences in radiation type, energy, LET, etc. on the biological response. The ICRU has distinguished between the quantities 'RBE dose' for radiobiological measurements and 'dose equivalent' (DE) for radiation protection measurements. Both of these have the unit rem, which is related to the unit for absorbed dose, rad, by applying appropriate dimensionless qualifying factors to the dose in order to weight it according to the biological effectiveness of a given radiation. For radiation protection considerations, DE is determined by using typical weighting factors, such as the quality factor (QF), which indicates how efficiently a given radiation spectrum produces a given biological effect, and the distribution factor (DF), which takes into account variations of dose at different positions in an irradiated specimen. In the case of radiobiological measurements the general weighting factor RBE, with which absorbed dose is multiplied to get RBE dose, must be determined experimentally. This leads to problems in trying to use the curve of survival fraction of a biological system as a function of the absorbed dose in order to come up with an RBE factor. The curve shape for electrons and X- and gamma-rays can change radically with dose rate, phase, oxygen enhancement ratio (OER), etc. As was indicated earlier, such complex factors are not always well established, especially on a microscopic scale for widely varying energy cascades occurring along radiation particle tracks at critical targets in living biological systems. They are established roughly for certain macroscopic effects, as in radiation protection, survival of various irradiated cells, etc.

4. CONCLUSIONS

In applied radiation biology, such as radiation sterilization, the interest is in producing a specific predictable biological effect resulting from a myriad of complicated reactions due to physical and chemical transfer processes, recombinations, etc. The main difficulty in our understanding
these reactions is not overcome by merely measuring the energy absorption in an aqueous or hydrocarbon system, rather it is in being able to project from the physical processes discussed above to unstable and highly complex and energy-dependent series of transient chemical and biological events. Unfortunately, this is not yet possible because the effects of some of the most reactive of energy deposition processes (~20 eV and below), e.g. delayed ionizations, excitations, electron attachments, molecular orbital disorientations, transfers of reactivity, and thermal dissipations, are so terribly complex and cannot be measured.

As an example, consider that in this radiation energy region there is undoubtedly strong energy and rate dependence of the absorption cross-section of highly conjugated molecular sites occurring at metabolic junctures. We cannot measure these adequately through the analysis of locally irradiated cell components or by pulse radiolysis or flash photolysis of DNA solutions. This is particularly true in those regions important to mitosis or, for example, at pyrimidine bases in DNA or at active sites in enzyme molecules because the sites are too small and the changes too rapid. The probability of these reactive sites incurring local damage by an ionizing or exciting energy deposition event is further complicated by in vivo protective influences of certain low-energy side events (e.g. energy storage by delocalization of $\pi$-orbitals, bond polarizations and relaxations, and heat conduction) or the presence of natural chemical protective agents (e.g. protonating aminothiols, such as cysteine or cysteamine). The radiation resistance of a biologically significant molecule can, in fact, be measured to some degree by determining the degree of conjugation and the concentration of other scavengers of DNA and enzyme-attacking agents (e.g. scavengers of solvated electrons and OH radicals, etc.). But this is not enough. We do not know the probability of a certain amount of energy being deposited within a certain small range of a critical site within a certain amount of time. Thus, the space-time relationship of energy distribution, charge distribution and distribution of electron density states, which strongly influences radiobiological end effects, remains something of a mystery. One type of useful low-energy physical measurement is that of energy transfer in very small volumes of conjugated hydrocarbon systems. Such measurements show to what degree exciton-exciton transfer in a strong coupling case and radiationless inductive resonance transfer in a weak coupling case can reach over distances from about 50 to 600 Å. If this sort of spectrometry and dose distribution measurement could be carried out in the unstable living cell, microdosimetry might indeed evolve to nano- or even pico-dosimetry and provide useful radiobiological information.

Since this is not yet possible, we should try in the meanwhile to elicit what sorts of local radiation energy deposition events are most likely to lead directly or through a chemical side-effect to a single chain break of an important biological macromolecule or to cause strand disorder, cross-linking, or to produce a chemical poison, so as to disrupt permanently the life function of the living cell. We see that this is not predictable purely from the knowledge of the physical behaviour of radiation interactions in the atomic constituents, C, H, N, O, S, P, Cl, Na, Ca, etc., or, for that matter, in the ordered molecules themselves. We are also reminded that radiation effects on the chemistry of biological molecules are not very well represented by absorbed dose measurements extracted from calorimeter readings (which represent power extracted from a radiation beam), from ionization chamber readings (which represent radiation energy converted
into free ion production) or in liquid chemical dosimeter readings (which usually represent free radical production from radiation energy release in the liquid phase). We need ideally to go to microscopic scales (~10 Å) in the biological systems themselves. We also need more extensive weighting factors to go adequately from dose in rads to RBE dose in rems.

To ascertain in detail the efficiency of the radiation damage of a critical part of a biological system, the following factors and their effects on each other will have to be known as a function of time: (1) the radiation spectrum of all interacting radiation particles; (2) the local distribution and flux density of events in and close to critical sites; (3) the probability of the many different types of events having given primary and secondary biological effects; (4) environmental effects (thermal, electrical, chemical), the latter of which especially includes the effect of pH, oxygen, water, salts, protective agents, etc.

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CHAPTER 2

THE EFFECT OF IONIZING RADIATION
ON SOME MOLECULES OF BIOLOGICAL IMPORTANCE

1. RADIOLYSIS OF WATER

Radiolysis of water has been studied by a relatively large number of investigators. At the same time, however, it can be claimed that the processes brought about in water by the effect of irradiation have not entirely been elucidated as yet.

The importance of following the transformations induced by irradiation in water is emphasized by the fact that water is known to be a component of every biological system and a constituent present in most chemical processes. The various products formed during radiolysis of water may in this way influence directly or indirectly the chemical processes and biological effects occurring in the individual compounds dissolved in water.

Experimental investigation of the interaction between ionizing radiation and water has revealed that the chemical processes in water are initiated by the dissociation of the primary products (excited molecules and ions) into free radicals. For these primary reactions, however, there are only hypotheses available since the shortness of their reaction times (10^{-12} to 10^{-3} seconds) means that the processes cannot be followed as yet for want of suitable methods.

The data on the basic products detected during radiolysis of water agree in showing that these products are molecules: H₂, O₂, H₂O₂, and free radicals: H, OH. However, the concentration and relative abundance of the products formed depend on the conditions of radiolysis. Accordingly, reaction products of different composition are formed, depending primarily on:

(a) The ionizing capacity of the radiation (LET effect)
(b) The purity of the water (quality and quantity of the substances dissolved in it)
(c) The pH value.

1. 1. Hypotheses on the production of free radicals

As the primary reactions have not yet been elucidated, various theories have been developed on the mechanism of formation of free radicals.

According to Lea's hypothesis, on irradiation water ions are formed:

\[ H_2O \rightarrow H_2O^+ + e \]
\[ e + H_2O \rightarrow H_2O^- \]
The water ions produced in this way, as claimed by Risse, are hydrated and dissociate while forming free radicals:

\[ \text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{H}^+\text{aq} + \text{OH} \]
\[ \text{H}_2\text{O}^- + \text{H}_2\text{O} \rightarrow \text{H} + \text{OH}^-\text{aq} \]

The above assumption means that the free radicals H and OH do not result from the same water molecule, nor are they spatially close to each other.

The theory of Burton, Magee and Samuel supposes the formation of two types of excited water molecules:

1. One of low energy, non-decomposing and transmitting its energy by radiation or by further excitation;
2. The other of higher energy and disintegrating into the free radicals H and OH.

This theory implies that the free radicals H and OH take their origin from the same water molecule.

1.2. Formation of molecular products

Part of the molecular products are formed by the recombination of the radicals:

\[ \text{H} + \text{OH} \rightarrow \text{H}_2\text{O} \]
\[ \text{H} + \text{H} \rightarrow \text{H}_2 \]
\[ \text{OH} + \text{OH} \rightarrow \text{H}_2\text{O}_2 \]

together with the interaction between the radicals and molecular products:

\[ \text{OH} + \text{H}_2\text{O}_2 \rightarrow \text{HO}_2 + \text{H}_2\text{O} \]
\[ \text{HO}_2 + \text{HO}_2 \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]
\[ \text{HO}_2 + \text{OH} \rightarrow \text{H}_2\text{O} + \text{O}_2 \]
\[ \text{HO}_2 + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{H}_2\text{O} + \text{OH} \]

The ratio of the reactions described above depends on the conditions of radiolysis and on the presence of other ions and molecules dissolved in the water.
1.3. Determination of the transformations

The possibility of various reactions and the diversity in the formation of the individual products render it very difficult to follow the alterations that occur.

Allen suggests determining the radical and molecular yields and then evaluating the amount of the radical products on the basis of the reaction equations. The concentration of the free radicals determined in this way does not correspond to all the radicals produced, as the recombination of the radicals starts almost simultaneously with their formation, before the reaction with captured radicals sets in.

Owing to the differences in concentration, this dead time is about \(10^{-9}\) s, during which about half the radicals produced are used up.

The amount of the molecular products formed from the recombinations achieved prior to the reaction with captured radicals has to be calculated from the results of molecular yields by taking into account the subsequent reactions, which are connected both with the formation and the diminution of the products measured. Reactions of this kind may proceed as described in the case of \(O_2\) formation; the following transformations may also ensue:

\[
\begin{align*}
O_2 + H &\rightarrow HO_2 \\
HO_2 + H &\rightarrow H_2O_2 \\
H_2 + OH &\rightarrow H_2O + H \\
H_2O_2 + H &\rightarrow H_2O + OH
\end{align*}
\]

<table>
<thead>
<tr>
<th>Radiation</th>
<th>(G_H)</th>
<th>(G_{OH})</th>
<th>(G_{H_2})</th>
<th>(G_{H_2O_2})</th>
<th>(G/-H_2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{60})Co (\gamma)</td>
<td>3.65</td>
<td>2.95</td>
<td>0.45</td>
<td>0.80</td>
<td>4.55</td>
</tr>
<tr>
<td>(^{60})Co (\gamma) in air</td>
<td>3.70</td>
<td>2.92</td>
<td>0.39</td>
<td>0.78</td>
<td>4.48</td>
</tr>
<tr>
<td>32 MeV (\alpha)</td>
<td>1.28</td>
<td>1.06</td>
<td>1.14</td>
<td>1.25</td>
<td>3.56</td>
</tr>
<tr>
<td>(^{10})B(n, (\alpha))\Li</td>
<td>0.23</td>
<td>0.41</td>
<td>1.66</td>
<td>1.37</td>
<td>3.54</td>
</tr>
</tbody>
</table>

Air-free 0.8N sulphuric acid

<table>
<thead>
<tr>
<th>Radiation</th>
<th>(G_H)</th>
<th>(G_{OH})</th>
<th>(G_{H_2})</th>
<th>(G_{H_2O_2})</th>
<th>(G/-H_2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{60})Co (\gamma)</td>
<td>2.78</td>
<td>2.28</td>
<td>0.42</td>
<td>0.87</td>
<td>3.70</td>
</tr>
<tr>
<td>32 MeV (\alpha)</td>
<td>1.55</td>
<td>1.13</td>
<td>0.66</td>
<td>0.87</td>
<td>2.78</td>
</tr>
</tbody>
</table>

Pure water, in air
Table I presents the molecular and radical yields formed in water as a result of various types of radiation. In pure water gamma radiation fails to produce water decomposition since ultimately water is reformed by recombination. Radiation of high ionizing capacity was found under similar conditions to cause decomposition of the water because the ratio of the free radicals and molecular products formed by the effect of radiation is altered. Decomposition of water takes place when the amount of the radical yield is smaller than that of the molecular yield.

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2. PROTEINS

According to the prevailing view on the radiosensitivity of the living cell, doses appreciably higher than the lethal dose to the cell itself would be required to influence or to inactivate the biological macromolecules isolated from the cell.

Recent developments in methods have provided suitable tools for studying the fine structure of macromolecules with the result that fresh knowledge of the biological activity of macromolecules and the means of measuring its degree disprove this belief. For the inactivation of viruses or bacteria doses of the order of megarads are required. Doses of this order influence both the physico-chemical properties and the biological activity of macromolecules when irradiated in aqueous media or even in the solid state. Still, the assumption that a difference really exists between the respective radiosensitivities of whole cells and of isolated macromolecules can be accepted for mammalian cells alone and even in this case with certain reservations. Since there is a close interrelationship between the structure and the biological activity of macromolecules, some alteration in their structure induced by ionizing radiation appears to be the most plausible explanation for the radiation effects.

The main trends of protein research in the past few years have been focussed on two major fields. Research on the composition and amino acid sequence, revealing the primary and secondary structure of proteins, constitutes one of these fields. Investigations into the tertiary and quaternary structure of proteins represent the other.
With regard to the primary structure of proteins, the amino acid composition and amino acid sequence of a fairly high number of proteins have already been elucidated. Present protein research is mainly concerned with the tertiary structure and with the 'fine' structure of proteins. Indeed, our present knowledge of the correlation between protein structure and biological function is due to such research and it is generally accepted that a given protein structural conformation is required for a given protein biological function. In other words, it is the given structural conformation that determines the biological function of proteins. High importance should be attributed to the elucidation of the allosteric properties of proteins, revealing the control mechanism at a molecular level, with special regard to the biocatalytic function of proteins. The currently available data on the structure of proteins enables one to do more than simply draw conclusions on the interrelationship between protein function and structure. In addition to the laboratory synthesis of biologically active peptides, these structural researches have also permitted synthetic proteins (such as ribonuclease and insulin) to be derived. The successful laboratory synthesis of biologically active peptides and proteins is convincing evidence of the correctness of the earlier analytical and structural data.

Research on the structural conformation of proteins has appreciably contributed to a better understanding of the effect of ionizing radiation on proteins. The use of ionizing radiation as a test method tool is no novelty in protein research. One example is illustrated by the X-ray diffraction technique, which provides one of the most reliable means of protein structure research. Besides, the target theory has proved to be a useful practical method for the determination of protein volume and molecular weight.

Certain changes may be induced in the conformation of proteins by ionizing radiation and the changes occurring in their biological function may be studied simultaneously. Accordingly, there is a possibility of influencing favourably their structural conformation by ionizing radiation. In other words, it is possible to modify the structure of proteins and by doing so to alter their existing biological activity or to initiate new useful biological functions. The biocatalytic and hormonal activities, and possibly the antigenic property, may be influenced in this way, as can also the conservation of proteins, a problem whose practical importance should be especially emphasized. Certain protein preparations, such as blood plasma and plasma fractions, are constantly used in therapeutic and diagnostic medicine. Considering the nature of these applications, sterilization of such products is a prime requirement. Proteins are, as a rule, heat-sensitive biopolymers so they cannot be sterilized by heat, but only by other procedures, e.g. by filtering. By choosing a filter with pores of adequate diameter the pathogen germs will be filtered out, with the exception of viruses.

Large radiation sources are also used for sterilization and radiation does not produce any significant heat denaturation effects. Consequently, such sources are suitable for the sterilization of protein products, too, provided the radiation dose applied does not impair either the biological function or the therapeutical effect of the protein in question. What has been said in the foregoing clearly explains why investigation of protein radiosensitivity has come into the foreground, as being equally significant to both theoretical and applied research on proteins.
Proteins are, in general, radiosensitive. However, within the protein molecule itself there are preferentially sensitive sites and structural elements. Such elements are the aromatic amino acids (tyrosine, phenylalanine, tryptophan) and, in addition, the amino acids with sulphur content such as cystine and methionine. The bonds maintaining the tertiary and secondary structures are the most radiosensitive. Among the primary structural elements the aromatic and sulphur containing amino acids are the most radiosensitive ones. On the other hand, doses of the order of megarads are required to split the peptide bonds.

Depending on the radiation dose level and the irradiation conditions, relatively small doses may be sufficient to induce structural changes in the proteins. As a result, the biological activity of the protein will alter, i.e. it will be denatured and lose its biological activity. The radiation hitting against the protein molecule target does not necessarily exert its effect at the site of hitting but, owing to intramolecular energy migration or migration of the charge, the effect may manifest itself at some other site. Most probably there exist preferentially radiosensitive sites within the protein molecule. This supposition seems to be confirmed by the observation that those proteins that have two or even more different biological functions lose only one of these as the effect of exposure to a given radiation dose, while they invariably retain the other function or functions, which will be impaired on the action of higher doses only. If we take, e.g., the enzyme proteins, the enzyme activity can be inactivated by lower doses than can the antigenic property.

The radiosensitivity of the enzyme ribonuclease, known to have a covalent structure, has been intensively studied. The following changes were observed upon irradiation: the reduction of the S-S bonds, changes in the optical rotation and the u.v. spectrum, and an increase in the number of exchangeable hydrogen atoms. Irradiation impaired in particular the hydrogen bonds maintained by the tyrosine groups. When using doses of radiation at the megarad level the amino acids are also damaged, in the first place, histidine, methionine and lysine. By irradiating glutamic acid dehydrogenase or chymotrypsin some enzyme kinetic parameters are changed earlier than the inactivation of the enzyme itself. As to chymotrypsin, X-radiation reduces the $K_m$ value, at the same time increasing the $V$-value. Accordingly, both the enzyme substrate affinity and the reaction velocity of the enzyme substrate complex change, while the function of the enzyme is maintained. This suggests that some changes must have occurred in the fine structure of the enzyme, without, however, destroying its catalytic function, although somewhat modifying it.

As a result of exposure to ionizing radiation the possibility of the induction of metastable intermediary states between the corresponding active and denatured states of the enzymes has been suggested. From this intermediary state the enzyme may denature or revert to the original active form. In connection with the allosteric properties of the enzymes it has been demonstrated that the regulatory function of the aspartate-transcarbamylase enzyme is more sensitive to the effect of X-rays than is its catalytic function; on irradiation the regulatory function of the enzyme was completely lost, while its catalytic activity remained unimpaired. As a rule, the absorption of as low as 50 to 100 eV energy per molecule may lead to the inactivation of the enzyme function, whereas for ribonuclease the $D_{37}$ value may reach as high as 27.4 Mrad.
### TABLE II. $D_{37}$ DOSES FOR VARIOUS HORMONES

<table>
<thead>
<tr>
<th>Hormone</th>
<th>$D_{37}$ (in air atmosphere) (rad)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>$2.0 \times 10^6$</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>$2.24 \times 10^6$</td>
</tr>
<tr>
<td>ACTH</td>
<td>$90.0 \times 10^6$</td>
</tr>
<tr>
<td>MSH</td>
<td>$77.5 \times 10^6$</td>
</tr>
<tr>
<td>Chorion gonadotrope</td>
<td>$25.6 \times 10^6$</td>
</tr>
<tr>
<td>Thyreotrope</td>
<td>$22.4 \times 10^6$</td>
</tr>
</tbody>
</table>

Of the protein and peptide hormones, the radiosensitivities of the insulin, chorion gonadotrope, vasopressin, oxytocin, ACTH and thyreotrope hormones were studied. These may also be considered as radiosterilized products for therapeutic purposes. Insulin can be radiosterilized in the solid state only. However, radiation resulted in discoloration and a certain reduction in the solubility of the product. The activity of the peptide hormones is impaired by the exposure to a radiation dose of several megarads (Table II). Data are available to indicate that the biological activity of MSH is increased when the irradiation exceeded a certain dose level.

A relatively high number of investigations have been performed on the radiosensitivity of blood plasma and plasma proteins, with special regard to the possibility of their radiosterilization. With regard to plasma protein fractions, numerous investigations have been performed with the serum albumins, which proved to be excellent experimental models.

When serum albumin was irradiated in an aqueous solution, polymerization, denaturation and splitting of the macromolecule were observed. Following the exposure of bovine serum albumin to ionizing radiation its physico-chemical properties changed. The solubility of the albumin irradiated in the solid state decreased with increasing radiation dose. Intramolecular changes were also observed, together with a certain degree of polymerization. Accordingly, interactions must have developed between the molecules. The aggregation of the preparations was observed after irradiation with high doses. After irradiation with doses of $10^8$ rads the cystine, monoaminodicarbonic acid, tyrosine, histidine and phenylalanine content of the serum albumin was found to be impaired.

$^{131}$I- or $^{125}$I-labelled serum albumin preparations are extensively used for diagnostic clinical purposes. On storage the radioactive $^{131}$I loses its radioactivity in the disintegration process and, as a source, it furnishes precious data on the radiosensitivity of serum albumin. As demonstrated, the 5% albumin solution with an activity level of $10 \mu$Ci/mg denatures within 8 days. After a dose of 56 krads $^{131}$I-labelled albumin at 2 to 3 mg/ml protein concentration denatured. The u. v. spectrum of lyophilized human serum albumin failed to change even after doses above 2.5 Mrad. In addition, the product can still bind iodine. Thus, irradiation of lyophilized human serum albumin did not deteriorate the iodine-binding capacity of
the aromatic amino acids, quite unlike their irradiation in an aqueous solution, when the u.v. spectrum and the iodine-binding capacity of the products were found to be markedly damaged.

The radiosensitivity of sera with immunoglobulins and of purified immunoglobulin products themselves was also studied. The products were irradiated in the lyophilized state at -20°C. The antibody titre of human plasma did not change significantly as compared with the control. The studies were made on the relative radiosensitivity of isolated human immunoglobulins both in solution and in the lyophilized state. The products irradiated in solution lost their biological activity, while no activity decrease was observed in the preparations irradiated in the lyophilized state. Nevertheless, physico-chemical changes could be demonstrated in all the irradiated preparations. The u.v. absorption of the irradiated preparations increased and differences were found in the course of fractionation on the Sephadex column; furthermore, the irradiated preparations could not be digested with papaine. Of the plasma proteins the radiosensitivity of isoagglutinin was studied. The anti-A isoagglutinin proved to be more radiosensitive than the anti-B isoagglutinin. Radiosensitivity studies on the plasma protein fractions and of other factors of tissue origin known to be responsible for blood coagulation including human and bovine fibrinogen and fibrin revealed that fibrinogen is much more radiosensitive than serum albumin or the globulin fractions when irradiated either in an aqueous solution or in the lyophilized state. At a dose of 0.5 Mrad fibrinogen decomposes into components of lower molecular weight and loses its capacity of transforming into fibrin. This impairment is clearly demonstrated by the prolongation of the coagulation time. The author had studied the radiosensitivity of prothrombin, thrombin and thrombokinase. When measured in the blood coagulation system the biological activity of prothrombin and thrombin appreciably decreases after irradiation with doses of 1.5 to 2.0 Mrad, while the biological activity of thrombokinase does not decrease even after irradiation with 2.5 Mrad of 7-rays and is still suitable for radiosterilization.

The toxins constitute a further group of proteins. Their radiosensitivity is an important problem, both from the medical point of view and from that of food preservation. The radiosensitivity of the toxins of diphtheria, tetanus, botulinus, aflatoxin and the toxin of snake venom has been investigated by several authors. The results of these investigations have been published by the International Atomic Energy Agency (1970).

The investigations designed to inactivate the toxic properties of snake venom by radiation and to maintain its antibody-forming capacity are of special interest. Should this attempt succeed, it would permit active immunization against snake bite accidents. The radiation-induced change in the biological activity of toxins is a problem similar to that solved in a chemical way for the toxin-toxoid transformation.

CONCLUSIONS

Because of the individual functions of the single proteins and of their structural conformational differences, no general, uniformly valid rule can be set up for the radiosensitivity of proteins.
(1) The structural differences of the various proteins also involve differences in their radiosensitivities.

(2) The radiosensitivity of proteins in the solid state is appreciably lower than that of the proteins in aqueous solutions.

(3) In aqueous solution the radiosensitivity of the proteins depends on the protein concentration of the solution.

(4) As regards the radiosensitivity of proteins, the radiation effect may be related to changes in either the physico-chemical properties or the specific biological functions of proteins. Accordingly, to characterize the radiosensitivity of a given protein it should always be clearly indicated whether radiosensitivity is related to the physico-chemical properties or to the biological activity (as enzyme activity, antigenic or hormonal activity) of the given protein.

(5) The change occurring in the protein structure may ensue in the primary structure, as impairment of the amino acids (aromatic, sulphur containing) and in the secondary structure (as measured by the alteration of the optical absorption spectrum, optical rotation, sedimentation property, viscosity, deuterium exchange).

(6) The radiation-induced products may be demonstrated by their behaviour during chromatography, dimerization, analysis of amino acids, etc.

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3. CARBOHYDRATES

Investigations dealing with the alterations induced by ionizing radiation in compounds of various composition comprise — as an important line of research — the study of the behavior of carbohydrates, the determination of the role of degradation products formed at various radiation doses, and the significance of these products in biological processes.

The presence of carbohydrates in tissues and food products participating in the active metabolism only underlines the necessity of studying radiation effects. It is of great interest to learn more about the composition of the compounds formed by the effect of irradiation on mono-, oligo- and polysaccharides. A knowledge of the physical state is none the less important since different changes will occur in compounds irradiated in the solid or the liquid state. The atmosphere (oxygen, nitrogen) and the solvent (water or organic compounds) are important factors, too. Further factors affecting the radiation effect are the concentration and pH of the solution. Moreover, for substances irradiated in the solid state any relatively low humidity present also affects both the degree of the changes and the secondary reactions.

When studying radiation-induced changes in the living organism as well as when radiosterilizing food products, in addition to the quality of the transformation products formed from carbohydrates, the quantitative relations of the single compounds are also of interest. Oxidative degradation is the most common radiation-induced reaction of carbohydrates.

3.1. Effect of ionizing radiation on monosaccharides

The effect of radiation will be discussed from two aspects:

1. Radiolysis of aqueous solutions of monosaccharides
2. Radiation-induced changes in solid-state monosaccharides.

3.1.1. Radiolysis of aqueous solutions of monosaccharides

Several earlier papers report on the compounds found on post-irradiation qualitative analysis of aqueous solutions of monosaccharides. The data confirm that, in spite of earlier attempts to reveal X- or gamma-ray induced changes in glucose- and fructose-solutions (Becker et al., 1956; Berry et al., 1965; Bonet-Maury and Lormand, 1959), no detailed and precise results could be obtained until the introduction of recent, more sensitive analytical methods. A variety of degradation products are formed in aqueous solutions of monosaccharides by the effect of ionizing radiation. However, owing to the small quantity of compounds resulting from the primary and secondary reactions, classical methods have failed to find out the results of the intricate reactions, which proceed simultaneously. The introduction of the isotope dilution method represented an important advance, permitting the analysis of the $5.5 \times 10^{-2}$ mol hexose solution. A G-value of 3.5 was obtained for this solution, notwithstanding its having been irradiated in the presence of oxygen or in vacuo.

Generally, identical post-irradiation degradation products were identified for hexoses (Bothner and Balázs, 1957; Ehrenberg et al., 1951), such as
glucose. The irradiation of fructose yields more optically active compounds and a higher accumulation of formaldehyde than that of glucose irradiated with the same dose (Fig. 1). The irradiation of D-mannose in the presence of oxygen results in the changes given in Fig. 2. In the absence of oxygen, owing to the lack of secondary reactions, the degradation process will be simpler than in the presence of oxygen (Fig. 3).

The \( G \)-values found for the primary products induced by \( \gamma \)-radiation in \( 5 \times 10^{-2} \) mol aldohexose solutions are shown in Table III [7, 8]. It can be seen from the data summarized in the table that the primary reactions are always accomplished, irrespective of irradiation in the presence or absence of oxygen. The presence of various post-irradiation end products suggests that the secondary reactions of identical initial radicals are different. This certainly does not exclude the possibility that some compounds are formed under both conditions. As shown by the results, the radiation-induced changes of the various hexoses appear to be of a similar nature.
FIG. 3. Reactions on the irradiation of D-mannose.

FIG. 4. Irradiation of D-mannose in the absence of oxygen.
### TABLE III. G-VALUES OF PRODUCTS FORMED IN $5 \times 10^{-2}$ ALDOHEXOSE SOLUTIONS UPON $\gamma$-IRRADIATION

<table>
<thead>
<tr>
<th></th>
<th>D-glucose</th>
<th></th>
<th>D-mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vacuo</td>
<td>In the presence of $O_2$</td>
<td>In vacuo</td>
</tr>
<tr>
<td>Hexose (-G)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Hexonic acid</td>
<td>0.35</td>
<td>0.4-0.5</td>
<td>0.45</td>
</tr>
<tr>
<td>Hexuric acid</td>
<td>-</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>sec.</td>
<td>sec.</td>
<td>0.5-0.6</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>sec.</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td>Glucosone</td>
<td>0.4</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>Product with 2 C atoms</td>
<td>0.85</td>
<td>0.8</td>
<td>0.95</td>
</tr>
<tr>
<td>D-erythrose</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Product with 3 C atoms</td>
<td>0.8</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Polymer</td>
<td>sec.</td>
<td>-</td>
<td>sec.</td>
</tr>
</tbody>
</table>

Irradiation, generally, induces the formation of acids and ring-splitting, the latter yielding aldehydes with 2 and 3 carbon atoms. In certain cases, however, formaldehyde and arabinose arise from, e.g., glucose in the presence of oxygen (if irradiated in the presence of oxygen). The changes occurring in the presence of oxygen show that the decomposition of the carbon chain with the oxidation of monosaccharides leads to the formation of acids, formaldehydes and endioles, with an absorption maximum at 265 mm. The degree of the splitting of C-C bond was found to be lower after irradiation in vacuo as compared with irradiation in the presence of oxygen:

\[
\begin{align*}
H - C = O & \quad H - C = O \\
\text{in vacuo} & \quad \text{HO - C - OH} \quad \text{HO - C - H} \\
\text{HO - C - H} & \quad \text{HO - C - H} \\
\text{HO - C - H} & \quad \text{HO - C - O}_2. \\
\text{O}_2 & \quad \text{H}_2\text{C - O}_2. \\
\text{H - C = O} & \quad \text{H - C - O}_2. \\
\text{HO - C - O}_2. & \quad \text{H} - \text{C}^+ \\
\text{HO - C - H} & \quad \text{HO - CH} \\
\text{HO - C - H} & \quad \text{HO - CH} \\
\end{align*}
\]
No uronic acids appear upon irradiation in vacuo and no polymerization can be observed in the presence of oxygen. The initial hexose concentration influences the degree of the ensuing changes. Knowing the primary products arising in the course of the radiolysis of water, this is, to some extent, comprehensible. At a certain hexose concentration and radiation dose the following transformations should, as a rule, be reckoned with:

\[
\begin{align*}
\text{R - CH - OH} & \xrightarrow{O_2} \text{R - CH} + \text{HO}_2 \\
\text{R - CH - OH} & \xrightarrow{\text{in vacuo}} \text{polymer}
\end{align*}
\]

for example, for aldohexoses at a \(5.5 \times 10^{-2}\) mol concentration of the solution. Increasing acidity and the slight change of reducing capacity both suggest the formation of glucuronic acid. Summarizing, it may be concluded that both in vacuo and in the presence of oxygen the G-value is 3.5 and, furthermore, that the radiation effect is not restricted to a certain part of the molecule. A thorough analysis of the degradation or transformation products reveals that all the bonds are affected to a certain extent.

Oxidation is the final process. Simultaneous chain-splitting yields compounds with smaller molecules.

According to some data reported in the literature, when analysing the radiolysis of the aqueous solution of D-glucose, appreciable differences are found between the products formed from the \(5 \times 10^{-4}\) and the \(5 \times 10^{-2}\) mol solutions. The failure of chain-splitting is, e.g., characteristic for the D-glucose solution with a \(5 \times 10^{-4}\) mol concentration. Moreover, the kinetics of the two concentrations are essentially different.

The data given in Table IV clearly show the effect of concentration on the G-values for the D-glucose solution.

**Table IV. Effect of Concentration on the G-Values (D-Glucose) in Solutions**

<table>
<thead>
<tr>
<th>D-glucose (10^4 M)</th>
<th>5,5</th>
<th>55</th>
<th>275</th>
<th>555</th>
</tr>
</thead>
<tbody>
<tr>
<td>(- G)</td>
<td>1.5</td>
<td>2.1</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>(G_{(acid)})</td>
<td>0.6</td>
<td>1.3</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>(G_{(H_2O_2)})</td>
<td>3.0</td>
<td>3.0</td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>In vacuo</td>
<td>Air</td>
<td>CO₂</td>
<td>Hydrogen Quantity not bound by the solid lattice</td>
</tr>
<tr>
<td>----------------</td>
<td>----------</td>
<td>-----</td>
<td>-----</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Crystalline</td>
<td>13.3</td>
<td>13.3</td>
<td>13.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Lyophilized</td>
<td>3.7</td>
<td>3.7</td>
<td>-</td>
<td>4.0</td>
</tr>
<tr>
<td>Monohydrate</td>
<td>5.2</td>
<td>5.0</td>
<td>-</td>
<td>4.1</td>
</tr>
<tr>
<td>Syrup</td>
<td>6.5</td>
<td>-</td>
<td>~ 5</td>
<td>4.75</td>
</tr>
</tbody>
</table>

When increasing the D-glucose content of the solution the G-value will also increase. At the same time the G-value for H₂O will not change and G for acids increases also only up to 5 × 10⁻³ mol D-glucose concentration.

3.1.2. Radiation-induced changes in solid-state monosaccharides

Phenomena observed on the D-glucose crystal focussed the attention on the importance of energy transfer in the radiochemistry of carbohydrates.

The physical condition in which the dehydrated α-D-glucose is irradiated basically influences all the radiation-induced transformations. α-D-glucose, dehydrated by deep-freezing, displayed an extraordinary high stability when irradiated with ⁶⁰Co γ-rays. The properties of the α- and β-D-glucose crystals are similar. However, the G-values for the D-glucose dehydrated by deep-freezing are appreciably lower. On the other hand, the chemical properties of the products do not show any difference. This is demonstrated in Table V, which proves that the G-value is a function of the original D-glucose.

G(H₂O) and the initial G (free radical) values are both 4. The processes yielding acid production probably differ from those resulting in the formation of hydrogen and long-lived free radicals.

The radiation-induced changes in the crystalline α- and β-D-glucoses show that energy transfer and the arrangement of the crystalline structure are correlated. The ensuing chemical changes are a function of the degree of energy transfer, known to be a maximum in completely arranged crystals.

The X-ray diffraction patterns of lyophilized and dehydrated α-D-glucose cannot be differentiated by either crystallographic or chemical methods.

The degree of decomposition in the crystalline forms studied is unusually high as compared with other organic molecules. Energy transfer and the utilization of transferred energy in the crystals have shown that this is connected with the network of hydrogen bonds within the crystals. Such bonds have proved to be effective in energy transfer. The structure of α-D-glucose monohydrate is rich in hydrogen bonds. In monohydrates the distance between the neighbouring D-glucose molecules is longer in certain directions than in dehydrated D-glucoses. Consequently, as shown
by the ESR spectrum, the efficiency of energy transfer is highly dependent on the intramolecular spaces. Provided it is the G-value that expresses the efficiency of energy transfer, the lower value obtained for monohydrates, as compared with α- and β-D-glucoses, may be the direct result of the intermolecular distances in monohydrates.

Pulse radiolysis

The analysis of the pulse radiolysis process immediately reveals that further degradation processes appear in solutions with a concentration higher than \(5 \times 10^{-2}\) mol. When eliminating the initial OH radicals intermediary compounds appear at the absorption maximum measured at 2600 to 2700 Å. Owing to combination reactions, the OH radicals tend to disappear at any higher D-glucose concentration.

\[
\begin{align*}
\mathrm{H}_2\mathrm{O} & \quad \mathrm{e}^-_{\text{aq}} + \mathrm{OH} \rightarrow \mathrm{H}_2\mathrm{O} + \mathrm{OH}^- \\
\mathrm{e}^-_{\text{aq}} + (\mathrm{H}_2\mathrm{O})^+ & \rightarrow 2\mathrm{H}_2\mathrm{O} \\
\mathrm{OH} + \mathrm{OH} & \rightarrow \mathrm{H}_2\mathrm{O}_2
\end{align*}
\]

Solid-state D-glucose is extremely sensitive to gamma radiation. An initial G-value of 20 was the maximum observed for polycrystalline α-D-glucose.

Investigating the direct effect, it has been established that complete decomposition runs to about 6% in the \(5 \times 10^{-2}\) mol solution, but may be appreciably higher when increasing the concentration. Water molecules, associated with the carbohydrates, may also interfere with energy transfer. However, their role in radiochemical processes has not been proved as yet.

Radiation-induced transformations are affected by changes in pH value as well, since this contributes to the formation of excited water molecules:

\[
\begin{align*}
\mathrm{H}_2\mathrm{O}^x + \mathrm{H}^+ & \rightarrow \mathrm{H}_2^+ + \mathrm{OH} \\
\mathrm{H}_2\mathrm{O}^x + \mathrm{OH}^- & \rightarrow \mathrm{e}^-_{\text{aq}} + \mathrm{OH}
\end{align*}
\]

Energy transfer from the excited \(\mathrm{H}_2\mathrm{O}\) to sugar molecules proceeds advantageously. Naturally, in aqueous solutions water molecules are associated with carbohydrates. However, owing to the sugar hydrates, a similar energy transfer may proceed in solid-state sugars, too. In support of this theory, reference is made to the observations that the energy required for the destruction of D-glucose decreases if the sugar concentration is raised from 1 to 60%.

3.2. Effect of ionizing radiations on oligosaccharides

As a rule, the oxygen bridge is split upon irradiation of oligosaccharides (maltose, lactose, raffinose). This accounts for the increase in reducing
capacity. Experiments with amylose complexes would promote a better understanding of this mechanism.

3.2.1. Cycloamylose complexes

As is well known, irradiation of D-glucose crystals results in a relatively high-grade excitation within the crystals. However, while the occurrence of this excited state has been proved for the aromatic crystals, no direct evidence is available on the possible excitations in carbohydrate crystals. Provided that the radiation-induced decomposition of various D-glucoses corresponds to what has been established for the monosaccharides and the excited state really occurs, this energy may be transferred to other molecules, too. To confirm this kind of energy transfer, research has been performed with some of these systems in which carbohydrate molecules are known to occur closely associated with other organic molecules. Finally, the cyclic oligosaccharides formed on potato starch by Bacillus macerans (Schardinger dextrins) were studied. These dextrins are unique with cyclic molecules and no free reducing groups.

Two aspects are valid for the radiation effect exerted on pure Schardinger-dextrin crystals.

First, the ESR spectrum of the gamma-irradiated cyclohepta-amylose displays a superfine structure with splits. 'Glycoseoxy radicals' may be supposed to account for the presence of the small, superfine splits:

\[
\begin{align*}
\text{CH}_2\text{OH} & \\
\text{H} & \\
\text{H} & \\
\text{O} & \\
\text{H} & \\
\text{OH} & \\
\text{H} & \\
\text{OH} & \\
\end{align*}
\]

where there is only a weak interaction with the proton and the G-value for C\textsubscript{1} (radical) in the irradiated cyclohepta-amylose is not less than 5.

Secondly, the main chemical change related to gamma irradiation is the splitting of the ring system. However, no glucose is formed, maltohexose being the main initial product. Glucuronic acid is the main acidic product, though, in particular after high radiation doses, other acids also appear. The effects observed can be shown schematically as follows:

I. cycloamylose

II. some intermediary radical transforms \rightarrow into

III. carbonium ion, the C\textsubscript{4} of which reacts rapidly with water and yields the adequate alcohol (IV).

Glucoseoxy is the most probable intermediary product in the formation of glucuronic acid. The maltohexose observed is merely some additional compound and the G-value of the pure cycloamylose is about 15.
TABLE VI. PROTECTION OF CYCLOAMYLOSE BY COMPLEX FORMATION

<table>
<thead>
<tr>
<th>Associated molecule</th>
<th>Ratio of associated molecule to cycloamylose</th>
<th>Electron affinity</th>
<th>$E_0$(eV)</th>
<th>$E_0$(eV)</th>
<th>$E_0$(eV)</th>
<th>G Radical</th>
<th>$-G$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azo benzene</td>
<td>0.4</td>
<td>9</td>
<td></td>
<td></td>
<td>2.80</td>
<td>11.4</td>
<td></td>
</tr>
<tr>
<td>Anthracene</td>
<td>0.2</td>
<td>12</td>
<td>1.82</td>
<td>0.82</td>
<td>3.26</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>Tetracyano-ethylene</td>
<td>0.3</td>
<td>7000</td>
<td>3.87</td>
<td></td>
<td></td>
<td>8.20</td>
<td></td>
</tr>
<tr>
<td>Methylmalthalene</td>
<td>0.6</td>
<td>2.62</td>
<td>1.21</td>
<td>3.83</td>
<td>2.55</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>p-Nitrophenol</td>
<td></td>
<td>3.87</td>
<td></td>
<td></td>
<td>4.45</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Nitrocyclohexane</td>
<td>1.0</td>
<td>4.45</td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>1.6</td>
<td>4.61</td>
<td>0.22</td>
<td>2.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluol</td>
<td>0.7</td>
<td>3.58</td>
<td>1.86</td>
<td>4.60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>1.4</td>
<td>4.37</td>
<td></td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iodine benzene</td>
<td>e</td>
<td>4.80</td>
<td></td>
<td>6.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-bromine naphthalene</td>
<td>e</td>
<td>2.56</td>
<td>4.70</td>
<td>7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylacetylene</td>
<td>1.2</td>
<td>4.76</td>
<td>1.65</td>
<td>8.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbon tetrachloride</td>
<td>1.3</td>
<td>~6.5</td>
<td></td>
<td>14.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetrachlor-ethylene</td>
<td>1.3</td>
<td>~7000</td>
<td>~7.0</td>
<td>3.4</td>
<td>16.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>0.4</td>
<td>0.01</td>
<td>7.8</td>
<td></td>
<td>16.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The effect of energy transfer in the complexes of cycloamyloses with organic compounds can be proved in two different ways. First of all, the ESR spectrum will be different and its intensity will be appreciably reduced, provided the proper complex is irradiated. Secondly, this property is related to the chemical protection of cyclohexa-amyloses. The changes occurring in the ESR spectrum are shown, e.g., by the complex consisting of benzene and cycloamylose.

In addition, the characteristic features of changes occurring in various other complexes have also been determined.

Table VI contains the characteristic results for the various cycloamylose complexes. As clearly appears from the results, in the case of electron transmission the affinity to the electron of the molecule actually participating in the complex may interfere with the efficiency of chemical protection. This, however, is not quite clear-cut.
3.2.2. Amylose complexes

The protection valid for the cycloamylose model system (see previous section) can be directly adapted to the polysaccharides occurring in nature. Amylose complexes are formed from starch in a similar way as the cycloamylose complexes referred to earlier. X-ray crystallographic analysis of the amylose molecule displays a helical structure. To determine the efficiency of energy transfer, the effect of gamma radiation on solid complexes has been studied in which the organic molecules were located in the inside of the amylose helix. In conformity with the previous experimental results obtained for other polysaccharides, degradation constitutes one of the major changes occurring upon irradiation of solid-state amylloses.

Glycoside splitting yields smaller saccharides. The degradation of amylose in corn is significantly less than the degradation of wheat amylose exposed to the same dose of irradiation. This difference in radiation responses may be a consequence of the differences in their crystallization patterns. In turn, molecular aggregation is highly influenced by the radiation-induced degradation products of D-glucose.

The intensity of the colour reaction of amylose with iodine (blue value) is directly related to the length of the amylose chain. The measurement of the blue value changes as a function of the radiation dose which serves as an adequate and sensitive method of determining degradation following gamma irradiation. The decrease in the blue value displays an exponential relationship with the increasing radiation dose, suggesting a progressive shortening of the polysaccharide chains. This exponential relationship permits a precise estimation of the change in the initial blue value through 100 eV.

3.3. Effect of ionizing radiations on polysaccharides

3.3.1. Effect of radiation on cellulose

Cellulose is one of the most commonly known and used natural polymers. The information on the effects of ionizing radiations on the changes in the properties of cotton-cellulose are of immediate interest for the textile industry. The following are of major interest in this regard:

(a) Refined cellulose fibres, which retain high molecular weight, can be produced directly from natural fibres;
(b) The effect of radiation on the chemical and physical properties of cellulose can be determined by both chemical and physical methods;
(c) The effect of radiation and radiation-induced reactions on the properties of macromolecules in cellulose fibres can be determined by the usual procedures for testing fibre polymers.

The effect of high-energy radiations on cellulose has two important practical application interests. One of these is the sterilization of medical supplies, the other is the possibility of influencing the macromolecular properties of irradiated cellulose fibres. In the latter case, the treatment is partly designed to retain the natural properties and partly to improve
them by producing grafted co-polymers. Research into the possible application of ionizing radiation in the fields of textile fibres, paper and wood products is in progress.

Cellulose is a high polymer, built of \((\text{C}_6\text{H}_{10}\text{O}_5)^n\) units where \(n\) represents the number of monomers.

\[
\begin{array}{c}
\text{CH}_2\text{OH} \\
\text{H} \\
\text{OH} \\
\text{H} \\
\text{H} \\
\text{O} \\
\text{H} \\
\text{OH} \\
\text{H} \\
\text{H} \\
\text{O} \\
\text{H} \\
\text{CH}_2\text{OH}
\end{array}
\]

Different methods have given different values for the molecular weight; \(n\) for cotton cellulose ranges from 4000 to 5000, while the respective molecular weight may reach as much as 800 000. As shown by X-ray diffraction studies, both crystalline and amorphous constituents occur in the cotton fibres: 70 to 80% of natural cellulose is crystalline and 20 to 30% is amorphous.

When irradiating the cellulose molecule, dehydrogenation and decomposition will be the primary effects in its constituent units, with the release of gaseous hydrogen, carbon monoxide and carbon dioxide. Table VII shows the distribution of the various gaseous degradation products formed in the gaseous atmosphere. Dehydrogenation results in the formation of both short-lived and long-lived free radicals retained in the cellulose lattice.

<table>
<thead>
<tr>
<th>Atmosphere at irradiation</th>
<th>Dose ((x 10^{20} \text{ eV/g}))</th>
<th>Gaseous product ((\mu\text{mol/g cellulose}))</th>
<th>(\text{H}_2)</th>
<th>(\text{CO}) (%)</th>
<th>(\text{CO}_2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vacuum</td>
<td>33.0</td>
<td>205</td>
<td>35</td>
<td>22</td>
<td>43</td>
</tr>
<tr>
<td>Oxygen</td>
<td>41.6</td>
<td>430</td>
<td>19</td>
<td>27</td>
<td>54</td>
</tr>
<tr>
<td>Air</td>
<td>13.9</td>
<td>127</td>
<td>13</td>
<td>59</td>
<td>28</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>22.0</td>
<td>163</td>
<td>22</td>
<td>56</td>
<td>22</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>32.1</td>
<td>217</td>
<td>24</td>
<td>51</td>
<td>25</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>37.6</td>
<td>247</td>
<td>24</td>
<td>51</td>
<td>25</td>
</tr>
</tbody>
</table>
Formation of free radicals

Under gamma-radiation exposures in air atmosphere long-lived free radicals are formed from the purified fibrous cotton cellulose (I) and cotton cellulose (II) at the normal humidity of about 7%. These free radicals also appear in the ESR spectrum. The ESR spectrum of the irradiated, purified fibrous cotton cellulose (I) consists of three and that of the cotton (II) of five lines.

As appears from the three-line spectrum of irradiated cellulose, dehydrogenation yields a free radical on C₅ atom:

![Free Radical on C₅](image)

The OH group splits on C₆ atom:

![Free Radical on OH](image)

or the H atom splits from the OH group on C₆ atom:

![Free Radical on H](image)

The compound so obtained may interact with the equivalent hydrogens on C₆ atom to give a three-line spectrum. The most important line of the spectrum may have an identical value for any of the free radicals.
Effect of oxygen

As shown by the respective ESR spectra, the atmosphere exerts a different effect on irradiated cellulose I and II. At the same dose the most important line in the ESR spectrum was more intensive after irradiating cellulose I in nitrogen than in oxygen atmosphere. No or only a slight change was found for irradiated cellulose II.

Effect of water

Increasing the water content generally decreases the number of free radicals. When dry cellulose is irradiated in vacuo and then brought into water vapour, the concentration of free radicals decreases to reach a certain equilibrium. In general, it can be established that, when some humidity is present in the cellulose prior to irradiation, the concentration of free radicals at a given dose is lower as compared to the situation when the vapour is added after irradiation. Both vapour absorption and the retention of liquid water decrease in cotton cellulose (I and II) as a result of irradiation.

Captured radicals

The long-lived free radicals, or at least part of them, are formed and captured within the crystal lattice of the cellulose molecule. This reduces their contact with oxygen and humidity. Various solvents, e.g. aqueous zinc chloride solution, interact with cotton cellulose, loosen the fibre structure and so make the free radicals accessible to humidity and other reagents.

Intramolecular energy transfer

When irradiating solid-state carbohydrates, the energy of ionizing radiations is at first captured at random and then distributed within the molecule as the energy associated with the high-energy electrons. Subsequently, during its fast localization this energy induces chemical and physical changes within the molecule. Compton scattering seems to be the fundamental mechanism in the $^{60}$Co gamma irradiation of carbohydrates. Energy transport in solid-state carbohydrates depends on the physical and chemical structure of certain complexes on the compounds involved (Phillips, 1968).

In the case of fibrous cotton cellulose the energy localized in part during irradiation induces the splitting of cellulose, with concomitant fragmentation of the fibres.

Investigations have been carried out on the radiosensitivity of celluloses that had reacted with aromatic groups. With the exception of some molecules, the aromatic groups in general have been found to be radioprotective. Fibrous cellulose undergoes morphological alteration on irradiation. The fibrous cellulose structure begins to alter and the long fibres start to disrupt.

When testing the physical properties it clearly appears that the fundamental change induced by irradiation is the depolymerization of the fibrous
cellulose, i.e. the chain-splitting of the molecule. The appreciable decrease in molecular weight in the presence of oxygen is accountable to this phenomenon as well. Besides the changes in the chain length, higher doses also induce appreciable transformations in the inner lattice structure. These transformations may be followed both by acidic hydrolysis and by changes in the iodine absorption values.

The increase in the quantity of the fraction soluble in 1% NaOH solution also suggests irradiation-induced depolymerization.

In the course of analysing the changes of chemical properties, in addition to the gaseous compounds released from irradiated cellulose, quite a series of other degradation products have also been identified by paper chromatography.

If cellulose is irradiated in an oxygen atmosphere, a slightly higher number of degradation products (cellobiose, glucose, 2-ketogluconic acid, arabinose, glyoxal, 2-ketocellobionic acid, 2-ketogluconolactone, four different acids and six other products) are formed as compared to its irradiation in a nitrogen atmosphere.
CHAPTER 3
THE EFFECT OF IONIZING RADIATION
ON BACTERIA

Irradiation with either gamma rays or electrons as a method of controlling bacteria has yet to be fully recognized alongside heating, drying, cooling and the use of toxic chemicals. It is the newest of these processes with some unique properties as far as the practical requirements of bacterial inactivation are concerned. Ability to penetrate, bringing lethal effect to even the most apparently inaccessible contaminating cell, is of particular attraction. This property, combined with insignificant temperature rise during treatment, is already exploited to full effect in the sterilization of medical devices, pharmaceuticals and biological tissues. Closely related in many ways is the application of the process to food preservation and the control of pathogens in food, although commercial implementation has been slow, mainly due to health authority requirements for long-term animal feeding studies to demonstrate the absence of toxic effects in the irradiated food itself. However, radiation microbiology is common ground to both fields, brought out in Table VIII, and it is relevant to draw on the broad data in assessing the effectiveness and limitations of the method. It is the intention in this chapter to bring out this point and to review not only the specific bacteriology upon which the medical sterilization process has been introduced but also to refer to similar work in allied fields, particularly food. For the final analysis of the microbiological safety of the process and its definition in terms of dose and suitability of use, a judgement based on broad experience must be made.

In contrast to the more applied microbiology carried out over the last 15 years, fundamental studies on bacteria had their beginning soon after the discovery of X-rays by Röntgen in 1895 and much has been contributed since in attempting to elucidate the mechanism of lethal action on the cell. The complexities of the influence of the biochemical and physiological state of the cell at the time of irradiation and of environmental conditions before, during and after treatment have been made apparent. Other contributions have established the quantitative relationship between reduction in viable numbers of a bacterial population and increasing dose. Such work is fundamental to the use of ionizing radiations in tumour therapy and in the same way it underlies the philosophy adopted in radiation sterilization. Although by far the most recently introduced physical sterilization technique, irradiation enjoys a very advanced and sophisticated scientific background of research, some of which will emerge here with reference to the bacteriological aspects of the process.

1. INACTIVATION OF BACTERIAL POPULATIONS

Criteria

Early experimenters trying to discover whether X-rays were bactericidal or not took as an end point the complete sterilization of a
<table>
<thead>
<tr>
<th>Objective</th>
<th>Means of attainment</th>
<th>Dose recommended (Mrad)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term preservation of meat, fish and other non-acid foods</td>
<td>Inactivation of spoilage organisms and pathogens, in particular Clostridium botulinum</td>
<td>4-6</td>
</tr>
<tr>
<td>Prevention of infection in medical use of devices, pharmaceuticals and biological tissues</td>
<td>Sterilization to standards required by health authorities</td>
<td>2.5-4.5</td>
</tr>
<tr>
<td>To attain public health standards required in animal hair etc. used in carpet manufacture</td>
<td>Elimination of pathogens particularly Bacillus anthracis</td>
<td>2.0-2.5</td>
</tr>
<tr>
<td>Supply of 'specific pathogen-free' diets for laboratory experimental animals</td>
<td>High degree of inactivation of all contaminants</td>
<td>1.5-2.5</td>
</tr>
<tr>
<td>To prevent contamination of contents of pre-packed products, pharmaceutical or food by packaging material itself</td>
<td>Sterilization of packaging material before use</td>
<td>1.0-2.5</td>
</tr>
<tr>
<td>To prevent contamination of food to which spices and similar ingredients are added</td>
<td>Reduction in population</td>
<td>1.0-2.0</td>
</tr>
<tr>
<td>Extension of refrigerated storage of various foods, i.e. 0°C-4°C</td>
<td>Reduction in numbers of relevant spoilage organisms, mainly vegetative</td>
<td>0.1-1.0</td>
</tr>
<tr>
<td>Prevention of food poisoning</td>
<td>Inactivation of salmonelllas</td>
<td>0.5-1.0</td>
</tr>
<tr>
<td>Preservation of fruits and vegetables</td>
<td>Reduction in population of fungi and yeasts</td>
<td>0.1-0.5</td>
</tr>
</tbody>
</table>
culture. The appearance of any colonies at all was recorded as indicating a negative result. Other tests were made by inoculating animals with irradiated bacteria and even if only a few remained viable, the animals became infected and again the conclusion was drawn that the rays were not bactericidal. It is not surprising that the subject became confused with quite different conclusions being reached from similar experiments.

The criterion of radiation-induced cell death or inactivation is now taken as the loss by the cell of colony-forming ability after inoculation on a nutrient medium. Effectiveness of specific treatment is measured in terms of numbers of cells surviving out of the original population. With such data we can now refer to the radiation resistance or sensitivity of a population and express it in quantitative terms.

Inactivation in quantitative terms

Dose/survival curves illustrate the relationship between numbers of organisms surviving and radiation dose. In practice the necessary data are usually obtained by exposing a number of equal sized populations to different doses of radiation and counting the number of survivors. This number, expressed as a fraction of the original number, when plotted linearly against dose gives rise to a curve which fits one of the shapes shown hypothetically in Fig.5. It will be observed that in all cases the surviving fraction decreases with increasing dose, becoming asymptotic with the X-axis. It follows that when choosing a sterilization dose for a population this choice must be based on some probability of the existence
FIG. 6. Semi-logarithmic plots of the hypothetical dose/survival curves shown in Fig. 5.

of survivors. It is convenient for mathematical manipulations to transpose the curves to a semi-logarithmic plot as given in Fig. 6. The four shapes were distinguished by Gunter and Kohn (1956) and by Alper (1961), who designated them A, B, C₁ and C₂ for convenience. However, it is interesting that the simple exponential curve represented by type A with its constant slope over the whole dose range was demonstrated for bacteria as early as 1912. A continually decreasing slope with increasing dose characterizes the type B concave curve, whilst with type C₁ a clearly defined shoulder is seen leading to eventually a simple exponential form. Type C₂ exhibits a continually increasing slope with increasing dose.

Populations of the majority of species of microorganisms yield dose/survival curves of either A or type C₁ with a shoulder at the beginning of the curve. Curves for Pseudomonas sp. and Salmonella typhimurium shown in Fig. 7 are of type A and the remainder of type C₁ illustrating shoulders extending in size up to that for Micrococcus radiodurans, the curve for which is produced from data of Krabbenhoft et al. (1967).

From the semi-logarithmic plot it is clearer to understand how a sterilization process must be based on the existence of survivors. For curves of type A and of type C, which exhibit small shoulders, the simple exponential relationship means in practical terms that if a given dose reduces the number of viable cells in a bacterial population by 90%, then a second exposure of the survivors to the same dose will reduce their number again by 90%, leaving 1% survivors; a third exposure leaves 0.1% survivors and so on. Expressed in terms of treatment of material,
if the initial degree of contamination is 100 organisms per gram, then, after a total exposure equivalent to three such doses or heat treatments, one organism would be expected to survive if 10 g of material were so treated.

The $D_{10}$-value

The probability of the existence of survivors can be calculated from survival curves, assuming that the linear exponential relationship holds at least over the functional part of the curve. First, the $D_{10}$-value, sometimes referred to as the decimal reduction dose, is obtained. This is defined as the dose that will reduce a given population by a factor of 10. The $D_{10}$-value is readily obtained from the linear part of a dose survival curve by reading off the dose required to reduce a surviving fraction through one log cycle. The linear plot that has been used here can be expressed mathematically as

$$\log_{10} \frac{N}{N_0} = -kD$$
where $N$ is the number of cells surviving a treatment $D$, $N_0$ is the initial number of viable cells, and $k$ is a constant equal to the slope of the curve. The slope can be seen to be $1/D_{10}$. The number of log cycles or powers of 10 by which a population is reduced by a given treatment is obtained simply by dividing the treatment dose by the appropriate $D_{10}$-value. If this quotient is designated $x$, then the term $10^x$ is referred to as the inactivation factor. A knowledge of this factor for a given species, together with a knowledge of the initial contamination level, allows a definition of the effectiveness of a given treatment.

The $D_{10}$-value, calculated from Fig. 8, for spores of B. pumilus irradiated in air, is 0.17 Mrad; the often used radiation-sterilization dose of 2.5 Mrad would therefore give an inactivation factor of $10^{15}$. In practice, this means that if articles were contaminated each with 100 such spores and then treated under similar conditions with 2.5 Mrad, an examination of $10^{13}$ articles should reveal that one organism has survived. This method of calculating inactivation factors does not take into account any initial shoulder, which often occurs on the curve, but the correction required is usually very small when considering the inactivation factor obtained by a sterilization treatment; the correction would, of course, be large with radiation for an organism such as M. radiodurans. In such a case the inactivation obtained at a particular dose must be read directly from the curve.

**Difference in resistance between species**

It is apparent from the use of inactivation factors that the initial numbers of organisms in a population influences the probability of the existence of survivors after a given radiation treatment. It follows that good hygiene in product manufacture, keeping the number of contaminants low, will improve the margin of safety achieved at a given sterilization dose. Also already apparent is the influence of the species of organism in the population. It can be seen in Fig. 8 that the 6 species cover a dose range from about 0.1 to 1.6 Mrad in achieving an inactivation factor of $10^5$. In general it is accepted that viruses are more resistant than bacterial spores, resistance increasing with decreasing particle size, and in turn spores are more resistant than vegetative organisms, yeasts and moulds. However, an exception is seen already with M. radiodurans, fortunately this organism is non-pathogenic and unlikely to occur as a contaminant on commercial medical products. There are other examples

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**Fig. 8.** Profile of resistance for spores of B. pumilus air-dried from water on to glass slides. Figures are $D_{10}$-values in Mrad.
of exceptionally high inherent resistance for example among the yeasts (Bridges et al., 1956). Erdman et al. (1961a), examining the comparative resistance of specific bacteria of public health significance, concluded that in broth suspension Streptococcus faecalis was more resistant than the staphylococci, salmonellae, coliforms and Micrococcus tuberculosis. It is important to note that because of the particularly low resistance to radiation of the coliforms, their absence after treatment should not be used as a measure of effective elimination of other pathogens as is sometimes used to check heat pasteurization. Similarly it is not logical to use cultures of Bacillus stearothermophilus as a monitor of radiation sterilization since it is not of particularly high resistance, although it is a good choice for monitoring heat sterilization.

A very important contribution to the introduction of radiation sterilization into commercial practice was made in a series of papers published in 1956. Although not based on dose/survival curve experiments, these papers give an insight into the comparative resistance of a wide range of bacterial spores (Pepper et al., 1956), vegetative organisms (Koh et al., 1956) and yeasts and moulds (Bridges et al., 1956). Similar experiments in the UK were designed to compare species, for example, Darmady et al. (1961) examined the inactivation of large populations of six vegetative and six spore-forming organisms. They were dried on to large numbers of paper discs, the vegetatives from glucose or glucose nutrient broth and the spore formers from distilled water. The results are given in Table IX and special attention was drawn to the comparatively high resistance of Bacillus pumilus E601 previously noted in US work.

Not only is there an obvious difference in inherent resistance between microbial species but also even between strains of the same species. As a background to the successful commercial process of radiation inactivation of anthrax spores in goat hair bales imported into Australia for carpet manufacture, Horne et al. (1959) examined eight strains of B. anthracis and noted significant differences in resistance. D10-values derived from survival curves for 21 strains of Lactobacillus ranged from 5 to 14 krad (Dupuy and Tremeau, 1961). Using whole egg as medium Comer et al. (1963) examined 18 serotypes of Salmonella and the D10-values ranged from 50 to 77 krad.

The question of the types of microorganisms involved in contamination is obviously important in defining the sterilization process as well as the initial numbers involved. It is to be expected that the process should deal effectively with even the most resistant species present. Food microbiologists have based a choice of radiation sterilization treatment for non-acid foods such as meat and fish on the resistance of the pathogen Clostridium botulinum, which is a known possible contaminant. In the medical field B. pumilus spores have been considered as a suitable reference, being typical of the more resistant spores. However, the importance of the high resistance of faecal streptococci has been stressed and attention drawn to Streptococcus faecium A21 (Christensen, 1964; Christensen and Sehested, 1964). Unfortunately the matter of defining the sterilization process in terms of numbers and type of contamination is further complicated by the fact that there are a number of factors concerned with the physiological state of the organisms themselves and with the environmental conditions in which the organisms are irradiated, which can markedly influence resistance.
<table>
<thead>
<tr>
<th>Organisms tested</th>
<th>Number of organisms per disc</th>
<th>Control</th>
<th>Percentage sterile after 21-d incubation at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0 Mrad</td>
</tr>
<tr>
<td>Ps. pyocyanea</td>
<td>$2.6 \times 10^7$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Staph. aureus</td>
<td>$1.1 \times 10^7$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Strep. viridans</td>
<td>$1.2 \times 10^7$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Strep. faecalis</td>
<td>$1.5 \times 10^7$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>E. coli</td>
<td>$8.7 \times 10^4$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>M. cryophilus</td>
<td>$4.2 \times 10^3$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>$8.1 \times 10^4$</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>B. globigii</td>
<td>$1.7 \times 10^5$</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>B. pumilus</td>
<td>$5.8 \times 10^7$</td>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>$1.6 \times 10^5$</td>
<td>0</td>
<td>99</td>
</tr>
<tr>
<td>Cl. welchii</td>
<td>$2.3 \times 10^5$</td>
<td>0</td>
<td>68</td>
</tr>
<tr>
<td>Cl. tetani</td>
<td>$3.2 \times 10^5$</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Note: One hundred paper discs per organism were irradiated at each dose level.
The oxygen effect

The role of oxygen in enhancing radiation damage is well established in radiobiology. With vegetative bacteria the $D_{10}$-values may be diminished by a factor between 2.5 and 4.5. The factor is often about 3.0, as seen in the results given in Table X for various serotypes of Salmonella. The absence of any difference between the aerated and non-aerated buffer suspensions is to be expected when it is appreciated that even the presence of small amounts of oxygen are sufficient to result in a marked reduction in $D_{10}$. Howard-Flanders and Alper (1957) demonstrated with Shigella flexneri that the concentration corresponding to a $D_{10}$-value halfway between the anoxic and fully aerobic values was 4.0 $\mu$M/litre, produced by equilibration with a gas mixture containing only 0.3% oxygen. With bacterial spores the $D_{10}$ reduction factor is expected to be in the range 2.0 - 3.0. A factor of 2.0 was noted by Burt and Ley (1963) for B. pumilus E 601 suspended in buffer, which is the same value recorded by Webb and Powers (1961) for spores in water suspension and similar to the overall oxygen effect of 2.6 observed by Powers et al. (1960) with air-dried spores.

The role of water

Extremely large differences in radiation resistance have been observed in experiments with a freeze-dried preparation of Bacillus megaterium spores mounted on aluminium silicate powder, reviewed by Tallentire (1967). The powder was chosen as substrate because it is inert to radiation and furthermore the system can be subjected to a high vacuum drying procedure designed to take the spores to a low level of water content. Spores were obtained with a water content equivalent to equilibration to a water vapour pressure of $5 \times 10^{-4}$ torr and higher water contents achieved by exposure to partial pressures of water vapour ranging up to 21 torr.

The very dry $5 \times 10^{-4}$ torr spores were subjected to three different gaseous treatments during and after irradiation, which resulted in three distinct levels of radiation resistance. Maximum resistance occurred with the spores irradiated in the absence of $O_2$ and treated with $H_2S$ gas immediately afterwards, i.e. no exposure to $O_2$. This resistance was a factor of 5 higher than for spores irradiated in anoxia but exposed to oxygen after irradiation and 15 times higher than for spores irradiated in $O_2$ and held in $O_2$ post irradiation. It requires at least 24 hours' storage at ambient temperature for the post-irradiation $O_2$ effect to reach a maximum. The results are interpreted to mean that there are at least three types of lethal damage induced by gamma radiation, as also concluded by Powers et al. (1960) in other experiments earlier for irradiation with X-rays. Firstly, damage can be caused that is completely independent of the presence of oxygen, secondly, oxygen can be involved in damage through exposure of anoxically irradiated spores to air and, thirdly, oxygen plays a full role in damage when present during and after irradiation.
TABLE X. THE $D_{10}$-VALUES (krad) FOR VARIOUS SEROTYPES OF Salmonella SUSPENDED in PHOSPHATE BUFFER (Ley et al., 1963)

<table>
<thead>
<tr>
<th>Media</th>
<th>$S.\ gallinarum$</th>
<th>$S.\ senftenberg$</th>
<th>$S.\ typhimurium$</th>
<th>$S.\ paratyphi\ B$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerated</td>
<td>13.2 (12.3-14.3)</td>
<td>13.0 (12.7-13.3)</td>
<td>20.8 (19.1-22.9)</td>
<td>19.0 (17.0-21.5)</td>
</tr>
<tr>
<td>Non-aerated</td>
<td>13.2 (11.3-15.7)</td>
<td>12.9 (11.6-14.4)</td>
<td>17.7 (15.5-20.5)</td>
<td>17.1 (16.4-17.8)</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>36.1 (34.8-38.1)</td>
<td>38.9 (35.5-42.8)</td>
<td>61.9 (58.9-65.3)</td>
<td>65.9 (62.6-69.5)</td>
</tr>
</tbody>
</table>

99% confidence limits in brackets.
The water content of such spores has an influence on these classes of damage. For the third type, with $O_2$ playing a full role, there is a seven-fold increase in resistance for a change from a very dry condition to a fully hydrated one. In the practice of radiation sterilization of medical products it might not be expected that natural organisms will be in as dry a condition as to show high sensitivity to radiation. Air-dryness is perhaps to be expected, but even so oxygen can play a role in damage during and after irradiation. The profile of resistance, expressed in $D_{10}$-values, shown in Fig. 8 is based on results with B. pumilus E 601 air-dried from water onto glass slides. The profile is modelled after the more detailed one described by Powers and Kaleta (1960). The lowest $D_{10}$ of 0.14 Mrad in the presence of the full oxygen effect compares with the anoxic value of 0.32 with post-irradiation oxygen limited by immediate suspension into Ringer's solution. Resistance is increased whenever this limited oxygen effect is removed by $H_2S$ treatment and finally a very high $D_{10}$ of 0.60 Mrad coincides with complete absence of oxygen-induced damage. A profile of resistance such as this will vary quantitatively with different species according to inherent resistance but the influence of oxygen and $H_2S$ will remain qualitatively the same.

Protection

Protective agents are chemicals which themselves reduce the lethal effect of radiation. The protective effect of hydrogen sulphide has already been mentioned. Aliphatic alcohols have been reported as effective by Hollaender and Stapleton (1953). Dewey (1960) studied protection by glycerol using Serratia marcescens and found greater protection under aerated than anoxic conditions. This compound acts with both spores and vegetative bacteria (Webb and Powers, 1961). Bridges (1962a,c) reported protection by dimethyl sulfoxide and by thiourea. A three-fold increase in resistance of B. megaterium exposed to the full oxygen effect is achieved with very dry spores by drying them in the presence of $3 \times 10^{-1}$ M thiourea (Tallentire, 1967).

Some chemical compounds exert apparent protection by using up oxygen causing depletion during irradiation. This can be done by purely chemical reaction induced by the radiation or by stimulating metabolic processes in the organism that are oxygen consuming. It is also possible that certain physical conditions imposed could result in oxygen lack.

In studies directed towards radiation sterilization interest has centred on estimating the protection afforded by chemically complex biological material that might form the natural environment of contaminants. In addition it has been supposed that the materials of the products themselves that are being sterilized might afford chemical protection. One approach was to simulate the situations that might arise. Burt and Ley (1963) air-dried B. pumilus spores from various suspending media, representing inorganic, organic and biological material, on to the surfaces of different materials representative of those used in the manufacture of disposable medical equipment. The conditions studied are shown in Table XI and the results expressed in $D_{10}$-values. Radiation resistance was only affected where local anoxic conditions might be expected to have been produced, i.e. with spores suspended in grease and with those deliberately trapped in a soluble film surface when a two-fold increase
### TABLE XI. THE D<sub>10</sub>-VALUES (Mrad) OBTAINED WITH SPORES OF Bacillus pumilus E.601 DRIED FROM VARIOUS SUSPENDING MEDIA ON TO DIFFERENT SUPPORTING SURFACES AND IRRADIATED IN THE PRESENCE OF AIR (Burt and Ley, 1963)

<table>
<thead>
<tr>
<th>Supporting surface</th>
<th>Buffer</th>
<th>Nutrient broth</th>
<th>Serum</th>
<th>PA&lt;sup&gt;a&lt;/sup&gt; solution</th>
<th>Greasy base</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>0.169</td>
<td>0.156</td>
<td>0.153</td>
<td>0.282</td>
<td>0.342</td>
</tr>
<tr>
<td></td>
<td>(0.159-0.180)</td>
<td>(0.143-0.170)</td>
<td>(0.147-0.158)</td>
<td>(0.266-0.300)</td>
<td>(0.321-0.366)</td>
</tr>
<tr>
<td>Perspex&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.159</td>
<td>0.151</td>
<td>+</td>
<td>0.321</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(0.152-0.167)</td>
<td>(0.141-0.161)</td>
<td></td>
<td>(0.299-0.346)</td>
<td></td>
</tr>
<tr>
<td>Polystyrene</td>
<td>0.178</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(0.171-0.185)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aluminium</td>
<td>0.165</td>
<td>0.144</td>
<td>0.154</td>
<td>0.283</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(0.158-0.171)</td>
<td>(0.136-0.151)</td>
<td>(0.149-0.160)</td>
<td>(0.272-0.295)</td>
<td></td>
</tr>
<tr>
<td>Stainless steel</td>
<td>0.165</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(0.148-0.161)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PA film&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.211</td>
<td>0.324</td>
<td>0.329</td>
<td>0.363</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>(0.196-0.228)</td>
<td>(0.293-0.361)</td>
<td>(0.296-0.370)</td>
<td>(0.348-0.389)</td>
<td></td>
</tr>
</tbody>
</table>

The figures in brackets are the 95% confidence limits.

<sup>a</sup> PA, polyvinyl alcohol.

<sup>b</sup> Perspex, polymethylmethacrylate.

*+ Not tested.
<table>
<thead>
<tr>
<th>Organisms</th>
<th>Approx. no. per thread</th>
<th>Radiation</th>
<th>Proportion of $\frac{1}{2}$-in threads fertile for doses of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>0.5 Mrad</td>
</tr>
<tr>
<td>B. globitii</td>
<td>$10^4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. globigeri</td>
<td>$10^5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. globitii</td>
<td>$10^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>$10^6$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl. tetani</td>
<td>$10^5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl. sporogenes</td>
<td>$10^5$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cl. tetani</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Infected by flushing through with blood containing spores of Cl. tetani $10^5$/ml; three needles, one metal adaptor, two lengths of Polythene tube tested at each dose.
TABLE XIII. RESULTS OF STERILITY TESTS ON INFECTED CATHETERS (Darmady et al., 1961)

<table>
<thead>
<tr>
<th>Organisms tested</th>
<th>Numbers of catheters examined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.0 Mrad</td>
</tr>
<tr>
<td>Cl. tetani</td>
<td>36</td>
</tr>
<tr>
<td>Dried soil</td>
<td>6</td>
</tr>
</tbody>
</table>

Results: Growth occurred in all controls retained in the laboratory. None of the organisms tested survived a dose of 2.0 Mrad and there was no evidence of photo regeneration after exposure to daylight at room temperature for 28 d. Cl. tetani appeared to be the most resistant of the organisms tested. All infected catheters were found to be sterile at all dose levels.

TABLE XIV. RESULTS OF STERILITY TESTS ON IRRADIATED SAMPLES OF GLASS SLIDES (Darmady et al., 1961)

<table>
<thead>
<tr>
<th>Organisms tested</th>
<th>Percentage sterile for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0 Mrad</td>
</tr>
<tr>
<td>B. stearothermophilus (a)</td>
<td>90 (90)</td>
</tr>
<tr>
<td>(b)</td>
<td>90 (10)</td>
</tr>
<tr>
<td>Cl. tetani (a)</td>
<td>89 (80)</td>
</tr>
<tr>
<td>(b)</td>
<td>100 (10)</td>
</tr>
<tr>
<td>Dried soil (a)</td>
<td>95 (90)</td>
</tr>
<tr>
<td>(b)</td>
<td>100 (10)</td>
</tr>
</tbody>
</table>

Note: Figures in brackets denote number of slides tested; (a) within a short time of irradiation and (b) after photoregeneration test.

was observed. Oliver and Tomlinson (1960) worked with both aerobic and anaerobic spores. Cotton thread was soaked in suspensions of the spores in horse serum and dried over calcium chloride at 4°C. The threads were inserted inside serum needles and also placed in rubber gloves. In further tests blood containing $10^6$ tetanus spores/ml was flushed through Polythene and nylon tubing, which was then vacuum dried before irradiation; the results are given in Table XII.

Darmady et al. (1961) used vegetative organisms, spores and soil as a source of microflora. In one test broth suspensions of B. stearothermophilus and Cl. tetani and soil in serum suspension were used to infect catheters, which were checked for sterility immediately after irradiation and again after 28-day storage; in another test glass slides were similarly infected before irradiation. Results are given in Tables XIII and XIV. Similar tests have been performed with surgical sutures; in one UK industrial laboratory organisms were introduced into sutures by drilling a hole down the axis of 3/8 in. diameter raw catgut. Sutures as substrate for B. pumilus,
B. subtilis and B. stearothermophilus were also examined in Hungary by Bartha et al. (1969). The spore suspensions in water were injected into sheep intestine and then dried at 40°C under reduced pressure; the results are given in Table XV. From the latter study it is concluded that a dose of 3.5 Mrad is needed for sterilization of sutures assumed to have an initial count in the order of $10^5$ organisms/g. This work contrasts with US studies on sutures reported by Van Winkle (1967) who used Str. faecium, M. radiodurans and B. pumilus and concluded that 2.5 Mrad is an adequate sterilization dose, but quoting only 3 to $4 \times 10^2$ organisms/suture as natural contaminants.

Data presented in papers by Christensen and Sehested (1964), Christensen and Holm (1964) and Christensen et al. (1967b) are particularly relevant to a discussion on 'protection'. They demonstrate well the way in which environmental conditions can influence radiation resistance. Some of their survival curves for various microorganisms isolated from dust and random samples of medical equipment are shown in Fig. 9, the organisms were irradiated dry and in the presence of organic matter. The behaviour of Strep. faecium A21 is noteworthy. When this organism after culture on blood agar was air-dried from a serum broth suspension onto Polythene foil, an inactivation factor of between only $10^2$ and $10^3$ was achieved at 2.5 Mrad, whilst at 4.5 Mrad it was $10^8$. However, when the organism was dried from buffer/saline the extent of inactivation increased to between $10^4$ and $10^5$ at 2.5 Mrad, the highest dose tested. Inactivation is much higher in buffer suspension in air, as seen earlier in Fig. 8, and also suspended in heart infusion broth irradiated in air (Matsuyama et al., 1964a). However, whether high resistance is inherent, as is the case with B. sphaericus (Christensen, 1970a), or M. radiodurans or due to protection by the chemical environment, the relevance of such resistance must be considered in relation to choice of sterilization dose, as discussed by Ley and Tallentire (1965). Certainly, environmental influence is well recognized in the food irradiation field where the effect has been so obvious when comparing the resistance of bacteria in complex food media with that in simple inorganic media as illustrated in the data given in Table XVI.

Sensitization

It is just conceivable that a chemical sensitizing agent might be of some practical value in reducing the radiation sterilization dose requirement for treatment of some pharmaceutical preparations but such application would be quite impractical in regard to medical devices, dressings etc. In food research Krabbenhoft et al. (1964) showed that the curing compounds in meat, nitrates and nitrites, sensitize Cl. botulinum spores to irradiation. Some considerable interest has focussed on vitamin K₅ (4-amino-2-methyl-1-naphthol-hydrochloride) demonstrated by Shehata (1961) to increase the sensitivity of E. coli, M. radiodurans, Ps. fragi and Torulopsis rosea. With the bacteria the action was demonstrated only in an anoxic situation, but with the yeast only in the presence of oxygen.

It could be that vitamin K₅ reacts with sulphhydryl groups, which seems to be the explanation for the action of N-ethylmaleimide reported by Bridges (1961) to sensitize a number of vegetative bacteria when
### TABLE XV. SURVIVAL OF SPORES DRIED ON TO PAPER DISCS OR GUT SAMPLES
(Bartha et al., 1969)

<table>
<thead>
<tr>
<th></th>
<th>Per cent samples giving positive cultures after irradiation with</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(control)</td>
</tr>
<tr>
<td><strong>Paper discs</strong></td>
<td></td>
</tr>
<tr>
<td>B. <em>pumilus</em> E 601</td>
<td>100</td>
</tr>
<tr>
<td>B. <em>subtilis</em> subsp. <em>niger</em> NCTC 10973</td>
<td>100</td>
</tr>
<tr>
<td>B. <em>stearothermophilus</em> NCIB 8919</td>
<td>100</td>
</tr>
<tr>
<td><strong>Gut</strong></td>
<td></td>
</tr>
<tr>
<td>B. <em>pumilus</em> E 601</td>
<td>100</td>
</tr>
<tr>
<td>B. <em>subtilis</em> subsp. <em>niger</em> NCTC 10973</td>
<td>100</td>
</tr>
<tr>
<td>B. <em>stearothermophilus</em> NCIB 8919</td>
<td>100</td>
</tr>
</tbody>
</table>
FIG. 9. Inactivation curves for various microorganisms isolated from dust and from random samples of medical equipment.

present during irradiation. Bridges (1962b) also reported the effectiveness of iodoacetic acid and phenylmercuric acetate, which also react with sulphhydryl. Investigations using such compounds, whether sensitizing or protective agents, contribute to the elucidation of the mechanisms involved in radiation damage, these are referred to later.

Temperature

The increase in resistance of vegetative organisms brought about by freezing is very striking. A 1.5 times increase is observed for *S. typhimurium* whether irradiated frozen in meat or buffer (Ley et al., 1963). Whilst this protective effect is also demonstrated for other vegetative organisms (Matsuyama et al., 1964a), low temperature appears to have little effect on the resistance of bacterial spores (Matsuyama et al., 1964b). However, the data presented by Grecz (1965) indicate some increased sensitivity for *C. botulinum* spores irradiated in buffer at -20°C compared with ambient temperatures when the dose given was 0.7 Mrad. Grecz et al. (1965), studying the influence of liquid nitrogen temperature on resistance of these spores in phosphate buffer, report $D_{10}$-values of 0.31 and 0.32 Mrad for 0°C and -196°C, respectively, whereas the corresponding values in beef were 0.46 and 0.68 Mrad; oxygen effects might well be involved here.

Low-temperature irradiation does play some part in the medical field. For example, whole 'ready to serve' meals, intended for hospital patients requiring isolation in germ-free wards following transplant operations etc.,
TABLE XVI. THE INFLUENCE OF VARIOUS MEDIA ON THE RESISTANCE OF S. typhimurium

<table>
<thead>
<tr>
<th>Medium</th>
<th>D$_{10}$-value (krad)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid egg</td>
<td>63</td>
</tr>
<tr>
<td>Egg albumen</td>
<td>49</td>
</tr>
<tr>
<td>Horsemeat</td>
<td>63</td>
</tr>
<tr>
<td>Desiccated coconut</td>
<td>158</td>
</tr>
<tr>
<td>Corned beef</td>
<td>80</td>
</tr>
<tr>
<td>Bone meal</td>
<td>91</td>
</tr>
<tr>
<td>Fish meal</td>
<td>174</td>
</tr>
<tr>
<td>Kaolin powder</td>
<td>21</td>
</tr>
<tr>
<td>Phosphate buffer (air)</td>
<td>21</td>
</tr>
</tbody>
</table>

are irradiated in the frozen state to inactivate contaminants, the low temperature before and during irradiation controls growth and also protects against undesirable flavour changes that might occur in some of the items. Again certain biological tissues or pharmaceutical products might require low-temperature handling to aid their stability. The nature and reactivity of free radicals formed during irradiation is thought to account largely for difference in radiation effect between the frozen and unfrozen state.

The use of elevated temperature during irradiation seems an unlikely commercial proposition, certainly with a gamma plant. However, it is interesting to note without giving detail that a synergistic action of radiation and heat has been observed for example with Cl. sporogenes spores (Licciardello and Nickerson, 1962) and with S. typhimurium (Licciardello, 1964). The practical value of the effect might be seen in producing 'commercial sterility' in canned hams. Hansen (1964) obtained a satisfactory result with 0.5 Mrad given to hams held at a centre temperature of 65 - 70°C. Farkas et al. (1966) used heat before irradiation to cause B. cereus spores to germinate and thus show less radiation resistance, suggesting applicability of the treatment to food.

Most other work involving heat has concentrated on the use of radiation to heat sensitize bacteria following up data published by Morgan and Reed (1954) and Kempe (1955). Grez et al. (1967) examined such effect in detail using Cl. botulinum and suggest mechanisms to explain their significant results.

Influence of factors concerned with cell growth

Having seen how susceptible radiation resistance in bacteria is to environmental manipulation, it is not surprising perhaps to find that alterations in the physiological and biochemical state of cells in a population can also change. Incorporation of certain chemicals into the growth media before irradiation can result in uptake into the cell, 'protection'
CHAPTER 2

Radiation dose (krad)

FIG. 10. Dose/survival curves for *S. typhimurium* irradiated in meat at -15°C: (○) following inoculation and growth in the meat at 37°C for 2 days; (×) following inoculation but without preirradiation growth in the meat.

...may occur as shown using α-alanine with *E. coli*, oxygen uptake having been stimulated (Hollaender and Stapleton, 1953). Time and temperature of incubation of *Salmonella* in meat prior to irradiation in the meat can also have an influence and Fig. 10 shows the straightforward comparison between growth and no growth in this complex media prior to irradiation. Apart from explanations based on oxygen depletion or cellular uptake of protective chemicals from the meat, the particular phase of growth at the time of irradiation might account for the observed differences; high resistance is expected in the stationary phase (Stapleton, 1955b) just reached in these experiments.

Finally, the ability of cells to recover and grow after irradiation will reflect on resistance since this is our measure of lethal effect. There are obvious implications too with respect to 'sterility testing' techniques still employed in some countries for checking commercial radiation plant operations. Brewer and Keller (1967) reported the detection of growth in 'sterility testing' tubes after 21 days incubation, whereas there was no growth observable after the 7-day period recommended in the US Pharmacopoeia. No such effect was seen when such testing was applied to naturally contaminated syringes (Ley et al., 1972). However, survivors of irradiation are known to exhibit an extended lag phase as observed with *Salmonella* (Ley et al., 1970), but cells of *Salmonella* inactivated by gamma radiation did not regain viability during storage. Christensen and Holm (1964) tested recovery of *B. subtilis* and *B. globigii* following doses between 1.5 and 2.0 Mrad and storage for up to six weeks; the results were negative. More negative results were obtained by Roberts (1970) with *Cl. botulinum* type A spores, in fact counts tended to fall rather than increase in storage at 35°C. The inactivation dose was 0.52 Mrad.
The choice of medium can influence the extent of post-irradiation recovery. *E. coli* B/r gave higher counts on media with added organic extracts than on basal agar (Stapleton et al., 1955). Alper and Gillies (1958) reported that suboptimal growth conditions were best for *E. coli* B after irradiation, suggesting that retardation of growth assisted in restoring imbalance in cell synthesis. Five recovery media were compared using six different organisms in a large experiment planned by Freeman and Bridges (1960). Only with *E. coli* B/r was a significant effect noted, blood agar giving the highest survival figures. Myasnik and Korogodin (1968) give data supporting the influence of recovery media on irradiated *E. coli* B and correlate increasing suitability of media with their suitability to maintain the growth of unirradiated normal *E. coli* B.

**Influence of factors concerned with the radiation itself**

In practice, the process of radiation sterilization is restricted to the use of gamma radiation from the radioisotopes cobalt-60 or caesium-137 or electrons from electrical machines, or in some specific instances to X-rays. The energy level differences between these radiations is quite unlikely to produce any difference in radiobiological effects; in fact the energy level is probably within the range 1-5 MeV, although in the food irradiation field the upper limit has been put at 10 MeV.

**Dose rate**

Dose rate differences between cobalt-60 sources are too small to be of any significance with respect to bacterial inactivation, but clearly the large rate difference between cobalt-60 sources and the electron machines could be significant. With cobalt-60 the rate of delivery is very often in the order of 0.1 Mrad/h, whereas the electron machine might be several Mrad/min. It is very difficult to draw a definite conclusion from published data (Ley, 1963) on the practical importance of any difference in lethal effect. The background work until then and since is based on specific situations, some of which show rate dependence and others none at all. Dependence is often explained in terms of 'oxygen effect', as demonstrated by Dewey and Boag (1959). At very high rate oxygen may be used up at the site of inactivation faster than it can be replaced from extracellular sources. Phillips and Worsnop (1969) demonstrated such a situation with *Ps. fluorescens* irradiated in the presence of various concentrations of oxygen. Experiments were carried out using a linear accelerator capable of varied pulse lengths from 10 ns to 4 µs and electron energies between 10 and 12 MeV. Dose rates were $3 \times 10^{10}$ rad/s or 3000 rad/min and the latter rate was also used with 300 kVp X-rays. At the lower rate both types of radiation gave the same response and there was no change in response at the ultra-high dose rate in air. However, with low concentration of oxygen in the gas the slope of the dose response curve showed a break and became equal to that of the nitrogen curve. The dose at which the O$_2$ depletion break occurs increases with increasing O$_2$ concentration. These and similar studies have potential usefulness in solving the oxygen problem in radiation therapy with reference to difference in sensitivity between normal oxygenated cells and anoxic tumour cells.
Of more direct application to radiation sterilization is the demonstration by Emborg et al. (unpublished data) of a difference in the response of *S. faecium* A21 to irradiation with electron accelerator compared with cobalt-60 as shown in Fig.11, the test organisms were prepared by drying from serum broth on to polyethylene foil. No such difference was observed with spores of *B. sphaericus* C1A prepared similarly.

**Dose fractionation**

Any influence of dose fractionation on inactivation could be relevant to radiation plant operation. Some cobalt-60 plants have been built to a design such that the sterilization dose is given in two equal fractions, the products travelling twice through the radiation chamber. Fractionation can also occur in any plant if the irradiation is interrupted for maintenance or similar purposes. The question of fractionation as occurs with a particle accelerator, where dose is delivered in microsecond pulses with relatively long time intervals (milliseconds) between pulses, is not within the context of this survey.
Decreased, increased and unchanged lethal efficiency from dose fractionation have each been demonstrated in laboratory experiments.

Decreased lethal efficiency

When *Micrococcus radiodurans*, a highly radiation-resistant microorganism possessing considerable repair capability, was irradiated in buffer suspension with two equal doses of 0.5 Mrad and held in broth at 30°C between doses, it showed higher survival than after irradiation with a single dose of 1 Mrad. The extent of increased survival depended on the inter-fraction time interval, a peak effect being seen at around 1 to 2 hours when no increase in total cell numbers was apparent, with a second steep rise in survival being observed at between 4 and 5 hours when a rise in cell numbers was also noted as a result of resumption of exponential growth. This second rise in survival continued for up to 7 hours inter-fraction interval, the maximum tested. In contrast, holding *Micrococcus radiodurans* cells between doses in a non-nutritive buffer solution resulted in no increased survival over that observed with a single continuously delivered dose. In fact, the lethal effect of fractionating doses under these conditions appeared to be slightly greater than that of a single dose (Fox and Hopkins, 1970).

Particular attention has been given to the possibility of causing increased resistance in *Salmonella* species by applying a series of sub-lethal treatments, survivors growing up in between. The investigations relate to possible public health hazards if irradiation was introduced to control this pathogen in food and animal feeds. Idziak and Incze (1968) increased radiation resistance in the survivors of several serotypes after 8 cycling operations as did Licciardello et al. (1969), but after 6 cycles. However, Erdman et al. (1961b) found no change in the resistance of *S. gallinarum* even after 14 cycles and Ley et al. (1970) actually noted reduced resistance after cycling of *S. typhimurium*.

It seems then that decreased lethal efficiency (i.e. reduced radiation effect resulting from inter-fraction repair of radiation-induced damage) is seen with microorganisms present in a substrate containing an appropriate energy source, present in an active metabolic state and having themselves repair capability. With regard to this latter point, it is now generally thought that the majority of microorganisms possess repair systems of one kind or another, but often for these to be operative and hence recognizable, very special circumstances must pertain.

Increased lethal efficiency

'Post-irradiation oxygen effect' has been referred to earlier with reference to spores and dependency on degree of hydration. With relatively dry spores the effect takes some time to develop fully, e.g. at ambient temperature 24 hours are needed (Tallentire, 1967). It has been shown that this effect is due to post-irradiation reaction of long-lived radiation-induced free radicals and oxygen. Similar reactions occur in dried spores irradiated in oxygen and a time-dependent increase in sensitivity is therefore also seen with spores held in oxygen following irradiation in this gas. For these conditions, increases in sensitivity are complete only
after holding in oxygen for about 40 hours at ambient temperature subsequent to the delivery of the radiation dose (Tallentire and Davies, 1961); scoring for survivors at any time up to 40 hours after cessation of irradiation gives a survival level for a given radiation dose that is greater than the minimum that is achievable. In other words, the longer dried spores are left in oxygen (air) up to 40 hours after irradiation, the more efficient is the radiation treatment. This effect has also been seen with dried Staph. aureus cells (Webb, 1964) and Aspergillus nidulans spores (Wilson and Powers, 1970).

Thus we expect that for very dry cells scored immediately after irradiation, survival from a dose given in fractions separated by a substantial time interval will be less than that from a dose given continuously. When tested this proved to be the case (Tallentire, personal communication). It should be stressed, however, that this 'beneficial effect' of fractionation is limited to cells of very low water content and that it is absent, or of little consequence, in 'air-dried' cells. Moreover, if cells given a single dose are held for a sufficiently long period after irradiation, they ultimately show as high a sensitivity as that seen with cells given the same dose administered as fractions.

Unchanged lethal efficiency

The absence of change in lethal efficiency is evident from the work of Burt and Ley (1963). They suspended spores of B. pumilus in M/15 phosphate buffer and prepared them as air-dried cells supported on a glass surface. Equal divided doses were given with intervening storage periods ranging from 1 hour to 1 week and in no case was the lethal effect different from the control for which the dose was delivered continuously. Greater degrees of fractionation also caused no effect. This negative result is confirmed by Borick and Fogarty (1967) using B. pumilus inoculated onto cotton sutures and also when suspended in agar; interruption periods between doses varied between 1 and 19 days.

The effects of fractionation of dose with spores suspended in liquid medium do not appear to have been reported. However, it is predictable, with a fair degree of certainty, that for spores in a non-nutritive medium fractionation will be without effect, and when suspended in a nutritive medium spores could germinate during the inter-fraction interval to give the considerably more radiation-sensitive vegetative cell, which would result in a greater lethal effect of the second fraction.

3. MECHANISMS OF INACTIVATION

Ionizing radiations impart their energy to molecules in a manner that depends on the atomic number of constituent atoms and not on the molecular configuration as is the case with ultra-violet radiation. It follows that chemical changes due to ionization occur in a more or less random manner in biological material. However, in a living cell it is to be expected that certain sites or systems will be more readily damaged than others. In dry cells ionizations occur at random in the molecules of which the cell is composed; chemical change ensues directly as a result of these ionizations. Such a process is known as the direct action of
radiation. In moist cells chemical change may occur not only in this manner but also indirectly through the ionization of water. The water molecules split to form free radicals, which are extremely reactive although short-lived.

Many workers have contributed to the search for the primary target of lethal damage yet there still remains some doubt as to the critical lesion involved. With irradiation the death of cells is not immediate and many cell functions such as endogenous respiration and motility may continue after treatment and cells may become enlarged (often elongated). Enzymes are noted for their high radiation resistance. For example, succinoxidase in liver tissue required 20 Mrad for complete inactivation (Marples, 1959) and the proteolytic enzymes of meat require between 20 and 70 Mrad (Schweigert, 1959). It follows that fresh meat stored at ambient temperature will deteriorate due to autolysis, even after radiation sterilization with between 4 and 5 Mrad. In bacteria the effect on the respiratory system has received considerable attention. With E. coli respiration was inhibited only after several hours post-irradiation, it was thought that enzyme replacement mechanism was involved rather than direct inactivation of enzyme (Billen et al., 1953). Again with B. subtilis no inactivation of respiratory enzymes was observed after 0.14 Mrad (Powell and Pollard, 1955).

Involvement of DNA

The most sensitive feature of cell physiology is cell division and the role of the cell nucleus as the target for lethal damage is now well established (Bacq and Alexander, 1961). Evidence reviewed by Moseley (1965) points to changes in deoxyribonucleic acid (DNA) as being responsible for inhibition of cell division in both bacterial and mammalian cells. For example, such cells have been grown under appropriate conditions in the presence of certain analogues of purines and pyrimidines (the base materials of which DNA is composed) and they incorporate into their newly synthesized DNA varying amounts of the analogues instead of their corresponding natural bases, for example, 5-bromouracil instead of thymine. As a result of this modification these cells are more sensitive to both ultra-violet and ionizing radiation than normal cells. B. subtilis cells grown in such a fashion and the transforming factor (native DNA) obtained from them are both sensitized to radiation to the same extent.

Another line of evidence is the correlation of radiation sensitivity of cells with the nitrogenous base composition of their DNA. A study of such base composition in 8 species of bacteria and their resistance to irradiation suggested an inverse relationship between radiation resistance and guanine-cytosine (GC) content, i.e. resistance increased with decreasing GC content. Preliminary experiments showed that the reverse might be true for u.v. light. However, M. radiodurans, the most resistant bacterium, is resistant to both types of radiation and has a high GC content, 67%; the same as the very sensitive Pseudomonads. The composition of DNA is not the determinant of radiation resistance in this bacterium. Suggestions that radiation energy might be dissipated in a 'sink' involving the pigment have also been shown to be incorrect by the isolation of a non-pigmented resistant strain.
The remarkable radiation resistance of *M. radiodurans* has been shown to be due principally to a repair mechanism, repair occurring in a period after cessation of radiation and before logarithmic growth begins. No such repair was evident with *S. typhimurium*. *M. radiodurans* is also capable of repairing u.v. damage to its DNA. Damaged bases, including thymine dimers, are enzymatically removed and the DNA molecule is reconstructed from information on the complementary strand. In the case of *M. radiodurans* repair of damage by u.v. and ionizing radiations has been shown to be similar, hence damage by ionizing radiation is also likely to involve DNA. An alternative theory to that based on damage at the cell nucleus is based on the possibility that enzyme systems within the cell become disorganized as a result of internal membrane damage.

**Non-nucleic acid target**

Alper (1970) postulates that DNA is only one of two important sites of primary lesion and that the non-nucleic acid target (type 0 damage) is the site at which radio-sensitization by oxygen occurs. It is argued that if in the cell the only primary lesion is in DNA, then it should be possible to demonstrate oxygen enhanced damage to DNA by irradiation of DNA extracellularly and the extent of such damage should be at least as great as observed with the cell. This was not observed (Hewitt and Read, 1956). The site or component involved in type 0 damage has been sought unsuccessfully in protein and in RNA. However, cell membranes have been implicated as the site by studies on a model system consisting of lysosomes, which are membranous sacs of enzymes (Watkins, 1970). The membrane can be envisaged as a unique target because it is known that in bacteria and other organisms the DNA is attached to a particular site on the cell membrane.

**Mechanisms involved in spore resistance**

Tallentire (1970), in presenting 4 commonly held notions that follow concerning the mechanisms responsible for high radiation resistance in spores, comments on each in the light of the results of relevant laboratory investigations.

1. The existence of a low level of hydration within the spore core that results in a low radiation yield — although water alone can play a prominent role in determining cellular radiation resistance, data do not show whether water or its absence is important in setting high radiation resistance.

2. The presence of innate chemical substances that protect against radiation damage — dipicolinic acid, unique to the spore, has been ruled out as a modulator of resistance and so too is protection by a S-S rich structure.

3. The existence of unique conformations in important spore components that are resistant to damage — spore DNA with its unusual structure is singled out as the primary target. The resistance of transforming ability of intracellular DNA in intact spores is greater than in vegetative cells, whereas its resistance after purification from spores is the same, indicating chemical similarity. There is also
evidence that the structure of the spore plays an important role in the resistance of spore intracellular DNA to ionizing radiation.

(4) The existence of spore-specific repair processes for radiation-induced damage. This concept requires further investigation but data available reflect the existence of repair mechanisms, latent in the resting spore but activated in the differentiating system.

4. IMPLICATIONS OF BACKGROUND KNOWLEDGE

It is obvious, perhaps, that with such a wide variety of factors influencing the radiation resistance of microorganisms superimposed on the broad spectrum of inherent resistance between species, it is impossible to calculate precise radiation doses to deal with the sterilization of individual products that are naturally contaminated. With the introduction of radiation sterilization on a commercial scale it has been necessary to make a judgement as to choice of dose, bearing in mind the requirement of an adequate margin of safety.

In considering the margin of safety achieved by a given sterilizing dose in terms of the number of viable organisms that might survive the treatment and hence the probability of an individual product not being sterile, three factors must be taken into account:

(1) The number of contaminating organisms on the products before sterilization; the smaller the number, the higher the margin of safety achieved – hence the need for hygiene in production.

(2) The resistance of the contaminating organisms in their natural environment on the products.

(3) The final use of the products, e.g. some products in use may be in contact with biological media for periods, which may give an isolated survivor the opportunity to multiply; a higher margin of safety may be desirable under such circumstances compared with those in which the majority of products are used.

Recommendations for a code of practice governing radiation sterilization have been proposed through the International Atomic Energy Agency (IAEA, 1967), and reference is made to the microbiological factors requiring attention when a suitable sterilization dose is approved by authorities. There is also reference to methods that may be employed to monitor the efficiency of the process. More recently a routine procedure has been recommended based on the use of the 'sterility testing' technique but applied to samples of commercial products given subprocess dose treatment, i.e. radiation doses less than that recommended for the sterilization procedure. A model describing the dependence of the proportion of items contaminated (P) in a population of items on radiation dose (D) has been developed (Tallentire et al., 1971). Curves relating P and D were constructed and the influence on curve shape of changing the initial average number of microorganisms on items and of varying parameters of microbial sensitivity to radiation has been assessed. A practical study on disposable plastic syringes using the subprocess dose method confirmed the possibility that a new microbiological quality control procedure might be introduced (Ley et al., 1972).
In an attempt to assist the reader in placing the radiation sterilization process and its microbiological background into perspective two final points are made:

(1) Other sterilization methods such as those based on heat or the use of chemicals such as ethylene oxide have similar complex microbiological backgrounds. In fact this point is stressed in the following introductory sentence of the IAEA Recommended Code of Practice, "In common with other methods of sterilization, the efficiency of radiation sterilization is dependent on the numbers and types of contaminating organisms presented to the process, and also upon the environmental conditions in which they occur".

(2) The ultimate aim in the application of any sterilization process is to minimize the risk of infection to patients in the use of such products. Choice of manufacturing materials, design of products and packs, hygiene of production, inactivation of contaminants by a sterilization treatment and finally, avoidance of post-sterilization contamination in storage, distribution and use are all factors which must be taken into account in approaching this aim. Inactivation of contaminants by sterilization is but one of many facets.
CHAPTER 4
THE EFFECT OF IONIZING RADIATION ON VIRUSES

1. INTRODUCTION

From the very early days of radiobiological work (Lea, 1955) it has been felt that there should be a simple relationship between the action of ionizing radiation and the loss of infectivity of a virus. In some cases this is the case, but the picture is clouded by the widely variable character of viruses. Viruses vary from extremely simple systems comprising a relatively short single strand of nucleic acid and two or three proteins, which form the capsid, to viruses that are larger than the smallest cells and that contain a variety of proteins and some lipid. To treat a virus such as *Herpes simplex* or *Vaccinia* in the same class with R17 bacteriophage is clearly dangerous and the danger is well illustrated when the nature of the action of radiation on the virus is seen.

Along with the complexity of some viruses goes a second factor, their ability to achieve some kind of repair of radiation damage. In the main, this process results from the occurrence of an infection by two virus particles in the same cell. If there exists a means by which the separate units can conspire to put together one whole virus, then a process of restoration has occurred and the virus infectivity is intact in spite of the radiation.

Viruses are now recognized to have many separate properties, including the property of attachment, of injection, of coding for a number of specific proteins, of achieving lysis of the cell, of interference with other viruses, and of eliciting antibodies in the immune reaction. Recent years have brought a clarification of many of these processes and the response to radiation can now be related to something more specific than was formerly the case. On the whole, each of these properties does react separately to the insult of ionizing radiation and the response is always informative.

A brief mention should be made that prophages can be induced by ionizing radiation. This may be a very important factor in the whole subject of the relationship between cells and radiation, but it may not be of great significance in the process of inactivation of viruses.

2. PHENOMENOLOGY

Characteristic inactivation curves for a virus are shown in Fig. 12. Here two viruses — influenza virus and R17 phage — have been chosen. In all such experiments the biological end point is of great importance. In the case of influenza the infectivity was measured by observing the development of the virus in chick embryos; in the case of the bacteriophages the infectivity was determined by the formation of plaques on host strains of bacteria. Both show the very characteristic behaviour that is nearly always observed for ionizing radiation, that of a 'single hit' type of kinetics which conforms to a relationship

$$\ln \frac{n}{n_0} = -MD$$

or

$$\frac{n}{n_0} = e^{-MD}$$
The ratio of surviving virus to unirradiated virus plotted as a function of dose for influenza virus and R17 bacteriophage. The statistical relation $\ln n/n_0 = -MD$ is obeyed.

where $\ln$ is the natural logarithm, $n$ the surviving virus titre after a dose of $D$ rads, $n_0$ the original titre, and $M$ a quantity dependent on the nature of the virus and related to molecular weight of the nucleic acid. The possibility of deducing knowledge about the structure of the virus from this relationship will be mentioned later, but all that need be said now is that nearly every virus conforms to this type of relation. Exceptions occur if there is present in the preparation a mixture of sensitive and insensitive strains of virus, or if there is a 'clump' of virus particles any one of which may infect, in which case the above type of relation is not seen until on the average the clump has been reduced to one single infectious unit.

Radiation can inactivate viruses by producing effects in the medium. In aqueous solutions, particularly those containing growth medium, ionizing radiation generates two classes of agents. The first is long-lived, namely hydrogen peroxide or an organic peroxide; the second has a relatively short lifetime and is composed of various kinds of active radicals that start out, as a rule, from water and are usually hydrogen and hydroxyl radicals and solvated electrons. These are rapidly followed by secondary radicals, which may be, for example, a very active phosphate radical if the solution contains phosphate for buffering purposes (Pollard and Weller, 1967). These active
agents normally work on the protein capsid of the virus and produce a type of inactivation that does not conform to the line shown in Fig. 12. Rather, it normally shows an ineffective initial stage followed by a rapidly increasing effectiveness, which can be associated with the presence in the capsid of many components, all of which need to be damaged in some way before the virus is actually inactivated. Figure 13 shows data taken by the author (Pollard, 1965) and Woodyatt. Bacteriophage T1, in the process of infection, not only produces more virus particles, but a quantity of lysozyme that lyses the cell wall. The presence of lysozyme can be observed by measuring the amount of cellular protein released. In these experiments the release of β-galactosidase was observed. The right-hand figure shows the effect of irradiation dry on the two properties, infectivity and enzyme release. The two do not go together and doses in excess of 10^6 rads are necessary to diminish the release of enzyme. On the other hand, in solution very much lower doses are needed and it is clear that both properties are affected equally. In the wet state radicals chemically alter the capsid and probably interfere with injection, which thus interferes with both infectivity and enzyme release. In the dry state ionization in a critical region of the DNA is probably needed to remove infectivity. While it is not yet known precisely what removes the ability to cause enzyme release, probability favours the loss of injection, which involves a smaller initial region than infectivity.
3. INACTIVATION OF THE VARIOUS ATTRIBUTES OF VIRUSES

A considerable number of studies have been conducted on the inactivation of the various attributes of viruses. These are continually being added to and yet no exhaustive table is available for even one single virus. A brief statement of the general situation can be made. The most sensitive property of a virus is invariably the infectivity; this is for a good reason because the infectivity usually requires all the attributes of the virus to be functional before the cycle of infection can be completed. For viruses of very simple structure having a capsid surrounding a short stretch of RNA or single-stranded DNA the lethal effect on infectivity is, in a vast majority of cases, a damage (strand break, for example) in the nucleic acid. Ginoza (1968) has shown quite clearly that the statistical relation (Hutchinson and Pollard, 1961)

\[ MW \times D_{37} = 6.0 \times 10^{11} \]

(where MW is the molecular weight of the nucleic acid in daltons and \( D_{37} \) the dose in rads to reduce surviving virus titre to \( e^{-1} \) or 37% of its original) correctly expresses the situation. On the other hand, a double-stranded DNA virus has two copies of all the molecular information needed and damage to only one strand may do no more than lengthen the time for the virus to mature. Therefore, radiation must either damage both strands or one segment of DNA that is crucial to the operation of the virus, as, for example, the segment from which is transcribed the special DNA polymerase that is used by the virus. Thus double-stranded viruses do not conform to the above relation and usually are less sensitive by a factor of 10-20. In addition, this loss of full sensitivity means that additional stresses, such as heat, can cause additional sensitivity.

Radiation also acts on the capsid and other aspects of the virus. These are usually made of an assembly of many protein subunits and damage to one or two of these may not be expressed. Thus radiation may produce considerable damage to the capsid of a virus, which may nevertheless retain its structure sufficiently to protect the infectious nucleic acid from outside agents, and the process of attachment may have escaped damage so that to all intents and purposes the virus may seem to be intact and the infectivity is not reduced. On the other hand, if by a lucky chance the damage is confined entirely to that part that is concerned with attachment, then such a virus will be damaged.

In general it is true that the effects on the capsid are far less sensitive than the effects on the nucleic acid. If the reduction of the ability to agglutinate red cells by influenza virus were plotted in Fig. 12, the line would show no slope. To see an effect doses that are 20 to 100 times higher must be used. This function is clearly far less sensitive than infectivity.

The effect of ionizing radiation on the process of mutual exclusion is extremely interesting. This process refers to the ability of one virus type to prevent the multiplication of another type, even if the infection of the former comes a little later than the excluded virus. Radiation destroys this ability with high sensitivity, but is nevertheless clearly possible to begin the infection of a cell with a virus and to develop that part of the machinery that will exclude the presence of another virus, even after considerable irradiation. In the case of the bacterial viruses this exclusion process is now understood; it consists of the transcription of an enzyme
### TABLE XVII. INACTIVATION STATISTICS FOR THE ACTION OF IONIZING RADIATION ON SEVERAL VIRUSES

<table>
<thead>
<tr>
<th>Virus</th>
<th>Property</th>
<th>$D_1$ (rads)</th>
<th>Radiation MW from Formula 2</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 phage</td>
<td>Infectivity</td>
<td>$4 \times 10^5$</td>
<td>$1.5 \times 10^6$</td>
<td>Pollard, 1965</td>
</tr>
<tr>
<td></td>
<td>Bacterial killing</td>
<td>$1.2 \times 10^6$</td>
<td>$0.5 \times 10^6$</td>
<td>Fluke and Pollard, 1955</td>
</tr>
<tr>
<td></td>
<td>Ability to attach</td>
<td>$3 \times 10^6$</td>
<td>$2 \times 10^5$</td>
<td>Pollard and Setlow, 1956</td>
</tr>
<tr>
<td></td>
<td>Ability to elicit antibodies</td>
<td>$&gt;3 \times 10^6$</td>
<td>$\sim 2 \times 10^5$</td>
<td>Yeisley, 1965</td>
</tr>
<tr>
<td></td>
<td>Ability to combine with antibody</td>
<td></td>
<td>$1.5 \times 10^4$</td>
<td>Pollard and Setlow, 1954</td>
</tr>
<tr>
<td>T2 phage</td>
<td>Infectivity</td>
<td>$10^5$</td>
<td>$6 \times 10^5$</td>
<td>Ginoza, 1968</td>
</tr>
<tr>
<td></td>
<td>Bacterial killing</td>
<td>$3 \times 10^6$</td>
<td>$2 \times 10^5$</td>
<td>Watson, 1952</td>
</tr>
<tr>
<td></td>
<td>Interference with T1</td>
<td>$7.5 \times 10^6$</td>
<td>$8 \times 10^5$</td>
<td>Pollard and Setlow, 1956</td>
</tr>
<tr>
<td></td>
<td>Ability to inject</td>
<td>$5 \times 10^6$</td>
<td>$1.2 \times 10^6$</td>
<td>Hershey et al., 1961</td>
</tr>
<tr>
<td></td>
<td>Attachment</td>
<td>$5 \times 10^6$</td>
<td>$1.2 \times 10^6$</td>
<td>Pollard and Setlow, 1956</td>
</tr>
<tr>
<td>Influenza</td>
<td>Infectivity</td>
<td>$6.5 \times 10^4$</td>
<td>$6 \times 10^6$</td>
<td>Jagger and Pollard, 1956</td>
</tr>
<tr>
<td></td>
<td>Haemagglutination</td>
<td>$2 \times 10^7$</td>
<td>$5 \times 10^5$</td>
<td>Jagger and Pollard, 1956</td>
</tr>
<tr>
<td></td>
<td>Interfering ability</td>
<td>$4 \times 10^5$</td>
<td>$4.3 \times 10^5$</td>
<td>Powell and Pollard, 1956</td>
</tr>
<tr>
<td>Polio</td>
<td>Infectivity</td>
<td>$1.4 \times 10^5$</td>
<td>$1.5 \times 10^7$</td>
<td>Benyesh et al., 1958</td>
</tr>
<tr>
<td></td>
<td>Complement fixation</td>
<td>$1.2 \times 10^6$</td>
<td>$1.3 \times 10^5$</td>
<td>Benyesh et al., 1958</td>
</tr>
<tr>
<td>Vaccinia</td>
<td>Infectivity</td>
<td>$4 \times 10^4$</td>
<td>$1.5 \times 10^5$</td>
<td>McCrea, 1960</td>
</tr>
<tr>
<td></td>
<td>Haemagglutination</td>
<td></td>
<td></td>
<td>McCrea, 1960</td>
</tr>
</tbody>
</table>

System that will degrade the DNA of the excluded virus. The fact that this property can be retained when the infectious property is not retained is a strong indication that radiation effects can be separately harmful to specific single functions. Table XVII gives a summary of the effects of radiation on the various attributes of several viruses, including infectivity.

The use of ionizing radiation to develop vaccines has been suggested many times (Hubbert and Miller, 1965). Not nearly enough work has been done on this quite interesting subject. An attempt was made by Helen Yeisley (1965) to study the residual antigenic potency of a bacterial virus after ionizing radiation. Even after considerable radiation, amply sufficient to destroy all infectious ability, rabbits are still able to produce antibody that is capable of neutralizing the virus.

Both heat and radiation may act synergistically in the destruction of a virus and this is a most important consideration in sterilization of viruses. As the character of the virus becomes more complex, in particular for viruses containing double-stranded nucleic acid of high molecular weight, there is ample experimental evidence that a combination of temperature and
ionizing radiation will give a much more rapid loss of infectivity than that found by either ionizing radiation or heat alone. This was discovered initially by Adams and Pollard (1951) and by Bachofer (1951) independently; it has been followed up by a number of studies that show this combined effect is confined to the more complex viruses and is not found in the single-stranded variety. But it is also clear that a very considerable advantage can be obtained by a relatively mild application of heat. It is possible that really very difficult viruses, such as serum hepatitis, for which adequate studies relating to sterilization have so far been impossible, may prove to be amenable to a combination of radiation at a moderate temperature such as 45°C plus the normal application of gamma radiation. The background knowledge of viruses would suggest this would be a method of sterilizing a blood serum in any kind of emergency. The problem, in the case of blood and hepatitis, is that a relatively small number of hepatitis virus particles in a pooled supply of blood can render the whole pool dangerous. The degree of inactivation necessary may not be as great as in other cases because the number of infectious particles is small. The use of a combination of moderate heat and moderate irradiation should remove the danger, without, in this instance, damaging the blood proteins appreciably.

4. RATIONALE FOR STERILIZATION

One of the most obvious requirements of sterilization is the elimination of every active particle. Very often if there is some reason why an agent cannot have access to the active particles (as is the case, for example, for ultra-violet light) or if there is a population of different sensitivities (as is very often the case for thermal inactivation), then the effect of the sterilizing agent is not uniform. In general, this is not the case for ionizing radiation. If the radiation source is properly designed, the penetrating character of gamma rays or fast electrons guarantees access and there are no well-documented instances of a resistant population of virus in a well-characterized preparation. Thus, the problem of sterilization does not lie in the inherent response of a virus to radiation, but may instead involve the way in which the virus is in groups in the original preparation.

If in fact the viruses occur in clumps so that there are two or three hundred that could be thought of as a single unit, then it will be necessary to eliminate every one in each unit before the preparation is considered sterile. This gives a 'multi-hit' type of inactivation. It is no serious problem because the final slope for inactivation is the same as would be the case if the viruses were distributed singly. It does mean that measurements have to be taken over a wider dose range in order to be sure that the infectivity is being lost.

In the process of sterilization it is essential to guarantee that there is full penetration of the material. There must be no pockets of untouched virus and partially wet material. Very interesting studies by Lewis (1961) showed that as water was progressively removed from a bacteriophage preparation the sensitivity altered markedly. The fully dry virus is the least sensitive and as the hydration proceeds sensitivity increases. It is quite possible that this phenomenon is not uniform and that sometimes, as in the case for heat, the fully dry virus is more sensitive. What is important is the realization that a preparation that contains some dry and
some damp virus may not be uniform with regard to radiation sensitivity and a resistant population may become apparent. If the method of inactivation chosen corresponds to the relatively low-dose inactivation that can occur in the wet state, then a virus that is at the edge of the liquid preparation and is dry may very easily not be destroyed.

Formulas developed as a guide in dose-response relationships for one virus cannot necessarily be applied to a second virus. They can be used as a guide to make the necessary experiments and checks more productive, but a direct test must always be made on each specific virus in each instance.

5. OUTLOOK

There is no question that for quite inert things like sutures sterilization with ionizing radiation should be quite satisfactory. There is not the same confidence about the sterilization of blood. The reason is that after heavy doses there may be products left in the blood that perhaps are harmful and contra-indicate its use. Very careful tests would be necessary to make sure that this was not the case. In cases where the necessity is very high (as is the case of eliminating hepatitis virus) this method could be tried for blood. The same considerations hold also for sterilizing food preparations.

In the preparation of killed vaccines there should be a very good opportunity for exploitation of ionizing radiation. It penetrates well; it kills primarily on the nucleic acid and leaves the protein antigen in reasonably good shape.
CHAPTER 5

THE EFFECT OF IONIZING RADIATION ON FUNGI

1. INTRODUCTION

Fungus spores, along with bacteria, represent an important portion of the flora-contaminating objects that need to be sterile for use. Factors affecting the radiation inactivation of fungi are generally closely related to those similarly affecting bacteria. Peculiarities in the irradiation of fungi, as contrasted with bacteria, result primarily from differences in habit of growth and the more complex cytology, morphology and life cycles of fungi. Since the radiation biology of bacteria has been discussed in detail elsewhere in this manual, a similar discussion of the radiation biology of fungi would be highly redundant. Consequently, this Chapter discusses only those factors that are of particular importance in the irradiation of fungi. Attention is not given, except in passing, to factors generally important in the irradiation of all cells.

2. NATURE OF FUNGUS CONTAMINANTS

Contaminants from airborne sources of objects requiring sterilization are most likely to be certain species of (1) Phycomycetes, especially the Mucoraceous genera Rhizopus and Mucor, and (2) certain species of the Deuteromycetes or conidial stages of the Ascomycetes such as Penicillium, Aspergillus, Cladosporium, Alternaria, Monilia and Stemphyllium. These species are extremely successful saprophytes usually capable of growth on diverse sources of organic matter. All are noted for profuse sporulation and the spores are readily airborne. Contact with the soil, including wind-blown soil particles or surface water, can result in the appearance, in addition to the above genera, of such common soil-inhabiting species as Fusarium.

The fungi are achlorophyllous plants which resemble green plants in that cells possess (1) a definite cell wall, and (2) organelles, including well-developed nuclei, mitochondria, ribosomes and vacuoles. Common contaminating fungi are filamentous, growing by elongation, by apical growth, of tubular, branching hyphae.

Dispersal and reproduction is commonly by non-motile spores, which may be single- to several-celled. Each cell always contains a nucleus but sometimes may have several. In some species two or more types of spores are dispersed simultaneously from the same fungus colony. Hyphal cells may round up and develop a heavy wall to form a non-deciduous spore (chlamydospore) that is resistant to unfavourable climatic conditions. Another climatically resistant structure, the sclerotium, is a firm, frequently rounded mass of hyphae commonly found in many fungus species. It may remain dormant for long periods before germinating by the outgrowth of hyphae. Further, the mycelium may form stromatic, pseudoparenchymatous tissue of various types. Portions of almost all parts of a fungus colony, including mycelium, may serve as propagules.
The life cycle of a typical contaminating fungus can briefly and simply be outlined as follows: Spores are transported by air, water, or animals (particularly insects). If moisture is present and the temperature favourable, they germinate by the formation of a germ tube. Organic matter, sometimes also required for spore germination, is necessary for the development of the germ tube into a branching hypha and ultimately into the familiar mould colony. From the mycelium of the mould colony are produced spore-bearing structures and the production and dissemination of spores completes the asexual cycle.

In addition to the asexual cycle, some common fungal contaminants produce a sexual spore stage. The sexual stage is not required to maintain the existence of the fungus and is seldom encountered. Consequently, the sensitivity of sexual spores to radiation has not been included in this discussion.

3. RADIATION SENSITIVITY OF FUNGUS SPORES

General considerations

Most kinetic studies of the inactivation of fungi by irradiation have been focussed on asexual spores, usually conidia of Deuteromycetes or sporangiospores of the Mucorales. The reason has been partly due to the importance of these spores as infesting propagules and partly because of convenience. Spores that have not yet started to germinate can be irradiated and their survival determined by plating on nutrient agar in Petri dishes by the same techniques widely employed in studies of survival of single-celled organisms, especially bacteria and yeasts. Dose-survival data thus obtained by irradiating non-germinating spores are completely valid only for those spores. Germinating spores, mycelia, sclerotia and other morphological structures of fungus colonies might exhibit somewhat different sensitivities.

Dose-survival curves (Fig. 14) of asexual spores of species of important genera of fungi show a wide range of resistance to irradiation (Durban and Grecz, 1969; Moseley and Mattingly, 1971; Ravetto, 1968; Sommer and Fortlage, unpublished data; Sommer et al., 1964a; 1964b; Tanooka and Hutchinson, 1965). No fungus spore yet studied, however, equals the resistance of certain bacteria such as Micrococcus radiodurans or Clostridium spp.

When a population of irradiated dormant spores is plated on a nutrient agar surface at a temperature conducive to germination, certain peculiarities can be noted. First, some of the spores fail to germinate and the size of this non-germinating fraction increases with increasing radiation dose. Among irradiated spores that germinate, germ tubes may appear several hours after the germination of non-irradiated spores. Secondly, one observes that among the germinants some germ tubes appear abnormal. Often the germ tubes are swollen and assume diverse shapes (Berk, 1952; Dimond and Duggar, 1940). Commonly, these germ tubes are highly evacuolated and cross walls are missing. Monster germinants of this type soon stop growth and ultimately die without forming a colony.

Although radiation-induced loss of the ability to germinate is dose related, the germination mechanism is much more radiation resistant than
FIG. 14. Dose-response curves for asexual spores of filamentous fungi. Similar curves for several radiation-resistant bacteria are included for comparison. Unless otherwise indicated, cells were suspended in water or buffer and irradiated in the presence of oxygen. A. Fungi: 1. Trichoderma viride (Sommer et al., 1964b); 2. Penicillium expansum (Sommer et al., 1964a); 3. Geotrichum candidum (Sommer et al., 1964b); 4. Mucor sp. (Sommer and Fortlage, unpublished data); 5. Botrytis cinerea (Sommer et al., 1964a); 6. Diplodia natalensis (Sommer et al., 1964b); Rhizopus stolonifer (Sommer et al., 1964a); 8. Alternaria citri (Sommer et al., 1964b); 9. Cladosporium herbarum (Sommer et al., 1964a); 10. Alternaria tenuis (Ravetto, 1968); 11. Bacillus subtilis (dry spores) (Tanouka and Hutchinson, 1963); 12. Clostridium botulinum 33A (irradiated in anoxia) (Durban and Grecz, 1969); 13. Micrococcus radiodurans (irradiated in anoxia) (Moseley and Mattingly, 1971).

colony formation. Consequently, germination cannot be utilized as an indicator for survival. Instead, germinants must have developed into definite fungus colonies before a spore can be said to have survived. It is true, however, that developing colonies and non-surviving monster germinants can be distinguished with a high degree of reliability with the aid of 10 X magnification while they are yet very small.

Dose-survival curves for fungus spores (Fig. 14) have usually exhibited a shoulder in the first log cycle followed by a straight line through subsequent log cycles. Occasionally, however, the shoulder is barely perceptible or may even be non-existent. The most striking difference in the shape of curves between relatively sensitive and resistant fungi is usually the width of the shoulder rather than contrasting slopes of the exponential portion.

At this juncture it should be mentioned that a false resistance or false shoulder results if spores have clumped together before they are plated.
When a fungus spore suspension contains many of the spores in clumps of 2 to 6 or more, the clumps behave as single units. The numbers of colonies developing from plated non-irradiated spores will be low because the colonies have been formed from several spores, not one. Among irradiated spores, the surviving fraction, in relation to the non-irradiated controls, will be abnormally high because a colony will develop providing one spore in the clump has survived. Although a constant hazard, clumping of non-germinated spores can usually be avoided by use of a surface-tension reducing agent. Germinating spores can be similarly maintained free of clumping in the presence of surfactants until germ tubes appear. Shortly thereafter, severe clumping usually results.

Effect of multicellularity on resistance

Some fungus spores are resistant, at least in part, because they are multicellular. If, as seems likely, individual cells in multicellular spores can germinate and function with a considerable degree of independence of other cells in the spore, there is a built-in clumping effect. Presumably, if one cell in the spore survives and germinates, the spore has survived as effectively as if all cells in the spore had tolerated the irradiation. The consequences of the multicellular condition is to increase the width of the shoulder of the dose-survival curve, thereby displacing the exponential portion to the right. The resistance of conidia of *Alternaria* spp. (Fig. 14) is almost certainly due, at least in part, to their multicellularity. Similarly, a 75-krad dose prevented germination of 50% of the single-celled microconidia of *Fusarium solani*, while a 500-krad dose left 70% of the multicelled macroconidia capable of germination (Buddenhagen and Kojima, 1966).

Effect of multinuclearity on sensitivity

A second cause for radiation resistance, very similar to the effect of multicellularity, is the presence of more than one nucleus in the cell. Norman (1951) compared the radiation sensitivity of uninucleate microconidia and multinucleate (average 2.27 nuclei) conidia of *Neurospora crassa*. The resistance of the multinucleate conidia was expressed by a survival curve with a distinct shoulder and it was displaced to the right in comparison to the curve for the microconidia. The difference in resistance was interpreted to be the consequence of the difference in nuclear condition between the two types of spores. At least in part, large shoulders on survival curves for the single-celled sporangiospores of *Rhizopus stolonifer* (Fig. 14) are believed to be the result of multinuclearity. Presumably some of the estimated 6-12 nuclei could be rendered non-functional by irradiation without inactivating the spore. On the other hand, the near exponential dose-survival curve for *Penicillium expansum* (Fig. 14) is probably not only a reflection of the single-celled condition of the spore but also the presence of only a single nucleus.

Effect of ploidy on resistance

Laterjet and Ephrussi (1949) showed that haploid yeast cells were less radiation-resistant than diploid. It appears that the greater radiation
resistance of the diploid condition is a direct result of the presence of a second set of chromosomes. The diploid condition presumably provides protection against recessive lethal mutations. Further duplication of chromosomes in polyploids, however, evidently does not impart added radiation resistance, judging from the results of Mortimer (1954) who found that hexaploid yeast cells were less resistant than triploids. Spores of most common fungi are haploid. Diplloidization evidently occasionally occurs spontaneously and can be induced by irradiation but stable diploid strains are evidently a rarity in nature.

Effect of spore germination on resistance

Because cells of yeasts and bacteria have exhibited striking changes in resistance to irradiation during certain stages of division, it has been generally believed that fungus spores become increasingly sensitive to irradiation during germination. Buckley et al. (1969) confirmed the reduced resistance of sporangiospores of Rhizopus stolonifer after incubation under conditions conducive to germination. When held in a liquid defined medium on a shaker at 25°C, many spores exhibited germ tubes after six hours of incubation. A gamma radiation dose of 0.25 Mrad reduced to 0.1% the survivors in a population of incubated spores, whereas a 0.4-Mrad dose was required to achieve a similar effect with non-incubated spores. When the spores were subjected to a uniform 0.15-Mrad dose approximately 90% of the non-incubated spores survived, while only 3% survived if they had been incubated for three hours.

4. RADIATION SENSITIVITY OF FUNGUS STRUCTURES OTHER THAN SPORES

Whether fungus structures other than spores are more or less sensitive than spores has important implications in using radiation as a fungicidal treatment. Obviously, fungus dose-survival data obtained by irradiation of spores can have validity with respect to the life-cycle of a fungus only if the sensitivity of other structures is reasonably similar. Unfortunately, the determination of the sensitivity of structures other than spores poses problems of technique and interpretation. First, the nature of mycelial growth precludes use of the familiar bacteriological plating technique. Secondly, the determination of cell numbers is difficult and frequently impossible in mycelial colonies. Indeed, just what portion of a hypha might be considered a 'cell' is difficult to discern with non-septate hyphae found in the Phycomycetes.

A comparison of the sensitivity of conidia, young mycelia, mature mycelia and sclerotia of Botrytis cinerea was made in the author's laboratory using dry weights as a means of equivocating the structures (Sommer et al., 1965). Mycelium grown on cellophane on the surface of potato-dextrose agar was removed and amounts of fresh mycelium required to equal 5 mg dry weight was determined. Similarly determined was the size of sclerotia and the number of conidia required to weigh 5 mg if dried.

Each structure was irradiated in the fresh state in amounts equal to 5 mg dry weight and 20 replications were provided for each dose. After irradiation, survival was determined by placing each sample on fresh potato-dextrose agar in a Petri dish and observing subsequent growth. Doses required to inactivate 50% of the samples were thus determined.
Young mycelium was the most resistant, followed by conidia and sclerotia, while mature mycelium was the most sensitive. A dose of 0.6 Mrad was required to inactivate 50% of the populations of densely protoplasmic young mycelia. Half the conidial populations equivalent to 5 mg dry weight each (ca. $5 \times 10^7$ conidia) were inactivated by 0.47 Mrad and sclerotia were only slightly more resistant. By contrast, only a 0.35 Mrad dose was required to inactivate 50% of the populations of mature mycelia.

5. SOME DOSE MODIFYING FACTORS

Conditions during radiation

Oxygen present during radiation strongly sensitizes fungi. For Rhizopus stolonifer, for example, the dose permitting 0.1% survival of sporangiospores irradiated in the presence of oxygen is about 0.3 Mrad, compared to about 0.4 Mrad if irradiated in anoxia (Sommer et al., 1964c). Substrate present during irradiation may contain protective substances and thereby increase the required dose. If fungus spores germinate and initiate colonies in such a substrate, the 'population' of fungus structures that can serve as propagules may be dramatically increased. Since inactivation is exponential over large portions of survival curves, severe initial contamination greatly reduces the effectiveness of a given dose.

Repair

Some fungus spores have demonstrated a high capacity for repair of potentially lethal radiation injuries. For example, to reduce the survivors in a population of Rhizopus stolonifer sporangiospores to 0.5%, a dose of 0.3 Mrad was required if the spores were plated immediately after irradiation. If spores were provided a 48-hour recovery period before being permitted to germinate, a dose of near 0.5 Mrad was required. The key factor in recovery of the fungus spores has been a period between irradiation and germination in which temperature conditions were favourable for the fungus without germination being initiated (Sommer et al., 1964c).

Heat sensitization

Fungus spores are readily sensitized to radiation by a prior mild heat treatment (Sommer et al., 1967). As a consequence, heating followed by irradiation results in greater inactivation than the additive effects of the component treatments. This increased sensitivity of survivors of heating to subsequent irradiation is expressed in the dose-survival curve as a reduction in the width of the shoulder and in some species by a change in the slope of the exponential portion. If, instead of heating before irradiation one heats after irradiation, the inactivation is usually much less than the former sequence.

Delays occurring between heating and irradiation may cause all or a part of the sensitization to disappear. Baldy et al. (1970) studied the recovery of conidia of Penicillium expansum after heating. He demonstrated that a holding period between heating and irradiation of about three days eliminated the heat-induced irradiation sensitivity of the spores. Since the
added sensitivity was not lost if the spores were held at 0°C, in anoxia, or were treated with respiratory inhibitors, Baldy concluded that desensitization was the consequence of metabolic recovery.

6. SUMMARY

Fungus spores are common contaminants of objects to be sterilized. Their radiation sensitivity is influenced not only by genetic factors but also by the number of cells in a spore, the number of nuclei per cell and by ploidy. Germination is not a reliable indication of spore survival in fungi because many irradiated spores die only after considerable germ tube development. Heating before or during irradiation may increase the sensitivity of fungus spores to radiation. The presence of oxygen or media during irradiation as well as the presence or absence of a recovery period between irradiation and germination may influence spore survival. The radiation resistance of fungus spores is usually much less, however, than the resistance of important spore-forming bacteria. Presumably a radiation treatment sufficient to inactivate bacterial contamination will normally eliminate fungal contamination.
CHAPTER 6
THE EFFECT OF IONIZING RADIATION ON ANIMAL PARASITES

1. INTRODUCTION

There has been a dearth of knowledge relating to the effect of ionizing radiation on parasites as radiobiological studies have only rarely been extended to these peculiar organisms. Although the discovery of the damaging effect of radiation on Ascaris eggs was made as long ago as 1904 (Perthes, 1904), the first experiment aimed at the medical utilization of this effect was carried out on Trichinella spiralis much later (Tyzzer and Honeij, 1916). The result of the latter attempt failed to furnish an experimental basis for the treatment intended, that of schistosomiasis in man by radiation. The idea of using radiation for the treatment of patients infected with parasites has not received much attention since but, mainly during the last two decades, other methods of employing ionizing radiation in the control of some parasite-borne infections have been sought.

A relatively simple method has been developed to sterilize trichinous meat by gamma radiation. The spectacular eradication of the ectoparasitic screw-worm, Cochliomyia hominivorax, from Curacao and the southeastern United States of America by the sterile-male technique has inspired a vast amount of work aimed at applying the same technique in the control of other insect pests. Experiments at the University of Glasgow resulting in the development of the first irradiated anthelminthic vaccine, successfully used in the control of Dictyocaulus viviparus infection in cattle, have encouraged similar work with further endoparasites and also accelerated research in related fields (Jarrett et al., 1958). Thus, a relative expansion of information on the effect of ionizing radiation has been produced, predominantly on the helminths of veterinary importance.

2. THE RADIOSENSIVITY OF ANIMAL PARASITES

Primarily the same, i.e. a 'bio-negative', effect is exerted on all parasites studied so far; nevertheless, the sensitivity to the damaging effect of ionizing radiation may be different in the representatives of various zoological species, genera, families, orders, classes and phyla with distinct morphology, complicated life-cycle, etc. A considerable scatter was observed in the radiosensitivity of unsegmented eggs derived from diverse specimens, even within the species, in both Ascaris suum and Ascaridia galli (Shikhobalova and Paruzhinskaya, 1961b). The sensitivity of the same parasite to the deleterious effects of irradiation may depend on the stage of development; the zygote and larvated eggs of Ascaris lumbricoides, A. suum and A. galli are more resistant than those at the morula, blastula or 'tadpole' stages (Shikhobalova et al., 1958). Cysts of Entamoeba histolytica are about ten times as sensitive to irradiation as are trophozoites of the same strain (Schneider, 1960).

The irradiation of dioecious nematode larvae causes a shift in the sex ratio of the arising worm population as in A. galli (Shikhobalova and
Paruzhinskaya, 1959), *Trichostrongylus axei* (Ciordia and Bizzell, 1960), *Haemonchus contortus* and *Trichostrongylus colubriformis* (Mulligan et al., 1961), *Dictyocaulus filaria* (Jovanović et al., 1961), *D. viviparus* (Jarrett and Sharp, 1963), *Ancylostoma caninum* (Miller, 1964), *Oesophagostomum columbianum* (Dhar and Singh, 1970), etc. since the males prove more susceptible to the detrimental effect of irradiation. This is not, however, the case with all dioecious nematodes, e.g. *Trichocephalus muris* (Shikhobalova and Paruzhinskaya, 1961a) and *Syngamus trachea* (Varga, 1964b) represent exceptions to that phenomenon. The small number or complete absence of males from a worm population developed from irradiated larvae of *A. galli* is related to the early elimination of males rather than to sex reversal (Ruff and Hansen, 1967). The absence of males is not the only reason for the vanished reproductive capacity of a worm population deriving from irradiated larvae, as the female worms do not produce eggs even when normal males are transplanted into their habitat.

3. FACTORS INFLUENCING THE EFFICACY OF IRRADIATION

There are some physical and environmental factors that presumably may influence the response of parasitic organisms to irradiation. The most commonly used sources for irradiating parasites are X-ray machines (operated mainly at 150-250 kV) and the pure gamma rays of radioactive cobalt ($^{60}$Co). Linear accelerators have also been used occasionally. The biological effect of X-rays proved superior to that of gamma rays from $^{60}$Co on *T. spiralis* (Gould et al., 1957), *A. lumbricoides* (Villella et al., 1958), *Nippostrongylus brasiliensis* (Kassai et al., 1966), etc., whereas gamma rays were somewhat less effective on *Dictyocaulus filaria* (Jovanović et al., 1961).

Investigations on *N. brasiliensis* (Jennings et al., 1963) and *D. viviparus* (Procházková and Tománek, 1968) suggest that, at least within certain limits, the effect of X-rays is independent of the dose rate per minute and dependent on the total dose of radiation. Fractionation of the dose does not alter the effectiveness of irradiation (Shikhobalova and Paruzhinskaya, 1959). The concentration of the larval suspension or the contamination of larval suspension with faecal matter failed to affect the degree of radiation attenuation, whereas the radiosensitivity of *N. brasiliensis* larvae markedly decreased as a result of increased temperature; this is probably due to an oxygen effect (Fitzpatrick and Mulligan, 1968).

In view of the foregoing and other factors possibly influencing the effectiveness of irradiation, a rigorous standardization of the radiation exposure has been recommended for reliability and intercomparison between various laboratories (IAEA, 1964). Accordingly, published reports should include the following information in order to permit the conditions of exposure to be assessed:

1. Radiation source and characteristics
2. Total dose
3. Duration of exposure
4. Geometrical conditions of exposed material and physical arrangement as related to contribution of scattered radiation
5. Method of calibration
6. Biological characteristics of the parasites.
CHAPTER 10

4. LETHAL EFFECT OF THE IRRADIATION OF PARASITES

The problem is to establish the dose of radiation that is sufficient to bring about the immediate death of any parasite at any stage of its life cycle. Larvae of *T. spiralis* lose their motility and die soon after an exposure to 750 kR of X-rays (Gould et al., 1953). The amount of radiation to kill the bulk of *A. suum* eggs in contaminated food would be more than 150,000 rep of *60*Co gamma rays or over 100 kR of X-rays (Villella et al., 1958). Scolices of *Cysticercus bovis* require 800 kR of gamma rays to be destroyed in meat (Kosminkov, 1966). The in vitro excystation of metacercariae of *Fasciola hepatica* is completely prevented by an exposure to a dose of 20 kR of X-rays (Wikerhauser, 1961). A dose of 600 kR of X-rays is needed to change the in vitro motility of *Trypanosoma gambiense* (Halberstaedter, 1938).

These are about the highest doses ever delivered to parasites and can be utilized for radiosterilization. Much lower doses of radiation are satisfactory to inhibit the reproductive capacity and lower still to decrease the pathogenic effect and the infectivity. Exceptionally, however, the effective dose is close to the lethal one; for instance, there is a small gap between the amount of radiation at which no effect can be observed on the irradiated trophozoites of *Entamoeba histolytica* and that at which no amoebae survive (Schneider and Porter, 1960).

In proportion to the dose of radiation, a range of morphological and developmental anomalies are noticeable in many parasites. A slight irradiation may be ineffective or result in a late effect only. Thus, larvae of *Syngamus trachea* exposed to 1 - 2 kR of X-rays at the third larval stage can develop into adults and pass normal-looking eggs, which, however, fail to form embryos. A few abnormal eggs or no eggs at all are laid by the worms originating from larvae exposed to somewhat larger doses (Varga, 1964b). A further increase in dose stops the development of larvae well before the copulation of juveniles would take place.

Radiation damage is irreversible. Irradiated larvae of nematodes can be stored at low temperature for as long as 18 months without any sign of recovery.

5. THE PATHO-PHYSIOLOGICAL EFFECTS OF IRRADIATION OF PARASITIC HELMINTHS

The damaging effect of radiation appears in a retarded cleavage and complete inhibition of eggs to reach the stage of embryo. The effect is dose-dependent and becomes noticeable after a short latency period. In species with a slow segmentation the first cleavage of the zygote, as in the case of *A. galli*, is performed in inverse ratio to the dose of radiation exposure (Varga, 1964a). The irradiated eggs undergo abnormal cleavage and differentiation. An increase in pronuclear fusion time was established in the X-irradiated eggs of *A. suum* (Bachofer, 1957), whereas changes in the cytoplasmic viscosity were observed in the irradiated eggs of *Parascaris equorum* (Neufahrt, 1960). Morphologically, irregular blastomeres, vacuoles and partly formed embryos are found in these embryos, as in those of other nematodes, *e.g.* *Heterakis gallinarum* (Ostlind and Hansen,
1966) and Toxocara canis (Casarosa and Favati, 1964). Though the cells in eggs cease dividing predominantly between the blastula and 'tadpole' stages, no evidence of how long they are metabolically still active has become available so far.

A small amount of radiation may stimulate embryonic development. For instance, eggs of the trematode, Cotylophoron cotylophorum, incubated in water containing $^{32}$P at 1.25 $\mu$Ci/ml or less, developed more rapidly and the hatching rate was greater than in the controls (Weber, 1953). Although slight irradiation may increase the infectivity and growth of some helminth larvae (Ciordia and Bizzell, 1960; Kassai et al., 1966; Ruff et al., 1965), the inhibitory effect prevails.

The in vitro survival of infective larvae is scarcely altered (Dhar and Singh, 1970), but a range of alterations can be detected in the post-embryonic development, including morphogenesis. A low-dose irradiation still enables the larvae to reach the adult stage but their eggs usually do not undergo normal embryonic development (Varga, 1964b; Wikerhauser, 1961). An adequate dose of irradiation allows the larvae of Trichinella spiralis to grow to maturity in the intestine but no offspring is produced and thus muscle invasion does not occur. Intestinal infection alone has proved suitable for producing immunity to a subsequent challenge with normal larvae (Levine and Evans, 1942); this recognition is considered as a basis for further experimentation to learn more about the immunological potential of irradiated larvae.

In addition to the absence of males from the worm population deriving from irradiated larvae of many species, stunted growth is frequently encountered in females. The arrested development of female gonads is conspicuous in some species of nematodes (Miller, 1964; Varga, 1964a), while cuticular thickenings in the body wall, oesophageal swelling and coiling of the intestine can be observed in others (Alicata, 1951; Babero, 1952). A wide range of malformations occur in the cestodes. For instance, absence of or abnormal suckers, rostellar hooks and rostellum; irregular alternation of genital atria; abnormal distribution of testes; cirri in abnormal positions; lack of cirrus in some proglottids, etc. have been observed in Hymenolepis nana (Schiller, 1959). Studies on Hymenolepis microstoma have revealed that the tissues of the neck region may be more radioresistant than those where proglottids are maturing since the damaged strobila may completely be replaced by a normal one as a result of mitotic division of resistant cells near the scolex (Tan and Jones, 1966). Specimens of Fasciola hepatica from metacercariae that had been X-irradiated at 3 kR have only some diverticula or sacculations instead of the branches characteristic for normal flukes (Dawes, 1964).

The higher the dose of irradiation, the earlier is the stage at which the parasite is arrested. Trichinella larvae exposed to 10 kR of X-rays 'mature' in the intestine but fail to produce progeny, while those irradiated with either 30, 100 or 700 kR stay in the intestine for 2, 1 or even less than 1 day, depending on dose, and are discharged in the faeces in a larval stage (Levine and Evans, 1942). Increasing the radiation dose produces a decrease in the infectivity of larvae, which appears as a smaller worm burden of any species. Small numbers of worms, arrested in postembryonic development, do not exert a serious pathogenic effect as most of the helminth infections remain unnoticed in those cases where only a few worms are present in the host. About one quarter of cattle lungworm larvae,
**CHAPTER 6**

D. viviparus, irradiated with 40 kR migrated through the mesenterial lymph nodes to the lungs but only a few of them survived to become stunted sterile worms there (Jarrett and Sharp, 1963). Calves given two doses of these irradiated larvae acquire immunity against subsequent challenge with normal larvae (Jarrett et al., 1959). Dictyocaulus larvae subjected to a dose of 60 kR or more are over-irradiated, i.e. they are unable to develop to that stage that would stimulate immunity. On the other hand, an exposure of these larvae to a dose of 20 kR is not enough to render the lungworms harmless. It would seem that a radiation dose-titration should be carried out on each parasite species that is intended to be used for immunization purposes.

Radiation attenuation is now an accepted means of studying immunological phenomena in relation to parasitic infections. Following the development of the first anthelmintic vaccine, consisting of irradiated D. viviparus larvae, a similar vaccine has been produced against sheep lungworm, Dictyocaulus filaria (Sokolić et al., 1961). Another vaccine, against canine hookworm disease, is in preparation. Irradiated larvae of Ancylostoma caninum were found to surpass the immunogenic power of normal larvae (Miller, 1966). Larvae of S. trachea irradiated at 5 kR migrate to and moult in the lungs of chickens but fail to attach to the tracheal mucosa. Administration of such larvae to chickens provides resistance against challenge with normal larvae (Varga and Kottlán, 1965).

As expected, other nematodes, some cestodes and trematodes can also be rendered less pathogenic by a suitable dose of irradiation. The immunogenic properties of irradiated oncospheres of Taenia taeniaeformis have been shown in mice (Dow et al., 1962), and irradiated scolices of Echinococcus granulosus, obtained from hydatid cysts, are suited to immunizing dogs (Movsesijan et al., 1968).

There has been a long period of doubt as to whether immunity to trematodes could be acquired by the various hosts. Extensive studies on schistosomiasis have proved that irradiated metacercariae are effective to produce immunity in some hosts including monkeys (Sadun, 1964). The pathogenicity of the metacercariae of F. hepatica can be reduced by an exposure to a dose of 3 kR of X-rays (Wikerhauser, 1961). However, attempts to immunize animals with irradiated F. hepatica metacercariae have not yielded yet unequivocal results (Dawes, 1964; Thorpe and Broome, 1962).

Although irradiated helminth larvae are a very useful means of performing immunological studies in many host-parasite relationships, there are some disappointing peculiarities that seem to present barriers to the practical application of further vaccines of this pattern. Irradiated larvae of H. contortus confer resistance in adult sheep but fail to do so in young lambs because of the immunological unresponsiveness at the age when the lambs are in fact most susceptible to the infection (Urquhart, 1968). Irradiated larvae of nodular worms, such as O. columbianum, are not practicable for vaccination purposes as this procedure and the immune response itself result in an increased nodule formation, which should really be prevented (Dhar and Singh, 1970). Moreover, some irradiated larvae, such as Metastrongylus in pigs, fail to elicit any discernible protection from challenge (Wikerhauser and Žuković, 1967).
6. IMMUNOGENIC PROPERTIES OF IRRADIATED PROTOZOA

Ionizing radiation has been shown to reduce the infectivity of many species of parasitic protozoa. Though some immunological capacity of irradiated oocysts of coccidia had been shown long ago (Waxler and Herrick, 1941), research on the possible employment of radiation attenuation for the control of protozoan diseases has only been resumed in recent years. A dose of 5-10 kR of X-rays decreases the pathogenic effect of *Eimeria tenella* considerably. Satisfactory protection from normal challenge can be achieved in broiler chickens by vaccinating them with irradiated oocysts (Hein, 1963).

*Trypanosoma lewisi* exposed to an X-ray dose of 50 kR remain motile and multiply in culture but fail to produce infection in rats. The irradiated trypanosomes are effective to produce immunity to a challenge with normal trypanosomes (Sanders and Wallace, 1966). The highly pathogenic *Trypanosoma rhodesiense* lose their motility when exposed to 640 kR of gamma radiation; nevertheless, the immunogenic potential of the latter parasites in mice and rats is comparable with that produced by motile trypanosomes exposed to smaller doses of gamma rays (Duxbury and Sadun, 1970).

The multiplication of malaria parasites and the subsequent invasion of the plasmodia into the erythrocytes can be prevented by a suitable dose of irradiation. Inoculation of rodents with parasitized blood cells exposed to 20 kR induced immunity to lethal challenge with non-irradiated *Plasmodium berghei* (Wellde and Sadun, 1968). Encouraging results have also been obtained with *Plasmodium falciparum* in monkeys, which, as a result of a successful immunization with irradiated forms, survived otherwise lethal challenge with non-irradiated plasmodia (Sadun et al., 1970).
PART II
GENERAL ASPECTS
CHAPTER 7

FACILITIES REQUIRED FOR RADIOSTERILIZATION

The first step in planning facilities for radiosterilization is to decide on the scale of operation — experimental, pilot, full production, or the possibility of starting in one category and moving towards larger scale work by modification and addition.

1. EXPERIMENTAL FACILITIES

For experimental work the emphasis is on versatility, i.e. the ability to treat a wide range of sizes of sample at doses chosen for each experiment. The efficiency of utilization of the ionizing radiation is unimportant.

If the facility has to service several groups of workers concerned with different projects, there is a choice between setting up a single radiation facility incorporating all that the various groups expect to require and can justify, or building separate facilities of a relatively simple nature for each of the working groups; a compromise between these two extremes is possible.

Theoretical planning of the number of radiation facilities required can be misleading because on average each research worker spends only about ten per cent of the working day irradiating samples and so by perfect programming one facility should serve up to ten workers. However, there is a great deal to recommend having several independent facilities. Time sharing of one installation between several groups slows down the work and can cause frustration and ill feeling. Moreover, research work frequently requires an experiment to be repeated under identical conditions, and if a radiation facility is shared, then the apparatus used by one worker will almost certainly be removed or disturbed in order to clear space for the next user of the facility.

Provided that the experimental samples are small and the ambient conditions required during irradiation are not critical, the cheapest and simplest type of installation is one in which radioactive source rods surround a cavity in the centre of a lead shield less than 1 metre in diameter and about 1 metre high. The sample can be moved into and out of the cavity without any dangerous escape of radiation by placing it in a close fitting plug, which passes through the cavity. Some designs incorporate means for stirring liquid samples and, less commonly, simple temperature control is included.

For more sophisticated experimental work irradiation cells having a working volume of about 2 m x 2 m x 2 m inside shielding walls have been found suitable. These dimensions allow room for the experimenter to set up the apparatus in comfort, which is conducive to accurate results. Access to the cell can be through a shielding labyrinth, which reduces the radiation at the entrance to a safe level; in these circumstances the entrance to the labyrinth only requires to be closed during irradiation by
a light-weight door or even a door constructed of a metal grille. There must be safety interlocks to prevent access when there is a high level of radiation in the cell (see Chapter 13).

The choice of the radiation source for experimental work is usually restricted to a gamma-ray or beta-particle emitting radioisotope because an electron accelerator of adequate energy would be too costly. When spent fuel elements are available from a local high neutron flux reactor they are worth considering, especially if the experimental facility is intended for material testing up to high total doses as well as the sterilization work. For experimental work up to total doses of about 5 megarads the cost of handling the spent fuel elements (including safeguards against setting up a critical assembly) and the inconvenience of monitoring the rapidly decaying activity makes them less attractive than cobalt-60, which is the most popular radioactive source for this purpose.

In an experimental cell the cobalt-60 source must be shielded while the apparatus is being set up. The source can be housed in a lead shield fitted with a remotely controlled means of exposing it during the irradiation; the lead shield can also be used as a transport container when the source is first delivered and subsequently when the source is to be changed or replenished.

When more than one experimental cell is to be used or when more than one source per cell is needed, it is preferable to shield the sources in a sand-filled concrete shield which forms part of the cell structure. A particularly convenient form of this type of shielding can be used when four cells are built around a central shield which serves all four cells.

2. PILOT PLANTS

The purpose of the pilot plant is to bridge the gap between experimental work and full production. The quantities to be handled should be large enough to make the process the same as full production in respect of dose rate, minimum to maximum dose ratio and temperature, which usually means adopting the same package size.

The quantities to be handled must also be adequate for the clinical trials or market tests of the product, which, when successful, may result in much larger quantities being required at short notice. It may be desirable to run the pilot plant long enough to accumulate reserve stocks, provided that there is no limitation of storage life, but the planning should take into account that about one year is needed for building and commissioning a full-scale production plant.

A pilot-plant design should aim at versatility so that it can be used for a wide range of products. In contrast to experimental cell designs, it is not economic to build several pilot plants on account of the cost both of the radiation source and the conveyer system needed for packages. An exception arises in pilot plants for chemical reactions involving fluid or gaseous reagents that can be piped into a simple reaction vessel incorporating means for introducing a small radiation source.

Now that industrial-scale processing by ionizing radiation is available in existing plants in many countries it may be possible to avoid the expense and delay in building a pilot plant by arranging a short-term contract for the treatment; it is even possible to obtain complete commercial security to cover an irradiation program in some plants.
The provision of a pilot plant is very much simpler for gamma-radiation processing than it is for high-energy electron irradiation. When an electron accelerator is envisaged for the full-scale production it is difficult and costly to match the dose rate and penetration of the electrons into the product in a pilot-scale machine; hiring time on a production accelerator is probably the only satisfactory solution. Working with gamma radiation from a specific isotope, e.g. cobalt-60, the penetration of the gamma rays into the product is only slightly affected by the differences in geometry between the pilot and full-scale plant. At the dose rates typical of gamma radiation an exact match is less critical but is easily achieved by using the same specific activity in terms of curies per unit area of the source.

If the pilot-scale operation is for a process that is already proven and the need for a full-scale plant at a later stage is a foregone conclusion, then time and money can be saved by building a full-size concrete cell, source frame and safe store for the source. The cell can then be fitted with part of the final product conveyer or some relatively simple support for the product for use during batch operation. Only part of the source frame need be loaded with cobalt-60 but the product may need to be circulated or moved in some way in order to achieve uniformity of dose; it is often convenient to carry out half of the irradiation period of a batch operation and then to re-arrange the position of the product before carrying out the second half of the irradiation.

If a full-size cell is used for pilot-scale work, it will usually be necessary to build loose block walls in each of the labyrinths that are ultimately used for feeding the product in and out of the cell. Unless this is done, the safety procedure will be invalidated because radiation scatter along the empty labyrinths could exceed the permitted level.

3. PRODUCTION PLANTS

The first step in deciding on a production plant using ionizing radiation is to choose between an electron accelerator and a gamma-radiation installation. The fundamental difference between the two types of radiation is that electrons are very limited in penetration in comparison with gamma radiation, and hence the suitability of either is controlled by the thickness of the product, its density and the importance of precise levels of dosage.

The design of a commercial irradiation plant is aimed at a minimum processing cost, which in turn is influenced by the following factors:

(a) The cost of the source of electrons or gamma rays
(b) The efficiency of utilization of the radiation
(c) The dose required
(d) The cost of the civil and mechanical engineering
(e) The cost of supervision, maintenance and material handling.

4. ELECTRON ACCELERATORS

The cost of the source of the electrons will be based on data supplied by the manufacturers, who will probably furnish a minimum guaranteed life for the expendable items. With careful operation it is often possible to extend the life to a period several times the guaranteed period, but this should always be used as a bonus in commercial accounting because there will be other unexpected causes of expense. The efficiency of utilization
of the high-energy electrons depends on several factors. The most advantageous conditions are when the density and thickness of the product are the same at all times because this enables the designer to optimize the penetration of the radiation. Further efficiency can sometimes be achieved by irradiating the product first from one side and then from the other, but again uniformity of product density and thickness is very desirable otherwise overdosing or underdosing can occur (see Fig. 15).

The design of the civil engineering must not assume that the scattered electrons are the only source of radiation that requires screening. It is inevitable that part of the electron beam will strike material of sufficient density to generate X-rays and the consequent screening is usually comparable with that required for cobalt-60 gamma rays.

Sterilization of large quantities of very low density material (less than 0.05) favours the use of electron accelerators. The energy will depend upon the package size but 4 to 5 MeV is a useful level as far as penetration is concerned and it does not raise the question of induced radioactivity, which begins to become important at 10 MeV.
There are several types of electron accelerator from which to make a choice. One class of accelerator generates an electrical potential sufficient to impart the requisite energy in the electrons as they pass a series of accelerating anodes. The resonant transformer accelerator generates the voltage by means of a high ratio air-cored transformer, which is operated in a resonant condition. The alternating voltage is fed direct to an accelerator tube, which, in consequence, conducts during less than half of the alternating cycle. A later system uses an insulated core transformer that has a series of secondary windings mounted on a central magnetic limb. The limb is broken up into sections insulated from one another in order to avoid large differences in voltage between the local winding and the magnetic core. Each core section has its own rectifier circuit so that the total output is d.c.

Another means of achieving a d.c. potential is used in the Dynamitron, in which a radiofrequency source of power is capacity coupled in parallel to a number of rectifier circuits connected in series. The linear accelerator is another type of machine that accelerates electrons progressively along a wave guide and, therefore, only needs to generate a fraction of the voltage used in the machines previously described.

In planning a commercial-production plant using electron accelerators two of the most important factors are the reliability of the machine and the type of staff required to run it. The more complex machines need qualified physicists to be available during all the running periods. Unlike plants using gamma radiation, it is sometimes feasible to operate an electron accelerator during the working day only because a large part of the cost of operation is attributable to the hours of operation of the accelerating tube, the electron window and the power-generating klystrons or magnetrons.

5. GAMMA-RADIATION PLANTS

For a commercial operation the factors that favour the use of gamma radiation are (a) the reliability of operation; (b) the ability to match the source strength with the current throughput; and (c) the low cost of running the plant.

The radioactive source is completely reliable in that there is no way in which the strength or life of the source can be influenced by external means. The lower dose rate that is always associated with gamma-radiation processing, compared with high-energy electrons, results in the product conveyer moving relatively slowly, thus subjecting the conveyer to much less strain and wear.

In designing the radiation cell it is possible to provide for five or more times the source strength for about ten per cent extra cost. When the plant is first operated the amount of cobalt installed should be appropriate with a margin of safety for the current throughput and extra cobalt can easily be added as higher throughputs are required. The limit of source strength is reached when either the shielding ceases to be adequate or the conveyer is not able to move fast enough for the product to be given the desired dose. An automatic gamma-radiation plant usually incorporates a short length of package conveyer outside the cell, which, when loaded, will keep the plant in operation for a few hours. The task of loading the cell with product therefore becomes a simple job of transferring the packages on to the conveyer discontinuously, leaving intervals for other
tasks to be performed, such as loading and unloading lorries. A man responsible for the plant must always be on call and he must be knowledgeable in health physics and dosimetry. He does not, however, have to be present at the plant apart from a normal working day.

To carry out maintenance on the plant it is necessary for a safe store for the source to be built into the floor of the irradiation chamber. This store may comprise a stainless steel water tank fitted with continuous circulation incorporating filters and ion exchange units. Alternatively, the safe storage can take the form of a dry pit that is sealed by a plug integral with the source frame.

The advantages of water storage are:

(a) A wider choice of transport container is possible
(b) More complicated arrangements of source units can be used
(c) The source with its Cerenkov radiation can be shown to visitors.

The disadvantages are:

(a) Initial and running costs are higher than for dry store; stainless steel tanking is desirable, circulating pumps, filters and ion exchange columns have to be maintained
(b) The time taken for loading, re-charging or re-arranging source units is considerably longer than with a dry loading system
(c) Welds on the source units receive a thermal shock each time they enter the water
(d) The humidity in the cell is greater
(e) Earth tremors are more likely to cause loss of shielding
(f) Local water authorities are concerned about the possibility of contaminating local water supplies
(g) There is a risk of a source being brought dangerously near the surface of the water during source loading or unloading.

Satisfactory operation has been obtained in many automatic plants.

The advantages of dry storage are:

(a) Lower capital cost and a minimum of maintenance
(b) Rapidity of source loading and unloading; it is common in practice for the loss of production during source replenishment to be less than one hour
(c) The store is more compact than a pond and interferes less with the structural strength of the cell walls
(d) Source handling is more tightly integrated with the safety system, resulting in a lesser risk to operatives loading or unloading sources
(e) Any chance of encapsulation failure is reduced to the minimum.

The disadvantages of a dry storage are:

(a) A special source transport container must be used
(b) The source pit must be cooled as the source strength approaches the maximum design capacity.
CHAPTER 8

ORGANIZATION OF INDUSTRIAL AND MEDICAL CENTRES FOR RADIOSTERILIZATION

1. INDUSTRIAL CENTRES

The organization of an industrial centre for radiosterilization is almost entirely dependent upon the throughput of the unit and, therefore, a careful market survey is an essential first step. The proprietors of the unit will know whether they wish to process their own products only, in which case their market survey will be already available. Otherwise, there is a possibility of combining the treatment of the house product with contract work from a number of other manufacturers or, in the extreme case, setting up the unit for contract work only. This latter arrangement is often a good way of attracting work from other manufacturers because the possibility of rival interests clashing can be more easily avoided and commercial security can be offered. One important detail in the survey is a determination of the package size or sizes that the radiation plant will be required to handle. This, in turn, depends to a large extent on the bulk density of the product being treated. The density sets a limit to the dimension of the package that will lie normal to the source of gamma radiation. The other two dimensions will be limited by the conveyer system and, in particular, the movement of the package round corners.

Another fundamental point is the siting of the plant in relation to the source of the goods, the places to which they must be delivered after treatment and the need for good transport facilities. The siting must also take account of the availability of staff, which will need to include management, accountants, secretaries, salesmen, porters and perhaps most important of all, a competent plant supervisor who must be trained in health physics and dosimetry. The accumulated information from the survey should be in sufficient detail for approaches to be made to the official bodies responsible for (a) planning of factory sites; (b) the safety of the general public — this usually includes the environment; (c) the safety of workers; (d) fire control and prevention; (e) health and medical matters; and (f) local politicians. With the possible exception of the last it is necessary to get the agreement of all the other bodies before taking any irrevocable steps in the planning.

The planning authorities will be interested in the amount of traffic coming to the plant, the size of the warehouse and any noises from warning sirens etc. There is no radiation hazard to the general public and the only effect on the environment is the generation of ozone in the cooling air, but this is of such low concentration as to be harmless. There are no special worries about the safety of the workers because the design of the plant will ensure that the radiation hazard is well within tolerance levels. The fire control authorities will wish to inspect the plant on account of the unusual nature of the shielding block, but the only real fire hazard is the inflammability of the product itself, which may include medical components
immersed in alcohol. The health and medical authorities will be interested in the cleanliness of the plant and the reliance they can place on the process. In particular, they will want to know that there is no risk of materials leaving the plant without having been treated as a result of some mishandling or accident.

The economics of plant design in relation to the throughput must be carefully studied to determine whether batch operation or a continuous system should be adopted. Other considerations include the efficiency of utilization of the gamma radiation and the dwell time in the plant. High efficiency of utilization of gamma radiation requires a large quantity of product in the irradiation cell and this in turn results in a long dwell time, particularly when the plant begins to operate and the initial throughput requires only a small source of radiation. A large quantity of product, if it is costly, may be an important capital investment. If the plant uses cobalt-60, there must be space and sufficiently strong roadways leading to the plant to accommodate a crane for handling the transport container.

In choosing the site there are several important civil engineering considerations. The ground must be capable of bearing the load of the concrete cell and the accompanying building. If a storage tank is to be incorporated in the plant, then the local water table must be ascertained. An adequate water supply, drainage and the availability of three-phase electric power are also necessary. There are several points to be considered in the field of mechanical engineering: (a) the conveyors outside the radiation cell must be capable of handling sufficient product to allow for the absence of porters while other operations such as loading and unloading lorries are taking place; (b) a system of handling that will minimize labour should be installed, e.g. use of pallets and fork lift trucks; (c) a regular maintenance and lubrication schedule should be laid down; and (d) a stock of spare parts should be in store.

The commissioning of the plant will require the use of sufficient dummy boxes to load the conveyer inside the radiation plant. At least one set of boxes of a given density must be provided so that adequate dosimetry throughout the volume of the boxes can be accurately determined. The tolerance on box sizes that has been laid down during the mechanical design of the plant can be effectively checked during the commissioning period. The actual handling of commercial product will usually involve boxes having a range of densities and the effect on dosimetry of different distributions of box density should also be checked. In a gamma radiation plant one of the important parameters is the average density of all the boxes under irradiation; it is possible to mix high and low density by spreading the high-density boxes uniformly.

2. MEDICAL CENTRES

The high capital cost of a radiation sterilization unit has militated strongly against the use of the process in medical centres. The vast majority of medical equipment that is suitable for radiation sterilization has already been treated as the final stage of manufacture. There are, however, a limited number of items that are not mass produced, such as pieces of equipment used in operating theatres, and that cannot be subjected to heat or chemical treatment. In addition, there is a steady requirement
for radiosterilization of materials for implantation — bone, homographs, heterographs and items made of plastic material.

In some areas it has been found convenient to run a weekly collection service amongst twenty or more medical centres for items of the above nature, and to have them sterilized in a single centre — usually a commercial radiosterilization unit. It can be argued that even the high cost of a radiation sterilization unit within a hospital can be justified and at least one hospital centre (London, Ontario) is already well into the planning stage for its own unit.

Some of the items put forward some years ago for radiosterilization in a hospital unit included complete heart-lung machines and some hyperbaric chambers, but much of the need has been reduced by the development of disposable components for the heart-lung machines.
CHAPTER 9

DOSIMETRY

1. INTRODUCTION

In general, an industrial radiation process is set up and defined in terms of source parameters, source-to-product geometry and product flow rate, and the integrated effect of the process may be checked on the product itself by conventional product-control means. In this respect a radiation process does not differ from any conventional technical process such as, e.g., curing freshly painted items on a factory assembly line or a continuous heat sterilization process at a hospital sterilization centre. Process control is maintained by, e.g., (1) continuous control of the process parameters, and (2) checking on random samples of the product to ensure that the appropriate physical, chemical or biological effect has been achieved. In the case of radiation processing this latter kind of control is sometimes termed 'effects dosimetry'.

In the radiation sterilization of medical and biological materials, however, the regulatory authorities usually specify restricted ranges of radiation absorbed doses for the purposes approved. In principle there should be no objection to treating these processes like any other industrial process, except that conventional product control of medical and biological materials has to be carried out by means of sterility testing, a procedure that can no longer be considered sensitive enough for testing whether a product meets the stringent bacteriological standards in use today (see, e.g., Chapters 11 and 17). As a substitute for the biological product control, the efficiency of the integrated sterilization process is challenged by inserting special biological indicators containing large numbers of highly resistant bacteria of pure cultures at product locations where the bactericidal effect is supposed to be minimal. This is done as a routine process in autoclaves and gas sterilizers. In radiation sterilization it has now been generally accepted that such minimum-effect locations can be adequately checked by absorbed-dose measurements. Once an inactivation efficiency versus dose relationship has been established for relevant test strains of bacteria in the given irradiation facility, further process control by means of biological indicators may often be dispensed with.

One may consequently define the role of absorbed-dose dosimetry in radiation sterilization of medical and biological materials as an indirect method of controlling the most vital parameter of the process: the inactivation efficiency under the given conditions.

Based on the specific dosimetry suggestions compiled in the IAEA's Recommended Code of Practice for Radiosterilization of Medical Products (IAEA, 1967b), this Chapter discusses some of the steps involved in setting up an irradiation procedure and elaborates on a number of practical points concerning choice and handling of various dosimeter systems.
2. THE DOSIMETRY PROCEDURE

To make sure that a radiation sterilization procedure meets the prescribed requirements with regard to dose or to inactivation efficiency, a set of procedures must be set up, including the following steps:

(a) To establish source, conveyor, geometry and package configurations that lead to technically and economically acceptable dose distributions in the products
(b) To establish control systems to maintain and record all relevant process parameters, thereby ensuring a reproducible exposure
(c) To establish inactivation versus dose relationships under the relevant process conditions
(d) To establish systems for routine dosimetry in product packages.

The successful execution of any of these four steps relies on accurate and reliable absorbed-dose measurement, i.e. reproducible systems, sensible usage and good laboratory practices need to be established.

Much guidance may be found in the section of the Code of Practice pertaining to dosimetry. The general requirement is that:

"Recognized methods of dosimetry must be employed and complete records of results obtained must be kept. Records should be kept of the various parameters controlling the plant operation."

Specific recommendations for dosimetry at gamma ray irradiation plants and at electron irradiation plants are given in two adjoining notes, which are reprinted as an appendix to this Chapter. For a more comprehensive treatment of industrial dosimetry and process control measures see, e.g., Holm (1968; 1969); Weiss and Rizzo (1970); Fielden and Holm (1970).

3. CRITERIA FOR CHOICE OF SYSTEM FOR ABSORBED DOSE MEASUREMENT

The ideal dosimeter may possess the following characteristics:

(a) Response (measurable effect per rad) independent of radiation energy
(b) Product-equivalent absorption characteristics
(c) Linear dose versus measurable effect relationship
(d) Sufficiently broad dose range
(e) No dose-rate dependence
(f) Accuracy and reproducibility
(g) Response independent of environmental conditions (light, temperature, humidity, storage, length of irradiation, etc.)
(h) Independence of side effects due to impurities
(i) Cheapness and simplicity in use.

The list, which is randomly ordered, does not pretend to be exhaustive. It may be stated without reservation that no existing system satisfies all these demands, and it is therefore necessary in any given situation to reach a reasonable compromise,
(a) The dose absorbed in a material for a given $^{60}\text{Co}$ $\gamma$-ray irradiation depends on the secondary radiation energy spectrum, which is influenced by the composition of the absorbing material. To obtain meaningful measurements it is therefore essential to select a dosimeter system with absorption characteristics and dimensions matching those of the products to be irradiated.

Table XVIII gives some data regarding the absorption characteristics under various spectral conditions of some commonly used dosimeters (Brynjolfsson, 1968). Table XIX gives some data for the absorption characteristics of various commercial plastic materials. Columns 3a and 3 in Tables XVIII and XIX respectively will permit straightforward calculation of product dose from dosimeter dose for a number of materials.

Spectral degradation is not a problem in electron irradiation. For a given incident energy fluence the ratio of absorbed doses in the product and in water (or the dosimeter) will closely approximate the ratio of their respective stopping powers at the initial electron energy. For practical purposes, the $D_p/D_w$ data from Table XIX may be applied with good accuracy.

(b) In routine dosimetry one often takes care of the problem of product equivalence by employing a plastic dosimeter similar in composition to the product. It should be noted that commercial plastics (PVC in particular) may contain substantial amounts of additives (fillers, plasticizers, etc.) which may alter the absorption characteristics.

(c) A linear dose versus effect relationship permits a direct conversion of the measured value to absorbed dose by means of a multiplication factor. Since most process dosimeters do not possess this quality, an experimentally determined calibration curve often has to be applied instead.

(d) A process dosimeter need only work in a fairly narrow dose range, e.g. from a factor of 2 lower than the minimum dose up to a factor 2 higher than the maximum dose do be applied in the sterilization process.

(e) The response of a dosimeter often varies with the dose rate. All aqueous chemical dosimeters are subject to changes in response at excessively high dose rates, i.e. at rates of the order of $10^8$ rads/s and higher. Dose-rate effects can be suppressed somewhat by using high solute concentrations, and this makes the oxalic acid dosimeter (Holm and Sehested, 1968; Draganic et al., 1967) and the 'Super'-Fricke dosimeter (Sehested, 1970) suitable systems for application at linear accelerators. Film systems are usually also dose-rate dependent at such high dose rates, but when calibrated under process conditions they can render quite accurate service.

(f) The accuracy of any particular dosimeter is of course dependent upon the accuracy of its calibration. Process dosimeters should be calibrated against a standard dosimeter at the particular radiation set-up. High reproducibility and low failure rate are of course very important in processes for which a narrow dose range is specified by the regulatory authorities.

(g) Many process dosimeters, particularly the plastic systems, are sensitive to environmental conditions. It is therefore advisable to test the system under extreme conditions that might be expected during processing in order to establish the margin of safety.

(h) Aqueous chemical dosimeters are often quite sensitive to impurities. An exception is the oxalic acid dosimeter. The film systems are sensitive to impurities in the sense that batch to batch variations for these systems
TABLE XVIII. $^{60}$Co γ-RAY ABSORPTION CHARACTERISTICS OF SOME DOSIMETERS COMMONLY USED IN RADIATION PROCESSING

$D_d/D_w$ is the ratio between the dose absorbed in the dosimeter and the dose absorbed in water at the same exposure with various absorbing thicknesses $l$ of water between the source and the sample. The longer the distance $l$, the more degraded is the gamma spectrum. Data calculated by Brynjólfsson (1968).

<table>
<thead>
<tr>
<th>1. Dosimeter</th>
<th>2. Density (g/cm$^3$)</th>
<th>3. $D_d/D_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$l = 0$</td>
</tr>
<tr>
<td>Fricke</td>
<td>1.024</td>
<td>0.997</td>
</tr>
<tr>
<td>Ceric sulphate</td>
<td></td>
<td>1.028</td>
</tr>
<tr>
<td>0.01M</td>
<td></td>
<td>1.066</td>
</tr>
<tr>
<td>0.1M</td>
<td></td>
<td>1.262</td>
</tr>
<tr>
<td>0.4M</td>
<td></td>
<td>1.001-1.03</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td></td>
<td>1.36-1.41</td>
</tr>
<tr>
<td>0.025-0.6M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PVC</td>
<td>1.18</td>
<td>0.939</td>
</tr>
<tr>
<td>Red Perspex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>and PMMA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE XIX. $^{60}$Co γ-RAY ABSORPTION CHARACTERISTICS OF SOME COMMERCIAL PLASTICS

$D_p/D_w$ is the ratio between the dose absorbed in the plastic and the dose absorbed in water at the same exposure under undegraded spectral conditions. Data calculated from Weiss and Rizzo (1970).

<table>
<thead>
<tr>
<th>1. Plastic material</th>
<th>2. Empirical formula</th>
<th>3. $D_p/D_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polystyrene</td>
<td>(CH)$_n$</td>
<td>0.97</td>
</tr>
<tr>
<td>Polyethylene</td>
<td>(CH$_2$)$_n$</td>
<td>1.03</td>
</tr>
<tr>
<td>Nylon</td>
<td>(C$<em>6$H$</em>{10}$ON)$_n$</td>
<td>1.00</td>
</tr>
<tr>
<td>Silicone</td>
<td>(C$_2$H$_5$OSi)$_n$</td>
<td>0.97</td>
</tr>
<tr>
<td>Saran</td>
<td>(C$_n$H$_3$Cl)$_n$</td>
<td>0.87</td>
</tr>
<tr>
<td>Thiokol</td>
<td>(C$_2$H$_4$S)$_n$</td>
<td>0.94</td>
</tr>
<tr>
<td>Teflon</td>
<td>(CF$_3$)$_n$</td>
<td>0.86</td>
</tr>
<tr>
<td>PVC</td>
<td>(C$_2$H$_3$Cl)$_n$</td>
<td>0.92</td>
</tr>
</tbody>
</table>
can be quite large. A special case is the rigid PVC film dosimeter, where the coloration used in the dose measurement is caused mainly by the impurities in the film.

(i) The requirement of low cost and simplicity is self-explanatory; taken together with the other requirements it is unfortunately also utopian.

In the opinion of the author, the most important requirements are: suitable absorption characteristics, accuracy, and low failure rate.

4. SOME COMMENTS ON PROCESS DOSIMETERS IN PRESENT USE

Aqueous chemical dosimeters

Because of the limited dose range (4-40 krad), the Fricke dosimeter (Sehested, 1970) and modifications of it are mostly used for research and for calibration of process dosimeters. For these purposes it is recognized to be the most reliable and accurate system in use today. It has tissue-equivalent absorption characteristics, a linear dose versus effect relationship, very good accuracy and may be used in the modified form up to the high dose rates obtained with linear accelerators.

The ceric sulphate dosimeter (Bjergbakke, 1970). The dose range of this system makes it potentially useful for medical sterilization processes. The dosimeter has proved useful in many laboratories, but it is generally experienced to be troublesome in routine applications. The yield is extremely sensitive to organic impurities, and consequently the water as well as the chemicals and the glassware must be very pure.

The oxalic acid dosimeter (Holm and Sehested, 1968; Draganic, 1967) is distinguished by having almost exactly the same absorption characteristics as water under all spectral conditions, which makes it a very useful dosimeter for use in thick targets at $^{60}$Co plants. An inconvenience is that the yield for a given dose is somewhat dependent upon the total irradiation time. The system is not sensitive to impurities and the storage stability before and after irradiation is excellent. The decomposition of oxalic acid versus dose proceeds approximately exponentially.

Film systems

Red Perspex. The red Perspex dosimeter (ICI Perspex type red 4043) is available in the form of sheets of approximately 3 mm nominal thickness. This system is widely used at commercial $^{60}$Co radiation plants (Whittaker, 1970). The dose range covered is $10^5$-$10^7$ rads and the accuracy is claimed to be ±2-3%. An ageing treatment is usually given in order to accelerate the processes of air and water-vapour absorption, which may otherwise change the response. The UKAEA Wantage Research Laboratory supplies dosimeters to users in a pre-aged condition and, if required, together with a calibration curve.

The clear PMMA dosimeter (Orton, 1970) is available in a particularly pure form (HX) from ICI in sheets of nominal thicknesses of 1 and 3 mm. The accuracy of the system is claimed to be ±2% and doses of 0.1-5 Mrad can be measured. Batch to batch reproducibility is reported to be quite poor and a pre-dosing is often desirable in order to get a more convenient dose versus response relationship.
PVC. The rigid PVC film dosimeter (Popović, 1966) has been widely used for dosimetry in medical radiation sterilization processes, particularly at accelerator plants where the small thickness of the film (0.25 mm) allows rather detailed information in spite of the limited penetration ability of the electrons. Apart from being cheap and simple in use, the system does not fulfill any of the demands listed. However, it has been one of the few systems that could be conveniently adapted to the industrial electron irradiation process to monitor continuously the dose received by product packages. By calibrating each sheet of film separately and by following a stringent routine including a well-defined heat treatment after irradiation, it is possible to obtain an accuracy of the order of ±5-10%. The workable dose range is from 0.5 to approximately 6 Mrad.

Blue cellophane (Henley and Richman, 1956) has been widely used for measuring the field characteristics of low-energy accelerator electron beams, for which it is well suited as the film is less than 0.1 mm thick. There is, however, some evidence that the response suffers from poor reproducibility.

5. CALIBRATION OF PROCESS DOSIMETERS

Any system used for industrial dosimetry should be calibrated against a standard dosimeter. Very careful calorimetric calibrations have been carried out in the case of the Fricke dosimeter and the G-value agreed upon for this system (15.6) is believed to be highly reliable. Other dosimeters may thus be calibrated against this system when the dose rate in the source is low enough to permit precise dosages in the range applicable (4-40 krad). In cases where this is not possible, a calorimetric calibration should be considered (Radak and Marković, 1970).

6. CONCLUSIONS

Based on extended personal experience, the author believes that the dosimetry guidelines (Section 4.3, notes 1 and 2) given in IAEA's Recommended Code of Practice for Radiosterilization of Medical Products are realistic and useful for the purposes intended. It is felt, however, that successful dosimetry is highly dependent upon personal experience and practice as well as on proper selection of dosimeter systems for the particular problems and conditions. Emphasis has therefore been given to provide supplementary information on dosimetry systems to facilitate some of the choices which meet the newcomer in the field.

Setting up a process parameter control for the plant itself may lead to quite different solutions from one plant to another and the space here does not permit any elaborations on this important problem. Interested readers are referred to, e.g., Holm (1989); Weiss and Rizzo (1970); Fielden and Holm (1970).
4.3. NOTE 1. Dosimetry at gamma ray irradiation plants

(a) General

The dose delivered at a particular location in a product package of a given size, conveyed through an irradiation field in a multi-pass mode, depends on the source strength, the conveyor speed, and the densities and atomic number of the materials passing through the system for irradiation.

The aim of dosimetry is to make sure that every unit receives at least the required minimum dose and that excessive doses are not given. The fulfillment of this requirement should be ensured on the commissioning of the plant, and whenever the process parameters are altered, and should be controlled during routine operation.

(b) Dosimetry on commissioning

For a given mode of conveyor operation and a given source geometry, the dose is inversely proportional to the conveyor speed. The conveyor speed is therefore an important parameter in the dose determination, and it should be ensured that the reproducibility of speed setting and the constancy of the system during operation are within the limits required for obtaining correct dosage.

It should be verified that positive indication is given when the source is in its correct operation position during processing, and that the conveyor is operating properly. Action should occur automatically when this is not the case.

In order to derive correct settings for the conveyor speed, the dose distribution in a standard package\(^a\) has to be evaluated for the given mode of conveyor operation. Because of the absorption characteristics of a multi-pass geometry, packages in any production run should be of known weight and composition. The dose distribution may be determined by placing not less than ten dosimeters in a homogeneous phantom material of similar density and chemical composition as the production packages, or dosimeters may be placed in a standard production package at specific locations representative of areas which might give the best indication of dose variability. This procedure should be repeated in order to test the reproducibility of the integrated process.

Recognized dosimetry systems should be employed, e.g. ferrous sulphate, Fe-Cu sulphate, oxalic acid, and film systems (polyvinyl chloride, coloured or uncoloured polymethyl methacrylate). Systems whose yield is not well known, or systems known to be sensitive to impurities (e.g. ceric sulphate), dose-rate, irradiation temperature, humidity or other external conditions, should be calibrated under actual working conditions against, for example, ferrous sulphate or by calorimetry.

\(^a\) The standard package is here defined as the standard volume unit fitting into the conveyor carrier.
Once the dose distribution is determined, conveyor settings should be fixed so as to ensure that at least the specified minimum dose is given to all the products. Provided it has been established that the speed can be set and controlled accurately, the dose distribution can be determined at doses different from those intended for use in production, and conveyor speeds may consequently be calculated by scaling.

When the process (including materials and packaging) is altered, those parts of the foregoing procedures which are influenced by the alterations should be repeated.

(c) Routine dosimetry

Once the plant is calibrated and the minimum absorbed dose in the production packages determined as a function of conveyor speed, the routine control may be limited to the following steps:

i. Positive indication of the correct operational position of the source, as described in 2.2.

ii. Continuous-line recording of conveyor speed, and/or the plant cycle should be monitored by a system mechanically locked to the conveyor drive. The recording paper should be date-marked so that the proper correction factor can be calculated to compensate for the source decay since the time of calibration.

iii. Either the packages should be labelled with a radiation indicator, or the entrance area to the source should be physically separated from the exit area to prevent irradiated and non-irradiated packages from being intermixed.

In addition to these measures, it is recommended that the dose be regularly checked by including dosimeters in the production packages.

4.3. NOTE 2. Dosimetry at electron irradiation plants

(a) General

The type of accelerator plant referred to in this section is one with the following characteristics:

i. A scanning system providing a sweep of the electron beam so as to give a homogeneous surface dose distribution over the width of the production package.

ii. A conveyor system which moves the production packages in a direction perpendicular to the beam and scanning directions.

iii. A sweep velocity high enough to ensure good beam-spot overlap on products passing at the highest conveyor speed.

With constant beam and scanning, the dose delivered at a particular location in a product package depends on the beam parameters (energy, average current and scan width), the conveyor speed, and the thickness, density and atomic number of the materials placed between the beam exit window and the location in the package.

The aim of dosimetry is to make sure that every item processed receives at least the required minimum dose and that excessive doses are not given. This should be ensured on the commissioning of the plant and
whenever the process parameters are altered, and should be controlled during routine operation.

Absolute standardization of the beam parameters is not required to obtain correct dosage, although the electron energy should be approximately known in order to evaluate the induced activity hazard.

The relative levels of the beam parameters, however, are to be monitored continuously whenever the process is to be carried out without dosimeters being attached at minimum dose locations in all packages.

(b) Dosimetry on commissioning

For a given set of beam parameters, and for a given geometry of the irradiation field, the dose is inversely proportional to the conveyor speed. The conveyor speed is therefore an important parameter in the dose determination, and it should be ensured that the reproducibility of speed setting and the constancy (or corrective regulation, see 3.3) of the system during operation are within the limits required to obtain correct dosage.

A system should be provided to deal with a failure of the beam operation (e.g., automatic and immediate shut-down of the product conveyor).

The beam parameters can be standardized as follows:

Absolute current measurements of the scanned beam can be made by having the electrons absorbed in, for example, an aluminium absorber thick enough to absorb all the electrons entering it, and by metering the resulting current flowing to ground. Correction must be made for electrons scattered back from the absorber. [Values for different electron energies and absorber materials may be found in NBS Handbook 97 (1964).] Routine current measurement may be carried out by means of a non-intercept monitor. A simple technique may be used, e.g., the scanner chamber of the accelerator might be provided with secondary emission plates mounted symmetrically around the beam and collecting electrons back-scattered from the accelerator exit window. It should be ascertained that the system preferred gives accurate readings which are not significantly influenced by other factors such as minor changes in beam focusing.

Absolute calibration of the beam energy may be carried out by various methods, such as

1. magnetic spectrometry
2. threshold-detector methods
3. film-stack dosimetry.

One way of obtaining a relative measure of the electron energy is readily available when the accelerator is fitted with a bending magnet located before the scanning system. In this case the current required to bend the beam by 90° (or any other angle chosen) is a relative measure of the electron energy. If the process parameters are to be integrated into the dosimetry procedure, it should be ensured that the energy remains constant during processing. A system of secondary emission plates such as referred to above may be used to control this parameter. If the energy varies, the beam, for a constant current in the bending magnet, will deviate from the angle chosen and thereby cause a greater response from one of the plates than from the other. This system can be made to trigger an alarm or to control an automatic energy-regulating circuit. Another system is based on the fact that the current necessary to scan the beam over a certain width is
a function of the beam energy. This principle can be applied to a monitoring system by installing two bars or wires at each end of the scanner house (under the exit window) and making the scanned beam strike the inner, but not the outer bars. Whatever method is used, it should be ensured that the response is not influenced significantly by minor changes in other physical factors.

The scan width can be controlled by the bar method referred to above or by an automatic feedback system to the driver circuit. Deficient operation should cause automatic shut-down of the process.

In order to obtain correct settings of the conveyor speed for a given set of beam parameters, the dose distribution in the standard production packages should be evaluated. Because of the absorption characteristics of high-energy electrons, packages in any production run should be of similar weight and composition, and the contents should be homogeneously distributed throughout the package. The dose distribution may be determined by placing not less than ten dosimeters in a homogeneous phantom material of similar average density and chemical composition as the production packages, or, better, the dosimeters may be placed at representative locations in the production package. It will normally be found that the part of the package farthest from the beam receives the smallest dose. This procedure should be repeated in order to test the reproducibility of the integrated process.

Recognized dosimetry systems should be employed. The low penetration of electron radiation as compared with gamma radiation makes it necessary to use film systems (e.g. polyvinyl chloride, coloured or uncoloured polymethyl methacrylate). As these systems are influenced by external conditions such as temperature, humidity, and dose rate, calibration should be carried out under actual working conditions (e.g. by calorimetry).

Once the dose distribution is determined for a given set of beam parameters, conveyor settings should be fixed so that the specified minimum dose is delivered to all product locations.

Whenever the process is altered, those parts of the foregoing procedures which are influenced by the alteration should be repeated.

(c) Routine dosimetry

Once the plant is calibrated and the minimum absorbed dose in the production packages determined as a function of conveyor speed, the routine control may be limited to the following steps:

i. The employment of an interlock system between beam current monitor and conveyor as described in 3.2.

ii. Continuous monitoring and continuous-line recording of current, energy, scan width, and conveyor speed, as described in 3.2. The electron current signal, as monitored by secondary emission plates or some other non-intercept monitor, may be used to feed the conveyor driver circuit, thereby ensuring a constant surface dose.

iii. Either the packages should be labelled with a radiation indicator, or the entrance area to the accelerator room should be physically separated from the exit area in order to prevent irradiated and non-irradiated packages from being intermixed.
In addition to these measures, it is recommended that the dose be regularly checked by attaching dosimeters to the production packages at the place farthest from the beam.

Whenever a mixed production (light and heavy packages together) is processed, or whenever monitoring of the production parameters is not carried out according to the requirements listed above, a dosimeter should be attached to each package.
The prime function of a package containing a presterilized medical item is to ensure that the sterility of the contents is maintained up to the time the package is intentionally opened and that provision is made for the contents to be removed without contamination. Failure to achieve this aim negates the efforts of bacteriologists to control sterilization methods and the standards of sterility that have been set. A recent recommendation (Christensen et al., 1969b) that any sterilization procedure should achieve a statistical probability of not more than one non-sterile article occurring in one million articles processed, loses its practical significance if the package fails to achieve a similar standard. To achieve such standards, many factors involved in the design and manufacture of a package must be carefully considered and the major ones are listed as follows:

(a) Suitability of packaging material for the sterilization method
(b) Resistance of the material to bacteria
(c) Type of package
(d) Type of opening
(e) Strength of package
(f) Testing of the package.

This is not a complete list of the factors involved but these are probably the major ones encountered. These factors have not been listed in any order of importance as this would be extremely difficult. Many of these items are interdependent and in many cases failure to overcome one problem will negate the whole package function.

1. SUITABILITY OF PACKAGING MATERIAL FOR THE STERILIZATION METHOD

Radiation sterilization allows a very wide choice of packaging materials. The process does not involve high temperatures so that many thermoplastic materials can be used successfully.

The gas and moisture permeabilities of packaging materials are very important when steam and gas sterilization processes are used, but these properties are not important for radiation sterilization since there is complete penetration of the radiation through all common packaging materials. Most packaging materials are not deleteriously affected by the radiation sterilization process so that materials commonly used in packaging such as polyethylene, polystyrene, polyesters, polyamides, cellulosics and aluminium foil can be used with confidence. Some materials can be affected such as polypropylene, which can lose strength and discolor, and polyvinylchloride, which tends to discolor and free hydrochloric acid may be produced. Nevertheless, radiation-resistant grades of these polymers are available. Certain strength properties of cellulosics can be reduced
by approximately 10% at dose levels required for radiation sterilization but this can be overcome, if necessary, by increased thickness or combination with other materials such as polyethylene.

Since no packaging material is a single chemical entity, many materials are available commercially in different chemical forms, although they may be classified as a single product. For example, a common thermoplastic material can contain different plasticizers, stabilizers and slip additives and in various proportions. These small variations may produce different end results when the material is irradiated. This is further complicated when packaging converters combine the basic raw materials in different ways using various adhesives etc. It is most important, therefore, that guidance is obtained from the packaging manufacturer and if necessary the packaging material should be irradiated and thoroughly tested before it is regularly used.

Although firm recommendations for packaging materials cannot be stated, nevertheless, a great deal of experience has been accumulated by packaging manufacturers during the last ten years and there is little problem in finding packaging materials that are suitable for radiation sterilization. The effects of radiation upon the materials commonly used in packaging are not normally deleterious enough to preclude the use of any particular material or to sufficiently destroy the effectiveness of any package.

A useful guide to the choice of materials for packaging and product has recently been published (Crawford, 1970).

2. RESISTANCE OF THE MATERIAL TO BACTERIA

A detailed examination of the bacterial permeabilities of packaging materials has not been carried out, probably because of the difficulties of measuring this property under practical conditions. The most important factor affecting bacterial permeability is the frequency and size of pinholes in the packaging material. Ideally the packaging material chosen for an application should be completely free of pinholes. Pinholes are frequently found in single-ply materials and they should only be employed in thicknesses where the occurrence of pinholes is very remote. Coatings will often effectively fill in pinholes that occur in a base material and when two plies of material are laminated together not only will the laminant tend to fill in pinholes but it is also unlikely that pinholes in one ply will occur directly opposite those in another ply to produce a continuous hole.

In theory only pinholes that are smaller than the smallest known bacterial cell can be tolerated but in practice this standard may be unnecessary. An investigation (Noble et al., 1963) using a size-grading, slit-sampler technique, of the size distribution of airborne particles carrying various species of bacteria and fungi found in hospital and office premises has shown that most organisms associated with human disease were usually found on particles in the range 4 - 20 μm equivalent diameter. Many fungi, however, appeared to be present in the air as single spores. There must also be a force to drive organisms through pinholes in a package to render the contents non-sterile. It is well known that paper packages held in humid storage conditions or in direct contact with water stand considerable risk of contamination by bacteria growing through the paper. The use of waterproof packaging materials is, therefore, always to be preferred and
sterile packages should, wherever possible, be stored in dry and good storage conditions, particularly if they are made in any part from uncoated paper; radiation sterilization allows a wide range of waterproof packaging materials to be used. Water is not, however, the only driving force that can promote the passage of bacteria through pinholes in a package. Pressure differences between the inside and outside of a package can cause ingress of airborne bacteria. These pressure differences can be caused by temperature changes, the opening of cupboards and drawers, impact during transport or simply by handling, so that a 'bellows effect' is produced.

Therefore, although no standards exist that control the frequency or size of pinholes that can be tolerated in packaging materials, it is important to develop and use techniques for determining pinholes. Also, it is necessary to measure pinholes in packaging materials that have been flexed, abraded and creased since these actions can cause pinholes in materials that are pinhole-free when examined in sheet form.

Paper is probably the most frequently used packaging material that is susceptible to pinholes. It is essential, therefore, that only high quality papers that have been specially developed for the medical market should be used for packaging sterile products and regular control measurement of pinhole size should be carried out. Paper is a very good filter medium because of its fibrous structure in which the cellulose fibres mesh into an irregular network. Actual holes should not occur in good quality papers but irregular-shaped passages through the fibres do exist and paper technologists call these pores, rather than pinholes. Measurements of maximum pore size give a good indication of the bacterial filtration properties of paper.

In the United Kingdom the Department of Health and Social Security has produced specifications for papers used in various hospital applications. These papers must pass a test procedure specified in British Standard B.S. 2577:1955, which measures the filtration properties of papers to a continuous flow of methylene blue particles. The apparatus is complicated and the standard is not expressed in terms of pore size. Nevertheless, paper mills can produce papers to a specification that is acceptable for hospital application.

A simple and rapid method of measuring the maximum pore diameter of a sheet of paper is based on the dioxane method described by Corte (1965). A sample of paper is clamped horizontally and covered to a small depth with an organic liquid, such as carbon tetrachloride or ethanol, that does not swell the paper fibres. The pores of the sample become filled with liquid and then a steadily increasing air pressure is applied to the lower surface of the sample, tending to push the liquid back through the pores against the surface tension force. When the liquid/air interface reaches the upper end of the pore at the paper surface, bubbles of air will form, provided the applied pressure is greater than the surface tension force acting on the curved surface of the liquid. For a liquid of surface tension $T$ and a pore radius $r$ the condition for the formation of bubbles (i.e. for airflow through the pore) is

$$p \geq \frac{2T}{r} + \rho gh$$

where $\rho$ and $h$ are respectively the density and height of the liquid. It is assumed that the bubble breaks away when its radius becomes equal to that
of the pore. From the equation it is seen that as the pressure $p$ is increased, bubbles appear first from the pore of largest radius. Smaller and smaller pores will pass air as the pressure is progressively increased.

A diagram of the apparatus is shown in Fig. 16. The paper sample is clamped between two flat flange glass joints (E) fitted with rubber gaskets to ensure a gas-tight joint. The two flanges are held together with a wire clip. The organic solvent is poured onto the sample to a depth of 1 cm and a mirror, set at 45° to the top of the glass tube (E), so enabling the upper surface of the sample to be viewed from the front of the apparatus, is swung into position. Air pressure from a cylinder is then applied to the lower surface of the sample through a pressure regulator (C), a reservoir (D) and a two-way tap (J). The pressure is measured by mercury and water manometers (A and B), a stopcock being provided to disconnect the latter when the pressure exceeds 76 cm water. The air pressure beneath the specimen is steadily increased using the regulator (C) and, when the first stream of bubbles appears in the liquid at the surface of the sample, the pressure on the manometer is noted. The pressure can be decreased and
then increased again and it is noted that the pressure to produce the first stream of bubbles is repeatable. When the test has been completed, a hole is made in the sample and the organic liquid drained into bottle (F) by opening the stopcock in the outlet tube.

The equivalent radius of the largest pore can be calculated from Eq. (1) by rearranging the equation so that the pore radius is given by

\[ r_{\text{max}} = \frac{2T}{P_0 - \rho gh} \]  \hspace{1cm} (2)

where \( P_0 \) is the air pressure at which the first stream of bubbles appears and the other symbols have the same meaning as in Eq. (1). It is usual to speak of 'equivalent' pore radius since the true shape of the pore is not known.

Equivalent pore radius may be defined as the radius of a capillary of circular cross-section that requires the same air pressure as the paper pore to force the liquid from it. This apparatus can be modified to measure pore size distribution by connecting a flowmeter to the upper chamber of the cell (E) so as to measure flow rate through the sample. The upper chamber is also connected to the free limbs of the two manometers, which will then indicate the pressure differential across the sample. This is necessary since the pressure in the upper chamber will be above atmospheric. To determine pore size distribution, the pressure is increased in equal increments and the flow rate, as indicated by the flowmeter, noted at each pressure level. A graph is plotted of the flow rate against pressure, from which the pore size distribution may be calculated as described in Corte's paper (1965).

The measurement of pore size distribution is a rapid method for screening paper samples when a grade of paper suitable for packaging sterilized products is being sought. The measurement of maximum pore size can be used as a quality control method when the maximum pore size that can be tolerated has been predetermined, and the paper samples are then subjected to a pressure, calculated from the equation, at which no bubbles should occur. The method as described can be used as a convenient control technique but it must not be assumed that the actual pore size as measured will have a direct relationship to the passage of bacterial cells. For example, papers with a pore size distribution of 10 - 14 μm have been tested as filters to carbon particles measuring 0.5 μm and have been found to prevent the passage of these particles.

The most objective test is to measure the bacterial resistance of papers. This can be done by drawing a bacterial aerosol through a paper sample subjected to a pressure differential. The disadvantage of this technique is that the air porosity of the paper sample dictates the volume of air passing through the sample. The porosity of a paper can vary significantly during manufacture, but this characteristic may have little effect on actual bacterial resistance. This porosity variable can, however, be overcome by introducing a flowmeter into the apparatus used.

A method for testing papers when wet for bacterial penetration has been described by Harbord (1968). Test samples of paper are subjected to direct contact with a bacterial suspension for 2 hours and any bacteria that have penetrated the samples are counted after incubation. It is considered that only papers with a NIL penetration rate are satisfactory since the test procedure simulates conditions that can be found in practice. This method
is used as a quality control technique, but suffers from the usual problems associated with bacterial testing, those of time and cost.

Materials containing films, coatings and laminations are commonly used for packaging disposable products because they are conveniently used on automatic packaging machines and they give good physical protection to a product as well as moisture resistance. There are several techniques available for detecting pinholes in these materials and a useful review of the methods used has been given by Becker (1963). The methods involve the penetration through the pinholes and the subsequent detection of liquids, light, electrical or corona discharges and gases. Liquids provide an efficient and rapid method for detecting pinholes. The liquids are generally coloured with a suitable dye and must have good wetting properties; benzene and ethyl alcohol have been found to be suitable and a small amount of detergent solution is often added when water is used. Becker quotes that pinholes with a diameter of 10 μm can be detected when ethyl alcohol is used at a test pressure of 2 mmHg. The method can be used with plastic coated papers, where a liquid that wets well will penetrate the plastic coating by capillary pressure and come into contact with the capillary system of the paper so that capillary force alone causes penetration of the liquid and no external pressure is required.

The most common gas method used is based on the diffusion of ammonia through pinholes in the test material and subsequent detection on a paper impregnated with an ammonia-sensitive reagent placed at the other side of the test piece. Pinholes with a diameter of down to 5 μm can be detected by this method. Davis (1969) has described this method fully and has used paper coated with ferrous ferricyanide (Turnbull's blue) as the ammonia-sensitive reagent, which changes to a white colour on contact with ammonia gas. The same principle can be used for detecting and measuring pinholes in plastic films by subjecting the film to ammonia on one side and hydrochloric acid on the other. Small white spots of ammonium chloride form where pinholes occur and these can be marked, the ammonium chloride washed away and the pinholes measured under a microscope.

Light can only be used for testing fully opaque materials. Large pinholes can be detected by observation against a bright light source or the material can be covered by a photosensitive paper on one side and exposed to bright light on the other side. Pinholes as small as 5 μm can be easily detected with this technique.

Electrical methods of detecting pinholes are based on a spontaneous electrical discharge in air and there are two variations of the technique. The first method utilizes a sparkover between a brush electrode and a plate or roller electrode and is particularly useful to packaging manufacturers since the pinholes can be detected in a continuously moving web of material. The material runs over a metal roller and a metal wire brush is positioned directly above the roller. A potential, provided from a high voltage supply, is passed across the electrodes. Pinholes are immediately detected by small sparks and instruments can be incorporated in the circuit to give visual or acoustic signals of occurrence of pinholes. It is thought that this technique detects pinholes with a diameter of less than 5 μm. An essential feature of the method is that the test material must have an adequate resistance; all plastic films have this property except films such as regenerated cellulose and cellulose acetate, which contain high proportions of water. The second electrical method operates at a lower voltage so
that sparkover does not occur but a corona discharge takes place through any pinholes. The pinholes are observed where a luminous discharge occurs and measurement of the corona discharge is approximately proportional to the number of pinholes.

A new technique has recently been described (Huldy, 1970) that relies on the electrolytic oxidation of a colourless redox indicator, benzidine, so that the black oxidation product formed gives a precise picture of pinholes occurring in a test film on a white millipore filter. Large pinholes can be seen with the naked eye but small pinholes down to a diameter of 1 μm are observed with a microscope. The method is described as a rapid technique for the detection and size determination of pinholes and could be a very useful additional technique to those already mentioned for determining pinholes in plastic films and coatings.

Although there are several methods for determining pinholes in packaging materials, it must be realised that only small samples are tested by these techniques. The statistical significance of results obtained by these methods compared with the amount of packaging materials in use is very limited but, nevertheless, they serve to eliminate certain materials that could otherwise have been considered for packaging sterile products and they also enable spot checks to be made on materials in current use as to the frequency of pinhole occurrence.

3. TYPE OF PACKAGE

Rigid containers made from fibreboard, plastics, metals and glass are all suitable for radiation sterilization, except that most glass containers discolor. Very few commercial applications of radiation sterilization, however, utilize rigid containers because of the relatively high unit cost and the difficulty of automatically filling. Rigid containers, nevertheless, have two useful characteristics, physical protection to the contents and methods of opening that enable the contents to be removed without contamination. They are commonly used for packaging biological tissues, for example, bone and arterial grafts, and liquids such as vaccines. Rigid containers are used on a commercial scale for packaging hypodermic syringes and needles and to a more limited extent for catheters. The pharmaceutical industry has found radiation sterilization a useful method for treating rigid plastic containers and aerosol cans and valves prior to aseptic filling; flexible packaging materials have also been sterilized for the same application.

Flexible packaging techniques are most commonly used for the commercial packaging of medical items. The main reasons for this are availability of automatic packaging machinery, wide choice of materials, ease of pack identification and low unit cost. The basic disadvantages are the amount of material required to pack a three-dimensionally shaped product, consistency of making seals, provision of methods to open the package, and the physical protection of the primary pack is not generally as strong as provided by rigid containers.

The main methods of packing medical items using flexible materials can be well illustrated by taking as an example the packaging of a disposable hypodermic syringe:
FIG. 17. Types of package.
(i) The simplest method is to package the syringe in a preformed tube of flexible material such as polyethylene lay-flat tubing (Fig. 17a). This type of package has the advantages of using a small area of material and only two seals have to be made. A disadvantage of the method is the limited number of materials available in this form and they are used generally in single-ply form and therefore have the inherent weaknesses associated with single-thickness materials. An additional disadvantage is that the method does not lend itself to automatic packaging.

(ii) Another type of package is sealed on four sides (Fig. 17b). This package style is suited for automatic packaging and a wide range of materials are available, both in single ply and combinations. The pack is made from two webs of material, which can be the same or dissimilar, allowing various types of 'peel-open' packages to be produced. A disadvantage of this package for bulky products, such as syringes, is that a large area of material must be used and since four seals are made, this increases the care that must be taken to ensure integrity of the seals. A variation on this method is to use a single folded web of material, which is wrapped around the product and then sealed on the remaining three sides (one long seal and two cross seals). This variation does reduce the area of material used but limits the types of peel-open devices that can be used.

(iii) An alternative method is to use one web of material, which is formed into a tube by producing a long seal in the direction of the material (Fig. 17c). This can be done by wrapping the material around a metal tube on a vertical filling machine, a seal is then made across the material, a syringe dropped into the tube formed, and a further cross seal made at the top to complete the package. This type of package is more commonly made by wrapping the material around the syringe in the horizontal plane, while the long seal is made and the two cross seals are subsequently produced. The latter method is normally carried out on a continuous flow system and lends itself to high packing speeds. This system utilizes less material than method (ii) and only three seals have to be made. Nevertheless, great care must be taken in making the cross seals where the long seal and cross seals meet because four thicknesses of material have to be welded together (see Fig. 17c). Since there is a change of material thickness across the sealing face, there is a danger of leaving a small channel at the point of change-over from two to four plies of material.

(iv) A further method is to pre-form one web of material to form a dish into which the syringe is placed, then a further web of material is fed over the top of the dish and heat sealed on all sides (Fig. 17d). The advantages of this method are the economy in use of material and that perfectly flat faces are presented for heat sealing. This type of packaging has always been attractive for packaging three-dimensional products but its general usage has been limited by the materials that could be easily thermoformed (these also were normally only available in single-ply form) and there were few automatic machines available to produce such a package. This situation has changed radically in the last two years so that the method is becoming very popular. Several types of machines are now available, laminated webs have been developed that can be thermoformed and materials are now produced that can be used as a peelable lid. The base tray can be rigid, semi-rigid or flexible and the top web is easily printed for carrying information. The forming tools for the base web can be changed very quickly on some machines so that a rapid change from packing one product
to another can be achieved. Stresses on the heat seals are limited by this technique because the package is formed around the product and although Fig. 17d shows a rectangular tray, this can be designed so that the dish conforms to the shape of the product.

As already stated, there is a wide range of flexible packaging materials available for use in the methods just described and combinations producing opaque, transparent or window packs can be used. The window pack, i.e., one side opaque and one side transparent, is very popular since the opaque surface can be used to print brand names and information for the user in a distinct form, whilst the transparent window enables the contents to be seen as an important check on the information printed on the other side. The size of the package is always an important consideration since sterilization cost using radiation depends a great deal on the space taken up by the product. It is sensible to do away with unnecessary material because of this factor as well as the cost of the material, but sufficient material must be used to prevent heat seals having to stand excess strain. Also, if a peelable type of opening is required, then some form of tab must be provided. Fortunately the problem of package size is usually self-solving since more automatic packaging machines are used and it is impossible for the machine to make an acceptable package with insufficient material.

The final type of package chosen for a product is dictated by many factors, such as materials, method of opening etc., and before a product is offered commercially on a large scale many of these factors can be investigated by a limited market test utilizing pre-formed packages and manually packing the product. Pre-made bags, pouches and trays are available and these can be used for market tests.

4. TYPE OF OPENING

There are various methods of opening packages and these are determined to a large degree by the style of package and packaging materials utilized. The final end use of the contents can also be extremely important in determining the complexity of the opening method. For example, a method that might allow the contamination of any part of an instrument intended for use in a surgical theatre when it is removed from its package could not be tolerated. For another product, e.g. a disposable syringe, it can be said that only that part of the item that may come into contact with the patient needs to be protected from contamination; a syringe can be removed from a package at the 'handle' end without too much danger of contaminating the 'business' end of the syringe, particularly if there is a needle attached to the syringe that is protected by a plastic sheath. The physical nature of the product can also dictate the method of opening so that a hard plastic syringe can be pressed through a flexible package wall, but this cannot be done with a soft product such as a catheter. The ideal situation is one in which the package can be easily opened without the aid of ancillary equipment and the article withdrawn from the package without any danger of contaminating the contents.

Some surgical items are packaged in rigid or semi-rigid containers incorporating methods of opening that are not applicable to flexible packages. For example, one catheter package comprises a long plastic semi-rigid tube sealed at one end into which the catheter is placed. A cap of the same
material is slipped over the open end of the container and a bacteria-tight
seal affected by binding the junction point of the tubes with adhesive tape.
The package is opened by removing the tape, slipping off the capping tube
and removing the catheter without passing it over a non-sterile edge. A
hypodermic needle is marketed in a small injection-moulded, rigid plastic
tube capped by a plastic disc that has been hermetically sealed around the
circumference to the open end of the tube. The disc can be prised off by
thumb, thus exposing the hub of the needle. A syringe is then introduced
into the needle hub and firmly attached to it by a screwing action. The
needle attached to the syringe is then withdrawn from the tube and does not
come into contact with a non-sterile edge.

For many years it was considered essential by several bacteriologists
(Hare et al., 1961; Cunliffe et al., 1963) that a sterile article should not
be drawn over a non-sterile edge so that cutting or tearing were not
methods of opening a flexible package that should be encouraged. Never-
theless, recent comments (Darmady et al., 1968; Christensen, 1970c)
indicate that cutting open a package, even with unsterile scissors, offers a
very slight risk in contaminating the sterile products when it is withdrawn.
Unfortunately, statistical data to show if the unsterile edge of a cut package
is of practical significance are not available. However, if a package is to
be cut open, then either sterile or non-sterile scissors must be provided.
The provision of sterile scissors for opening every package does not appear
to be practical considering the very large number of prepackaged sterile
items that are in current use and it is inconceivable to suggest to a nurse
that she may use non-sterile scissors to start a sterile procedure.

Probably the easiest way of opening a flexible package is to provide
a method of tearing and this technique has been widely used, particularly for
packaged syringes. This method may require the introduction of starting
aids such as a notch, but an essential feature for this type of opening to be
successful is that the material should have a low degree of stretch on
tearing. A tear tape or string incorporated into a package might be
considered as a further tearing aid but such a method inevitably increases
the risk of imperfect sealing. An alternative approach is to break the
package open by pressing the object through the wall of the package. This
method cannot be universally employed, but has been used successfully
for needle packages where the hard hub of the needle facilitates the
breaking of the material. A bacteriological assessment of this type of
package has been carried out and it has been found that out of two series of
needles with 400 needles in each series from two different manufacturers,
7% and 1.5% were contaminated (Darmady et al., 1961).

It has been suggested (Darmady et al., 1968; Christensen, 1970c) that
the most important factor in opening a sterile package is the bacteriological
aerosol that is created when a package is torn or pulled apart and this may
contaminate the contents. The aerosol is particularly important when the
outer surface of the package is covered with dust particles. The practical
significance of the aerosol effect has not been determined since there are
no published bacteriological experimental data to enable this factor to be
assessed, but the effect has been illustrated by dusting the outer surface of
packages with a fluorescent powder, opening the package, and then observing
under ultra-violet light how the particles settle on the contents (Fallon,
1963). It is very difficult to envisage any method of opening a package that
does not cause an aerosol effect since the movement of hands and the
'Bellows' effect that is produced when a package is held in the hand and opened will certainly produce an aerosol of some degree. Nevertheless, since the aerosol effect almost certainly exists, a sterile package and the method of opening should be designed in such a way that the dangers of an aerosol should be limited. One of the most popular ways of avoiding this and also limiting the possibility of contaminating the sterile item by handling is to employ a double package. It is preferable that the inner wrap should not only protect the contents during the opening of the outer wrapper, but it should be easily opened without tearing or cutting before the contents are withdrawn; in many cases the inner wrap need only be folded over like an envelope. An outer wrap where the packaging material can be peeled away from the article so that at no time does the content touch the outer surfaces of the package is a distinct advantage. Nevertheless, peelable packages must be provided with tabs that can be held to facilitate the peeling action of the package. There is a distinct danger that dust can collect in the tab opening and come into contact with the contents when the package is peeled apart. This danger can be largely overcome by folding over both edges of the tabs so that dust cannot collect in between the tabs; this may not be necessary if other ancillary packaging is used that limits dust contamination. In principle, therefore, it would appear that an initial target for pre-sterilized items should be a package designed with a folded inner wrap and securely sealed peelable outer wrap with the opening tabs folded over.

Several methods of producing a peelable package have been employed. The most common method is the controlled rupturing of paper fibre. A heat seal is produced between a paper surface and another web of material, which is coated with a thermoplastic or other heat-sealing medium. The two webs of material are peeled apart and paper fibres in the area of the heat seal are broken so that the package is opened. It is very essential with this method that the paper surface is carefully selected so that limited fibre rupture occurs and not paper tearing. Also, rigid control of the heat-sealing medium and the sealing conditions must be maintained. This method of opening has been criticized in that a cloud of paper fibres might be created, which, although sterile, should not be allowed to contaminate the contents. The importance of this factor has not been determined but its significance should be limited if suitable papers are used and also double packaging utilized.

A heat-sealable lacquer coating that can impart peeling qualities to polyethylene to polyethylene heat seals has been developed (Douglas and Scarrott) and this method of producing peelable seals is used extensively for radiation-sterilized packages. The lacquer coating can be applied to only one or both polyethylene surfaces and the composition of the coating can be altered to give the seal strength required. Care must be taken to apply the correct amount of heat when sealing since excessive heat will produce strong polyethylene to polyethylene seals, which will not peel, and insufficient heat produces physically weak seals; the lacquer coatings, nevertheless, have a wide heat sealing range and problems do not normally occur. A range of peelable packages can be produced using this lacquer system such as completely opaque packages of polyethylene-coated paper or foil, transparent packages of polyethylene-coated polyester or cellulose films, or a window package comprising, for example, polyethylene-coated paper on one side and polyethylene-coated polyester film on the other side.
These lacquer coatings are very versatile and they can be sealed to poly-vinyl chloride and polystyrene; polyethylene-coated paper that has been treated with lacquer is a commonly used peelable lidding material for polystyrene trays. This method of peelable lacquer heat seals is incorporated in many packages for radiation-sterilized items such as catheters, surgical gloves, surgical suture overwraps, needles and ward procedure tray packs. The system is particularly useful for radiation-sterilized items since the packaging materials can be very protective in terms of moisture resistance, physical strength and freedom from pinholes but the packages can be peeled open in a smooth controlled way. A further advantage is that the lacquer-coated materials can be processed on a wide range of packaging machines.

Another method of producing peelable heat seals has been developed that takes advantage of the deleterious effects of radiation on polypropylene film (Powell). The method has been found particularly useful for packaging surgical scalpels where the package is made of cast polypropylene film laminated to aluminium foil. The blade is sandwiched between two plies of the laminated polypropylene film, which are heat sealed together around the blade on four sides. The plies of polypropylene cannot be peeled apart if the heat seals have been made efficiently but, after the package has been sterilized by radiation, the films can be successfully peeled apart at the heat seals. The physical strength of the package has been found to be acceptable after irradiation but the radiation has sufficiently embrittled the polypropylene heat seals so that they can be peeled apart. The package can also be autoclaved after it has been radiation sterilized if necessary; this sometimes occurs in hospitals when the blades are incorporated in procedure packages that are sterilized in an autoclave. It can be seen that there are various ways in which peelable seals can be made but it is often forgotten that such seals are a compromise between a complete weld and no seal at all and in an effort to produce good peelable seals the strength and integrity of the seals are sometimes ignored; a well-sealed package is the first essential and peelable seals are a secondary requisite.

5. STRENGTH OF PACKAGE

It is very difficult to define the physical strength characteristics required for a sterile package but the ultimate aim is to ensure that the sterility and function of the contents are not impaired by damage to the package. There is no doubt that many sterile items are insufficiently protected against physical damage. A visual examination of packaged sterile items distributed to a group of hospitals has shown that of 2546 packages inspected from various manufacturers 6.4% were found to be defective and of 2400 syringe packages examined 1.2% were similarly imperfect (Darmady et al., 1968). It was found that approximately half these defects were due to bad seals and it is not known if these faults were due to non-sealing at the time of manufacture or whether the seals had opened during subsequent transit; the subject of sealing will be discussed later. Christensen (1970c) has also stated that it is not uncommon to find approximately 10% of packaged sterile items with holes in the packages caused by various means.

There are many factors which have to be taken into consideration when determining the physical strength characteristics required by a package. The size, weight and shape of the product are extremely important.
considerations. For example, Crawford (1970) has described an experiment in which a hundred 20-ml syringes, each weighing approximately 20 g, were packaged in thick polyethylene bags and then packed in a strong transport carton. The carton was dropped on the floor from a height of a metre and almost all the polyethylene bags were found to have been punctured by the syringe. This type of problem can largely be overcome by restricting movement, which may be done by using smaller bags to limit the movement of the product in the primary pack and also containing the packages in a smaller outer container or placing more packages in the containers. The product can also be designed so that sharp edges that could damage the package are eliminated. The movement of packages within a container is extremely important since the packages move against one another and can cause abrasive perforation. Because of this, the ancillary packaging, such as dispenser cartons and transport containers, must be carefully considered, bearing in mind the product, its primary package, the method of transport and the storage conditions that are likely to be encountered. The final end use of the product must also be considered since the product can be used under various circumstances. For example, it is likely that a syringe used in a hospital will be issued from its ancillary package such as a dispenser unit and, therefore, the primary package is unlikely to be subjected to the physical stresses that could be experienced by a package being carried around in a doctor's car or bag.

All these factors must be computed before the primary and ancillary packaging can be selected and a final decision can only be made after thorough distribution and market tests.

6. TESTING A PACKAGE

There are several methods of testing the integrity of a package and, since there are no officially recognized methods of determining this, manufacturers tend to use methods that they consider are best suited for testing their own products. There are few non-destructive test methods so that most tests are carried out on a selected number of packages that has statistical significance.

A simple method for detecting faults in packages made of water-resistant materials is to immerse the pack under water and slowly squeeze the package by hand. Air bubbles will appear if a leak is present. A variation on this test is to immerse the package in water, which preferably contains a wetting agent, contained in a desiccator and a vacuum then drawn on the desiccator; again air bubbles appear from the package if there is a hole. The air-bubble technique for detecting holes in packages has been made more sophisticated by utilizing the apparatus (Fig.16) previously described for measuring the maximum pore size in paper. A special connector (Fig.18) has been designed so that a test package can be attached to the apparatus. A circular hole, slightly smaller than the threaded brass disk (extreme left Fig.18), is cut in one side of a package and the disc inserted through the hole by folding the side of the package at this point. The threaded stem of the connector is then screwed into the brass disc; a rubber gasket is pressed into contact with the outside of the package wall and
clamped by tightening a knurled locking ring. A Perspex plate (I of Fig. 16) is inserted beneath the locking ring before it is finally tightened and this serves to prevent the package floating to the surface of the test liquid held in a plastic tank (G). The connector, with package and Perspex sheet attached, is connected to the air line (Fig. 16) and clamped vertically so that the package is held at a depth of about 1 cm below the surface of the test liquid. The two-way tap (J) is turned to connect the air pressure line with the connector (H) and the same pressure regulating and measuring system is used as described previously. The air pressure in the package is increased steadily using the regulator (C) until the first stream of air bubbles appears from the package. The pressure is noted and the size of the hole calculated from the same equation used for measuring maximum pore size in paper. If a package is made in part from uncoated paper, then a test liquid that does not cause swelling of paper fibres must be used. This test method is very useful because not only can the location of the hole, in package material or seals, be seen, but the actual size of the hole can be measured.
Another gas technique for detecting holes in packages utilizes ammonia as a test medium. A piece of cotton wool or tissue impregnated with ammonia is inserted into a package which is pre-made and finally sealed or as it is being made on a packaging machine; care must be taken to ensure that the ammonia-impregnated tissue does not contaminate the seals when they are formed. The package is then placed in a desiccator containing hydrochloric acid and a vacuum drawn; the formation of ammonium chloride fumes indicates faults in the package.

A very common test method used is to subject a package to a vacuum of 20 inHg in a desiccator. The vacuum is held for a set time, usually a minute, and the behaviour of the package is observed. A perfect package inflates, remains turgid as long as the vacuum is held, and returns to a normal appearance when the vacuum is released. Packages with gross faults will not inflate and those with minor leaks will possibly inflate but will appear shrunken when the packages are returned to atmospheric pressure. Only packages made of air-impermeable materials can be satisfactorily tested by this technique. The volume of air in the package, the rate that the vacuum is drawn, and the time that it is held are variables which can affect the result of the test but these can be controlled and the technique can be used as a regular test method for the production of a standard product.

Coloured liquids are also used and one technique is to inject the liquid into the package, tape over the injection hole and then shake the liquid, which normally has good wetting properties, around the package; faults are detected visually where the liquid has 'searched out' holes. A slight variation on this technique is to cut the package in half and then partially fill both halves of the package with the test liquid, which again indicates faults. Frohnsdorff (1968) has described a non-destructive test using a dilute fluorescein solution for examining rigid needle containers. The plastic needle cases are immersed in the fluorescein solution in a vacuum chamber and a vacuum of 10 inHg drawn and held for 5 minutes. The vacuum is released and the containers are held for a further 5 minutes before they are washed and lightly centrifuged. The containers are placed on a conveyor, passed through a darkened chamber illuminated with ultra-violet light and faulty units are detected by bright fluorescence and rejected. Solid particles, such as carbon black and anthracene, have also been used as a test media. The method normally used is to place a package in a desiccator containing a layer of the test media, a vacuum is drawn and then suddenly released. The package is removed from the desiccator, the outer surface of the package carefully cleaned and then opened for examination. Any carbon black particles that have entered the package can be seen visually and anthracene particles are observed under ultra-violet light.

The methods that have been described for testing the integrity of a package may indicate failures in the seals of the package. Frequently these weaknesses are seen to occur at the same location of a seal area and they may be caused by a fault in the sealing mechanism. It is useful in these instances to make thin sections of the faulty area of the seal and examine them under a microscope; the sections may be stained to aid examination. For example, a thin section of a package made from paper/foil/polyethylene has been taken at right angles to the direction of the seal (A-A, Fig.19a). In this instance the seal has been well made so that the interface of the polyethylene surfaces are completely fused (Fig.19b). To produce a seal of this type, the materials must be brought together in the heat sealing
plates under the correct conditions of temperature, pressure and dwell, and, if the plates are of the crimping type as in Fig. 19a, then the crimping jaws must be perfectly aligned. This is one of the most important factors to examine when a packaging machine is set up to ensure good heat seals; if the jaws are out of line then no adjustment of temperature, pressure or dwell will produce efficient seals. Figure 19c shows a section through a seal made with jaws that are not aligned. It will be seen that the material has been so badly sheered that it is almost fractured and holes have been produced between the polyethylene interfaces. Although penetration of bacteria is not possible through these holes because of the direction in which the crimp seal has been made (see Fig. 19a), some seals are made with the crimp seals at right angles to the package and in this case great care must be taken with jaw alignment to prevent holes.

It is desirable that the two faces of material to be sealed should be presented completely flat to the heat sealing plates. If this is not done, creases may be produced. Figure 19d shows a section taken in the direction X-X through the crease which is seen at point C in Fig. 19a. A large hole is visible through the heat seal and it may be possible for bacteria to enter the package by this means. It may not always be possible to avoid creases completely and it is then desirable to use a heat-sealing medium that will flow out and fill in minor irregularities (e.g. Polythene and ionomers). If pre-forming is not used, it is an advantage to use material with good flexibility for packaging bulk items since, to avoid an excessively large package, sufficient material must be available to stretch over the product to allow seals to be made without too much creasing.

The ultimate test of a sterile package is to subject it intentionally to bacteriological contamination under stress and determine if the contents have been contaminated. Two methods that have been used to carry out such a test have been described (Darmady et al., 1968). The first method involves subjecting a test package contained in a respirator to alternating positive and negative pressures to simulate the changes in temperature and atmospheric pressure that a package might experience during storage. A pump, connected to the respirator, produces an alternating pressure at a rate of 20 cycles per minute and the test package is subjected to this procedure for two hours in a suspension of an organism, Serratia marcescens, that has been injected into the respirator. After this cycle the contents are tested for the ingress of the organism. The second method simulates rough handling that packages might experience during transport by subjecting packages in rotating drums that contain the same microorganism; this organism is used because it is easily recognized. The drums, constructed with three right-angled plates inside, are rotated at two cycles a minute for two hours. After this process the packages are carefully removed from the drum and the outside surfaces of the packages are swabbed with a solution of 0.5% chlorhexidine in 70% alcohol and left for thirty minutes. The contents of the packages are then removed aseptically and incubated in a suitable culture media.

This second method of bacteriological testing illustrates an important factor in testing sterile packages since most of the test methods previously described are almost solely used when packages are actually manufactured and not after the packages have been subjected to a transit test. Packages that appear satisfactory at the time of manufacture can often develop faults by the time they reach the user. It is very important, therefore, that not
only should sterile packages be subjected to a transit test when they are produced and then examined for faults, but a manufacturer of prepackaged sterile products should also institute a program of testing package integrity of a proportion of his products that are regularly returned from their normal storage and user outlets.

It can be said that the sterility of a packaged product is dependent on the integrity of the package in which the sterilized item is contained and packaging is, therefore, of the same order of importance as the method that is used to sterilize the product. Unfortunately, there is no such thing as a perfect package into which a range of products can be contained; the packaging requirements for each item must be independently assessed. Many of the problems that are encountered in selecting and testing a sterile package have been considered in this Chapter but it must be recognized that even a perfect package will probably not satisfy the standards that bacteriologists would like to achieve. It is most difficult to remove an item from a sterile package, even under ideal conditions, without some degree of contamination occurring; 3.8% contamination has been reported (Alder and Alder, 1961) and a 1-4% contamination rate is often experienced. Despite this major problem, it is the responsibility of packaging and product manufacturers to ensure that only packaging materials of the highest standard are utilized for packaging sterile products and that the final package integrity is maintained.
CHAPTER 11

HYGIENIC REQUIREMENTS, STERILITY CRITERIA, AND QUALITY AND STERILITY CONTROL

1. INTRODUCTION

Over the past twenty years sterilization by means of ionizing radiation has developed from a promising idea whose future applications could only be loosely estimated to one of the routine methods of sterilizing ready-to-use medical equipment. This new method of sterilization, however, has not yet reached its optimum utilization. The choice of sterilization method for a given product is still often influenced by inadequate knowledge of the full potential of radiation sterilization on the part of the producers, and of the special conditions of the manufacture of medical equipment on the part of the radiation experts. Moreover, the number of irradiation facilities with sufficient capacity for industrial sterilization of medical equipment is still limited. In spite of the rapid increase in the application of radiation sterilization, a very substantial quantity of medical equipment is still being sterilized by other methods such as various toxic gases, although the equipment could provide better bacteriological and toxicological safety for the patients and better storage stability if radiation sterilization were applied instead.

The demands made on the quality of radiation-sterilized products should naturally be the same as those made on products sterilized by other methods. The control methods to be applied are therefore also in general the same as those applied in the control of medical products sterilized by autoclaving or by ethylene oxide, although the special conditions of radiation sterilization necessitate some modification.

The many breakthroughs in the past decade within medicine and surgery have led to an intensification of the demands on the control of medical equipment to be used in therapy.

An increasing number of patients with decreased resistance to infection are being treated in the hospitals and the border line between pathogenic and non-pathogenic microorganisms is becoming more and more difficult to define as we get new clinical evidence indicating that organisms, which hitherto we have felt justified in considering harmless, have been the cause of infectious complications. This has led to an increased interest in the hygienic conditions in hospitals, to demands for better bacteriological control of sterile drugs and to demands for better control of sterilization procedures for medical equipment in general.

It is well known that the conventional sterility testing of the end product yields very little protection from contaminated products. One of the conclusions at a round-table conference on sterility testing in London in 1963 was: "Sterility tests employing reasonable sampling detect only gross contamination" (Council of the Pharmaceutical Society of Great Britain, 1963).
FIG. 21. Inactivation curve for a mixed population of microorganisms. The shape of the curve reflects that different microorganisms with different resistance are inactivated. The individual inactivation process is not significantly influenced by the presence of the other microorganisms.
This conclusion is reflected in more recent international recommendations and national pharmacopoeias, where it is stressed that the entire manufacturing process should be designed with due regard to the quality of the end product when producing sterile medical products. The emphasis in the microbiological control of medical products is no longer placed on sterility testing of the end product, but rather on continuous control of the entire manufacturing process including the sterilization procedure.

2. STERILITY CRITERIA

2.1. General principles

The purpose of a sterilization procedure is to inactivate all microorganisms on or in the material exposed to the procedure. In this context the term 'microorganism' includes bacteria, fungi, other single-cell organisms and viruses. The term 'inactivation' must also be defined specifically with regard to sterilization. The presence of microorganisms is demonstrated by their reproductive ability and inactivation in relation to sterilization is therefore synonymous with destruction of this ability. The term 'viable' consequently becomes synonymous with 'able to reproduce'.

Inactivation of pure cultures of microorganisms by means of heat, chemical action or irradiation usually proceeds in such a way that the same fraction of the population is inactivated per unit time or dose. Figure 20 shows that the inactivation curve becomes a straight line when such an interdependence is plotted in a semilogarithmic system. It is therefore not possible to express in quantitative terms the action necessary to ensure 100% inactivation but only to determine the probability of finding viable microorganisms after a given treatment.

Under practical conditions one should of course not expect a contaminating microflora to be made up of a pure culture. Normally a mixture of microorganisms will be present. The inactivation curve for a mixed population will usually appear as a curved line in a semilog system where the sterilizing action is given on the equidistant axis (Fig. 21). The shape of the curve will reflect that various microorganisms with differing resistance are inactivated and that the individual inactivation process is not significantly influenced by the presence of the other microorganisms.

The straight-line inactivation curve for the pure culture (Fig. 20) and the convex curve for the mixed culture (Fig. 21) represent coarse simplifications of the conditions experienced in practice for the various sterilization methods. The inactivation curve for a pure culture is quite often not linear and the natural microbial contamination may vary considerably from one product unit to another, even when all units have been produced under similar conditions. Moreover, large variations in the number of organisms per product unit and in the composition of the contaminating organisms may be experienced from one day to another. Furthermore, it should be noted that the physical conditions during a sterilization procedure cannot be made completely identical in all product locations. In autoclaving or in gas sterilization the microorganisms will be much more protected in some locations than in others, and in radiation sterilization some parts of the material may receive a lower dose than others. The
complete picture of the inactivation of microorganisms on a given piece of equipment exposed to a given sterilization procedure is therefore very complicated. Simplified models may none the less serve to demonstrate some few, but very important rules that are valid for all sterilization methods:

(1) Everything else being equal, it requires a larger amount of the treatment to inactivate a large number of organisms than to inactivate a small number. An attempt should therefore always be made to minimize the contamination of the equipment to be sterilized, and to prevent the possibility for microbiological growth on the equipment.

(2) Whenever an attempt is being made to obtain a high degree of safety against surviving organisms on the sterilized equipment—and this, of course, is the case when sterilizing medical devices—the initial number of the most resistant microorganisms is decisive for the result of the sterilization procedure.

When calculating the radiation absorbed dose to be applied in order to obtain a given margin of safety against surviving organisms on radiation-sterilized medical equipment, the most resistant organism in the given environment should be considered the determining factor. The inactivation of a mixed population of microorganisms on the equipment does not proceed exponentially and radiation-sensitive bacteria, which often occur in large numbers, will dominate the initial part of the inactivation curve. If the calculation of dose is based on this initial part, the efficiency of the sterilization method will be grossly overestimated.

(3) A given dose or a given sterilization time will lead to a given probability of finding surviving microorganisms on the equipment. Under practical conditions, however, some of the important parameters cannot be very accurately determined—even under the best conditions—and they may change from case to case. Determination of the risk for a given unit of the sterilized equipment of containing a viable organism is therefore a fairly inaccurate venture. The most unfavourable conditions that may occur in the given case should be taken into account so that the value calculated will express the minimum efficiency.

It is the responsibility of the regulatory authority to decide the maximum tolerable risk. The Scandinavian requirement (see later) is: less than one viable organism per one million product units.

2.2. Sterility tests as a basis for a sterilization standard

If the criterion for designating a given product with the term sterile is that the product shall be able to pass a conventional sterility test such as that according to, e.g., WHO’s recommendations or to a current pharmacopoeia, one must conclude that the demands on the microbiological efficiency of the sterilization procedure are very modest indeed. If 40 product units are tested under ideal conditions, more than 7% of the units must be contaminated for contamination to be demonstrated with better than 95% probability (Council of the Pharmaceutical Society of Great Britain, 1963). The inadequacy of random sampling of equipment where the microbiological contamination is not evenly distributed among the products is demonstrated by the fact that a batch where, e.g., 2% of the product units are contaminated cannot be distinguished from a batch where only 0.01% of the units are contaminated, unless an excessively
large number of units is tested. The necessity of using several sets of growth conditions — also for equipment that cannot easily be divided — certainly further increases the number of units to be tested in order to obtain an adequate sensitivity of the control. In addition the laboratory testing results may be compromised whenever laboratory contamination during the investigation causes 'false positives'.

With the number of samples that can be drawn from a batch without unrealistically high costs, all that the conventional sterility test may be expected to reveal is that less than approx. 10% of the units in a given batch may be assumed to have been contaminated with bacteria and fungi. This is unsatisfactory when one takes into account that possibly contaminated units could be heavily contaminated without this influencing the laboratory results.

The conventional sterility test is therefore entirely inadequate as a basis for a definition of sterilization if it is maintained that 'sterile' should be interpreted as 'without viable microorganisms'. A sterilization standard must be based on other control methods if the distinction between disinfection and sterilization is to be meaningful.

2.3. Biological indicators as a basis for a sterilization standard

Sterilization by saturated water vapour or by dry heat has been controlled for decades by means of biological indicators. A natural product such as fertilized soil, which contains a large amount of highly heat-resistant endospores of bacteria, has been widely applied in the sterilization control as a 'go-no go' test. Weighted samples have been placed in the product locations considered most difficult to sterilize and the procedure has been considered successful whenever these samples were inactivated by the sterilization procedure. The biological indicator contained a larger amount of microorganisms than the product to be sterilized and as the microorganisms it contained could be assumed to have a higher resistance to the given sterilization method than the microorganisms usually found on the equipment, a very high degree of safety was obtained by this control. The generally accepted recommendations for the time-temperature relations in autoclaving and in dry heat sterilization of medical products have been based on experience with such biological indicators.

Natural products, such as fertilized soil, cannot be recommended today for use in the control of medical products. Standardization of the test pieces is difficult if not impossible. Furthermore, the fertilized soil contains pathogenic or potentially pathogenic bacteria such as, e.g., Clostridia. Moreover, the shape of the inactivation curve (see Fig. 21) is not favourable to accurate measurement as the resistance of the various organisms in the test pieces is quite different. Soil samples are therefore being substituted with preparations of pure cultures of spore-forming strains of high resistance. In the control of autoclaving spores of Bacillus stearothermophilus are usually applied and spores of Bacillus subtilis are applied in the control of dry-heat sterilization.

In the Scandinavian countries hospitals traditionally control their sterilization procedures by means of biological indicators. Whenever autoclaving or dry-heat sterilization is performed and controlled according to the given recommendation, it may be calculated that even under the most unfavourable conditions no more than one surviving organism per one million
units may be expected for the surgical equipment generally used (Christensen et al., 1969b).

Biological standard preparations should also form the basis for the choice of dose in radiation sterilization of medical products (Christensen et al., 1967a; 1967b) and biological monitors should be applied in the control of the microbiological efficiency of the irradiation plants (IAEA, 1967b). The inactivation curves for microbiological standard preparations should be used as reference in the minimum requirements recommended by the regulatory authority (Christensen et al., 1967b). If desired, biological indicators may be applied as go-no go tests in routine process control (see later) (Christensen, 1970b).

2.4. Sterilization standard based upon evaluation of the risk for surviving microorganisms

The very rapid increase in the application of disposable sterile medical devices in hospitals has given rise to a strong wish in the Scandinavian countries for medical equipment to be controlled by the health authorities in a similar way to that of the production and marketing of pharmaceutical products in operation for many years. All the experts appear to agree that conventional sterility tests are useless as a criterion for sterility and it has also been agreed that a recommendation for sterilized equipment should be valid for all sterilization methods. The recommendation that is expected to be used by the health authorities in the Scandinavian countries and that will be included in the next addition of Pharmacopoeia Nordica is phrased as follows: "Sterile medical equipment is to be manufactured and sterilized under conditions by which it is attempted that less than one viable organism may be found per one million product units" (Public Health Service of Denmark, 1967).

Such a standard can be controlled if it is possible to obtain a realistic estimate of the number and the resistance of the microorganisms that can occur on the equipment under the most unfavourable circumstances prior to sterilization. Furthermore, the efficiency of the sterilization procedure should be measured at the locations in the products where the inactivating action can be assumed to be least. These measurements should be made by means of one or a few approved test strains in standardized preparations, or with reference to such preparations. Due consideration should be given to the sterilization method and to the type of medical supplies involved when the choice of standardized preparation is done.

As mentioned above, this standard is based on experience in Scandinavian hospitals in the application of autoclaving and dry-heat sterilization and is met in the daily hospital sterilization routine. It must be considered reasonable to demand the same degree of safety, regardless of which sterilization method is applied and whether the sterilization is performed at a hospital or in industry. Taking into account that the demands on hospital hygiene are continuously being increased and that the control of sterile pharmaceutical products is being expanded, it would not be realistic to suggest decreasing demands on the safety of autoclaving or dry-heat sterilization procedures. Such decreased demands would have very little chance of acceptance by the medical world. Accordingly, it is necessary to demand that sterilization by radiation or toxic gases shall
provide the same margin of safety as lege artis autoclaving when these methods are applied to medical products. Radiation sterilization and sterilization by means of ethylene oxide are almost exclusively used under industrial conditions in the Scandinavian countries. These methods may lead to the same degree of safety as autoclaving and dry-heat sterilization if the production procedures of the medical products are designed with due regard to the given sterilization method and to the fulfillment of the recommendations.

The standard expressed above is not a unique Scandinavian idea. It parallels the 'D-12 concept' applied in the food industry whereby an inactivation factor of $10^{12}$ for pathogenic bacteria is required in the sterilization of food commodities (Hannan, 1955; Schmidt, 1963). Nor is the standard new for sterilization experts outside the Scandinavian area. Professor Cook expressed it in 1964 as a desirable objective during the first discussions on a Code of Practice for Radiation Sterilization of Medical Products (Cook, 1965).

3. STERILITY CONTROL MEASURES

3.1. Production hygiene at the premises
E. A. Christensen and O. Ringertz

All medical products required to be sterile at the time of application should be produced from raw materials offering the least possible risk of microbial contamination and the manufacturing process should be designed so that the products are well protected against microbiological contamination during all production stages.

Aseptic manufacturing of products that do not tolerate a sterilization procedure is the extreme situation where all precautions that may serve to protect the product against accidental contamination must be taken into account in order to make sure that the end product deserves the designation sterile.

However, a product manufactured under aseptic conditions will not often satisfy the demands for safety from viable organisms that are made on sterilized products in general. Sterility tests on the end product can only reveal gross contamination and the efficiency of the safety precautions taken during production cannot easily be translated into a quantitative evaluation of the contamination risk. It is therefore generally recognized that a device that has been exposed to sterilization in the final container offers better safety than an aseptically produced device.

That a product may tolerate a sterilization procedure does not imply that the degree of contamination prior to sterilization is without importance. Whenever a certain degree of sterility is to be obtained more extensive sterilizing action is required for a large number of microorganisms than for a small one. In many cases a large amount of microorganisms on the equipment will also imply that more different types of microorganisms of various origin may occur, and this again increases the risk that both pathogenic organisms and organisms extremely resistant to the inactivation procedure applied may occur. Furthermore, the microbiological contamination may lead to risks other than those caused by surviving organisms.
Bacteria and fungi often contain pyrogens and toxins and they always contain antigens. These microbiological products are not destroyed to the same extent as the ability of the microorganisms to reproduce. Finally, a heavy microbiological contamination may serve as an indicator for the possibility of other kinds of contamination. Contamination with toxic compounds or with insoluble particles is hindered by the same hygienic precautions as those that limit the possibility of microbiological contamination. None or only slight microbiological contamination does not, of course, preclude that the equipment could be contaminated in a physical or chemical sense, but if gross microbiological contamination is demonstrated, the possibility of contamination in general must have existed.

It is therefore to be emphasized strongly that even when the product is exposed to an efficient sterilization procedure as the final step of the manufacturing process, this must not lead to negligence in the hygienic conditions during production. An improvement in the sterilization procedure is not acceptable as a substitute for maintaining good hygienic conditions.

One should of course not go to the other extreme and demand that all medical equipment should be produced under aseptic conditions. Such a demand would increase the product costs very considerably without significantly improving the safety. The demands on the hygienic conditions in the individual case will depend on many factors. Other requirements are made of the facilities and staff when the product is complicated and has to be assembled manually than when it is simple and may be manufactured and packed automatically. Experience shows that some products may be produced with very little microbiological contamination without unreasonable extra cost, but there are other examples where a satisfactory solution to the contamination problem has not yet been achieved. It is often a matter of judgement whether or not the hygienic conditions are acceptable in the individual case. This decision must be left to a regulatory authority.

Minimum requirements can hardly be presumed to be identical in different countries. However desirable an agreement on quality standards for medical products seems to be for the international trade in these products, it must be acknowledged that the most important task of any national health authority is to improve the health conditions in their own country as much as possible. Economic possibilities are never unlimited and it is therefore necessary to establish priorities for the problem to be solved and there may be large variation from country to country as to how much the individual country finds reasonable to invest in the control of medical products.

It is, however, possible to give a number of general recommendations concerning manufacturing conditions. Such general recommendations particularly aim at limiting three important sources of contamination:

1. The medical products should be kept physically separated from the unpredictable sources of contamination present in the 'outer environment'. Dust and dirt from industrial premises, agricultural products, city trash etc. certainly constitute a number of known risks in connection with medical products. In addition, there are probably a number of unknown risks.

The medical products should therefore be physically separated from the outer environment and from all other production processes during the entire manufacturing process inclusive of packaging and sterilization. The cleanliness of raw materials should be controlled and it should be
ensured that contamination is not being channelled into clean areas by raw materials, packaging materials or other supplies necessary for the production process.

(2) Man releases steadily a large amount of particles to his environment and these particles often carry viable microorganisms. The particles are released from his clothes, from his skin and from mucous membranes. On contact the skin may release large amounts of microorganisms.

The products should therefore be protected to a reasonable degree from contamination from the personnel and the personnel should be given adequate opportunity for good personal hygiene.

(3) Some of the individual procedures in a production process may release large amounts of dust or in other ways lead to a particular risk of physical, chemical or biological contamination.

Such single procedures should be limited in space and time whenever possible. The contamination to be found on the end products depends not only upon the intensity of exposure but certainly also upon the length of exposure.

The space allocated for the production process should be adjusted to the size and type of the production so that the work may proceed under conditions of good hygiene. Floors, walls and ceilings should have surfaces that are easy to clean. The floors should be impermeable to water and they should join tightly to the walls. The production premises should be maintained in a clean condition and ventilation should be adequate. Access of noxious animals should not be possible and the premises should be kept free of animals and plants in general.

There should be no direct access from the outer environment into the production premises. The production premises should be protected by access corridors containing facilities for changing clothes and maintaining personal hygiene and for the disposal of transport crates, etc.

Only personnel involved in the production may have access to the production premises. While working personnel should wear suitable clothes that remain at the factory after working hours and are cleaned by the factory. Every person involved in the production should have one closet for his private clothes and another for his working clothes.

No person may be involved in the production when suffering from a disease that might directly or indirectly provide infection or contamination of the products.

Process equipment that gives off dust particles or chemically active gases should be supplied with local exhaust systems or the like, so that contamination of the products and risks to the personnel are diminished to a reasonable degree.

The production rooms should be utilized only for the manufacture of medical products and if the same rooms must be employed for the production of different types of medical products, care should be taken that no risk of cross-contamination between products can arise.

Unsterilized, but otherwise finished products, should be kept physically separated from sterilized end products.

3.2. Production hygiene at the radiation facility
E. A. Christensen and O. Ringertz

Whenever the irradiation plant is an integrated part of a production layout that only deals with the manufacture of sterilized medical products,
the hygienic conditions in the irradiation plant will not give rise to problems that differ from those in the other steps of the manufacturing process. Shielding from the outer environment and protection against contamination from personnel, noxious animals, etc. and the maintenance of cleanliness on the production premises are as necessary in the irradiation plant as in other production locations.

Physical separation of unsterilized and sterilized equipment should also be maintained in the irradiation plant and the application of a radiation-sensitive chemical indicator to each individual box passing through the irradiation facility is recommended to ensure that irradiated and unirradiated equipment are easily identified.

In situations where the equipment is irradiated in several steps, i.e. whenever one may at some stage have equipment that has been irradiated, but has not yet received the total minimum dose required, it must be kept physically separated from both non-irradiated and fully sterilized equipment.

In radiation plants that perform service irradiations for various producers the hygienic problems are more complicated. Such plants often irradiate products ranging from onions, potatoes, meat products and fish products to plastic materials for the electrical industry and medical equipment.

If the irradiation cell and the working and storage rooms are not originally designed to meet the requirement of the physical separation of the medical equipment from all other products, the only efficient solution then requires that the medical equipment is brought to the plant in a transport container and that the products are processed in the transport container without this container being opened at any time while it is at the plant. In this case the irradiation facility may be considered as part of the outer environment in the same sense as the 'vehicle' of transportation from producer to consumer. The producer must solve the problem of efficient protection against contamination during transport anyway and due consideration to the optimum dimensions of the transport crate with respect to the demands of the radiation plant need not increase the costs.

The crate referred to above is the transport crate proper, i.e. the physical protection against damage and contamination during transportation from the clean areas on the production premises to the clean areas on the consumers premises. The path from the production premises to the consumer, however, often goes via a wholesaler or a distribution centre where the goods are divided up into smaller packages for use at the individual hospital departments or by the individual medical persons. This division into smaller units necessitates a safe smaller-unit wrapping, e.g. for internal transport at the hospital. It would be irresponsible to use the same outer packaging for internal transport at the hospital as that used during treatment in the multipurpose irradiation plant. Even careful cleaning of the irradiation facility between treatment of various types of products would not solve this problem.

It should be emphasized though that if separate rooms and separate irradiation channels are applied for the medical products, the hygienic problems of the multipurpose irradiation plant will be limited to those described in the previous section concerning irradiation plants integrated into the production line.
3.3. Total counts on medical products prior to sterilization
E. A. Christensen and N. W. Holm

Introduction

The number of microorganisms on medical supplies prior to sterilization is important for the evaluation of the microbicidal effect necessary for the end product to meet a given standard (Christensen et al., 1967a; Cook and Berry, 1967; Tattersall, 1964). Besides, knowledge of the initial contamination allows an evaluation of the hygienic conditions during production (Christensen et al., 1967b; 1968; 1969a) and this information may be of value to the controlling health authorities.

The need for information on the number and types of microorganisms present on disposable equipment prior to radiation sterilization was strongly accentuated when in January 1967 the International Atomic Energy Agency published a Recommended Code of Practice for Radiation Sterilization of Medical Products (IAEA, 1967b). According to these recommendations, control of the number and types of contaminating microorganisms on equipment prior to radiation is a condition for lege artis radiation sterilization.

General conditions for total counts on a mixed population of microorganisms

It is well known that no procedure and no set of growth conditions can ensure that all viable units in a contaminating flora of microorganisms will grow into visible colonies.

However sophisticated the method of investigation, the probability will always remain that some of the microorganisms initially capable of growing will be inactivated by the procedures applied, while other organisms may not grow under the given conditions and therefore escape discovery.

Advanced microbiological techniques may enable a large variety of the microorganisms present on the equipment investigated to be detected, but with such techniques one must either content oneself with information on the microbial contamination of a few samples, or make an excessive number of investigations to obtain data from a reasonable number of samples. On the other hand, by applying a simpler experimental technique for the investigation of a large number of samples, one may get erroneous results if some of the microorganisms of importance in the sterilization process have been given no possibility of growth. It is necessary to find a practical compromise that gives the information desired without excessive costs. Similar difficulties are well known in connection with the traditional tests for microbial contamination of pharmaceutical products and sterilized medical products.

While total counts are thus not strictly total, they may be of considerable practical value in connection with the industrial sterilization of medical products. The counts may be used as a measure of the hygienic conditions during manufacturing and thereby facilitate comparisons between equipment of different origin and make them more objective; further, the count may be used in the evaluation of the irradiation dose necessary for the sterilization of a given product. Of course in both cases it is a condition for the application of this count that it has been determined by a
standardized technique that permits growth of a reasonable fraction of
the microorganisms of practical interest in the given case, and gives
results that are reproducible in the hands of various investigators.

It is often seen that the individual parts of disposable equipment are
sterile when they come from the casting process, as the latter has taken
place at temperatures that efficiently sterilize the parts. Whenever
equipment is produced under good hygienic conditions, the degree of
contamination from assembly and packaging is low (Christensen et al.,
1967a; Cook and Berry, 1967; Tattersall, 1965). Not infrequently, how-
ever, a piece of equipment is composed of several individual parts and if
just one of these is contaminated with a large number of microorganisms,
this part will of course be decisive for the judgement of the microbiological
quality of the assembled product.

The microbial contamination to be found on the end product immediately
before the sterilization may have come about in one of the following
three ways:

1. Through raw materials or parts that enter the production line in an
   unsterile condition
2. As dust or droplet contamination at the production premises
3. By contact: workers' hands, dirt from the machines, etc.

This broad classification can often be of value when the cause of high
counts on unsterilized equipment is to be located. The microflora extracted
from raw materials or unfinished parts may often be easy to recognize
in samples from the unsterilized end product and similarly dust and handling
contamination often gives characteristic patterns on the agar plates, e.g.
in cases where the contamination on the equipment has been reasonably
constant for some time but suddenly changes in an undesirable way.

A suitable method for counting microorganisms on disposable equip-
ment prior to sterilization should provide a good likelihood of the dis-
covery of small numbers of staphylococci, aerobic and anaerobic spore-
forming rods, micrococci, coryneform rods, streptococci and fungi. Of
these groups the first three are numerically by far the most significant
and will therefore give the most sensitive measure of the hygienic conditions
during production. It should be emphasized, however, that for the end
quality of the product the most resistant microorganisms are of much
greater significance than the most commonly occurring organisms
(Christensen, 1967; 1970a). Under the conditions prevailing for dry
disposable equipment the most resistant microorganisms are to be found
among the micrococci, the streptococci and the coryneform rods
(Christensen, 1964; Christensen and Holm, 1964; Christensen et al., 1967a).
Highly radiation-resistant sporeforming organisms also exist (Christensen,
1970a). It is essential, of course, that a method of determining the initial
count on disposable equipment leads to the discovery of the known types of
radiation-resistant microorganisms whenever these are present in large
numbers. Minor mishaps or small variations in the production process
might result in such contamination and it is of the utmost importance that
such changes in the contaminating flora on the equipment are detected as
soon as possible.

Even under optimum growth conditions some of the most radiation-
resistant microorganisms known today need two to five days to grow into
colonies large enough to be easily recognized. For routine investigations
of the initial count on disposable equipment to be irradiation sterilized
the period of observation for cultures of contaminating organisms should be long enough to permit the presence of such rather slowly growing radiation-resistant organisms to be proved.

The initial count is to be used for the evaluation of the sterilization effect necessary for a given product, and for the evaluation of the hygienic conditions during all steps of the production process. Therefore it is necessary that the random samples for bacteriological assay are taken immediately prior to the sterilization, i.e. at the time when it may be assumed that the equipment will be subject to no further contamination. As the number of viable organisms present on dry disposable equipment may be assumed to decrease in the course of time, the microbiological assay should in principle be carried out shortly after sampling. Both these rules have to be followed so that the optimum information may be obtained from the assays.

It must be assumed that the rate of spontaneous inactivation of the contaminating microorganisms will vary over a broad range. It is well known that resistance to drying may vary greatly from one type of microorganism to another. Several pathogenic organisms do not withstand drying at room temperature in atmospheric air, whereas some bacterial spores may remain viable for years under the same conditions. The 'lifespan' of microorganisms may change significantly with temperature, water-vapour tension, exposure to light and the presence of damaging or protecting compounds.

Continuous control of the initial contamination on medical products can supplement, but never replace, the inspection that should be carried out by an independent microbiologist. In the control of sterilized disposable medical products it is important to know whether the initial count on the product has been excessively high and/or greatly varying. If a high count has been determined, it is relatively unimportant whether the hygienic conditions, the raw materials, etc. seem to have been adequate; the presence of microorganisms, as demonstrated by the initial count, directly proves that the production hygiene has been insufficient. It is, however, equally unimportant whether one knows that the initial count is low if it has been ascertained that loopholes exist in the hygiene measures taken at the production premises. The hygienic conditions may be completely unacceptable from the microbiological and toxicological points of view without this manifesting itself as high initial counts. Finally it should not be taken for granted that unaesthetic production conditions may be tolerated for equipment to be used in patient therapy only because a definite risk cannot be established. Too often it has been experienced that a risk was indeed present, although it had not been recognized for a long time. Inspection of the hygienic conditions by an expert should therefore form an integral part in the overall control of medical products.

Methods of determining the initial count

One cannot devise a standard technique for determining microbial contamination that is valid for all kinds of medical products. Even when the objective is limited to proving the presence of viable organisms, as in sterility testing where the number of viable units is not determined, the individual technique must be tailored to whether the object is liquid or solid, soluble in water or other liquids that do not damage the microorganisms,
or whether it is insoluble in all such media. Furthermore, modifications are necessary whenever the object possesses bactericidal qualities or if it has unusual physical or chemical characteristics.

When the task goes beyond demonstrating the existence of microorganisms on or in medical products and includes a count of the number of viable units and a coarse classification of the organisms isolated, it is even more necessary to develop individual techniques.

A complete review of the officially accepted bacteriological testing methods will not be given in the present manual. Reference must be made to the national pharmacopoeias (United States Pharmacopeia, 1970; Pharmacopeia Nordica, 1963; WHO, 1960). Further reference is made to NASA's recommendations (National Aeronautics and Space Administration, 1966) and Risø Reports No. 122 (Christensen et al., 1968) and No. 194 (Christensen et al., 1969a).

When determining the initial count on medical products according to the IAEA's Recommended Code of Practice for Radiation Sterilization of Medical Products (section 3.2, note 1), the determination should include the contamination on the equipment as well as the contamination on the inside of the packaging. For most types of disposable medical equipment the first step of such a determination is a careful flushing of all surfaces with physiological saline water or with peptone water to which 0.1% Tween 80 has been added. Such flushing procedures often require relatively large volumes of liquid. As the number of viable units per unit product is often low, a concentration of the suspended microorganisms is recommended. One may either perform such a concentration process by membrane filtration, in which the organisms will be collected on the filter, or by a centrifugation process.

The object of routine control of the contamination on medical products immediately prior to sterilization is to ensure that the maximum number of viable units acceptable per product unit is not exceeded, the limiting value being specified by the relevant regulatory authority. Because the number of organisms per product unit varies substantially from unit to unit, even under very good production conditions, it is preferable that the limiting value be based on the average count rather than on the individual counts for the units investigated. When the production can be divided into batches in a relevant way, one should use the average count per batch. For continuous production the IAEA's Recommended Code of Practice (section 3.2, note 1) suggests that the average initial count shall be calculated on the individual counts from the date of production and from the nine preceding days of production. For this reason the routine control may be based on counts each comprising several units of the given product.

If, for example, the regulatory authorities request that the average count for hypodermic syringes prior to sterilization should not exceed 50, this implies that 20 syringes from the same product line should not give off more than 1000 viable units. The flushing liquid from 20 syringes may in this case be divided into three portions, each being membrane-filtered individually, and the three filters may, after being placed subsequently on a suitable agar plate (e.g. blood agar or Tryptone-Glucose-Yeast extract agar), be incubated in atmospheric air at 30-32°C, under anaerobic conditions at 30-32°C and in atmospheric air at 18-20°C respectively. The evaluation of the number of colonies daily for at least 7 days is recommended. In this example the number of colonies per filter should not exceed approx. 300 so as not to exceed the limiting value. In cases where one or more sets of these growth conditions have given rise to growth of organisms that do not grow or only manifest themselves at a lower frequency under the other growth conditions applied, the total number of organisms for all sets of growth conditions should not exceed approx. 300.
The number and type of microorganisms on the equipment to be sterilized are decisive parameters for the end result of the sterilization procedure, regardless of which method of sterilization is applied. So far, however, radiation sterilization is the only process where control of the initial count has been applied in the industrial production of sterilized medical products. It is important in radiation sterilization that the dose applied be as low as possible in order to minimize material damage. The demands on the safety from surviving organisms on the sterilized equipment must, however, be the same as those traditionally made on medical products sterilized by autoclaving. Consequently, it is necessary in radiation sterilization to utilize the possibility of prescribing the minimum dose on the basis of the initial count.

The microbiological efficiency is well defined in radiation sterilization whenever the dose is well defined, and a well-defined dose is obtainable within reasonable limits in a routine procedure. The efficiency of the procedure may be calculated for the severest case on the basis of the minimum dose if we have a reasonable knowledge of the initial contamination. The maximum dose determines the maximum material damage caused by the procedure.

4. BIOLOGICAL EFFICIENCY MONITORS

Standardized preparations made up by highly radiation-resistant microorganisms may be applied for several purposes in relation to the microbiological control of radiation sterilization. In the sterilization of medical products such standard preparations may be used for controlling the microbiological efficiency of the process, as reference standards and as biological dosimeters and biological indicators.

4.1. Control of the microbiological efficiency

According to the IAEA's Recommended Code of Practice for Radiation Sterilization of Medical Products (section 3.1) the efficiency of a facility shall be tested on commissioning and whenever the process is altered by means of microbiological reference standards. These microbiological reference standards should include at least one with bacteria in the vegetative form and one with endospores of bacteria.

The inactivating effect of ionizing radiation on microorganisms is not determined by the absorbed dose alone. The physical characteristics of the radiation, e.g. the dose rate (Christensen and Kallings, 1966), may influence the microbiological effect; the conditions under which the organisms are kept before, during and after the irradiation may also influence the radiation resistance. The resistance of various strains of bacteria may also be influenced by the temperature and the relative humidity during irradiation. A direct comparison of inactivation curves for approved test preparations irradiated in various irradiation plants is therefore important in order to make sure that the microbiological quality of equipment irradiated in any given plant is the same. It may be mentioned in this connection that the health authorities in the Scandinavian countries have applied the electron linear accelerator plant at Risø as reference plant. Cobalt-60 plants for which it has been demonstrated that a given absorbed
dose yields 10–12% higher microbiological efficiency towards the reference preparation of *Streptococcus faecium* strain A₂1 than does the same dose in the electron accelerator plant have been allowed to use doses 10–12% lower than the Risø doses for radiation sterilization of medical equipment.

For testing the microbiological efficiency of various doses in a new plant, standard preparations of *Streptococcus faecium* strain A₂1 (Christensen et al., 1967a), endospores of *Bacillus sphaericus* strain C₁A (Christensen, 1970a), and *Coli phage* T₁ (Kallings, 1967) (Fig. 22) are applied. These standard preparations may be obtained through the International Atomic Energy Agency.

The choice of *Str. faecium* strain A₂1 (Christensen, 1964; 1965; Christensen and Kallings, 1966) is based upon the fact that enterococci are present in great numbers wherever human beings are present. Furthermore, enterococci must be considered as potentially pathogenic for man. Finally, this strain is highly resistant compared to the majority of bacteria. As mentioned above, this strain is also applied as reference by the Scandinavian health authorities in establishing doses for radiation sterilization of medical equipment.

In inexperienced hands standard preparations of *Str. faecium* strain A₂1 may give rise to a number of problems. As mentioned already, radiation resistance is influenced by dose rate, but this can also be changed by humidity (Emborg, 1972) and temperature. Furthermore, quite a pronounced post-irradiation effect (Christensen and Kjems, 1965) may
give rise to misinterpretation. The storage life of the standard preparations with maximum radiation resistance is only 6-8 weeks under the best conditions.

In the IAEA's Recommended Code of Practice for Radiation Sterilization of Medical Products (1967b) (section 3.1, note 1) spores of Bacillus pumilus strain E 601 are given as an example of a microorganism that could be applied as test strain for the microbiological efficiency of a plant. This strain has, however, to the knowledge of this author not yet been applied in practice for such a purpose. Biological indicators based upon spores of B. pumilus strain E 601 have been applied by various companies in their routine control of radiation sterilization of surgical sutures and medical equipment (Artandi, 1965; Ley et al., 1967). However, spores of B. pumilus strain E 601 have, when prepared in the usual way, a $D_{10}$-value of approx. 0.30 Mrad. The strain is therefore useless for testing the microbiological efficiency of doses exceeding approx. 2.4 Mrad where the inactivating factor is $10^8$ and it is not very useful as a 'go-no go' indicator at doses exceeding approx. 2.7 Mrad corresponding to an inactivating factor of $10^9$. The radiation resistance of B. pumilus is lower than the resistance of the most radiation-resistant Clostridii (Schmidt, 1963), which presumably have $D_{10}$-values about 0.37 Mrad, and also lower than the numerous other strains of bacteria (Christensen, 1970a) of high radiation resistance that have been isolated in more recent years. Even if the bacillus strain were used in preparations of a very large number of spores per test piece, this large number would not compensate for the relatively low radiation resistance of the strain. Whenever the number of viable units per test piece is to exceed approx. $10^9$ the preparation problems become very substantial. The radiation resistance of B. pumilus strain E 601 might be increased by various protective modifications (Ley, 1965) so that the strain could be useful as test strain in the relevant dose range from 2.5-6 Mrad but it does not seem worthwhile to work in this direction as several other spore-forming strains of bacteria are known whose radiation resistance is so high that they are directly applicable within the dose range mentioned above.

Radiation-induced mutants of Bacillus sphaericus (Christensen, 1970a) are well suited as test strains for testing the microbiological efficiency. Some of these strains have $D_{10}$-values between 0.8 and 1.0 Mrad.

A substrain (local isolation indication C7 A) is used at present. Spores of this strain keep their radiation resistance for a long time and within wide limits the resistance is not influenced by dose rate, humidity or temperature during irradiation. The batch-to-batch variation in resistance has been small and post-irradiation effects have not been experienced.

To ensure that the irradiation treatment of a given facility does not possess unexpected characteristics towards microorganisms without these characteristics being demonstrated by the control, the two bacterial strains Str. faecium strain A21 and B. sphaericus strain C7 A have been supplemented with a Coli phage T1 (Kallings, 1967). This means that three different test organisms are now applied to control the microbiological efficiency.

The commercial irradiation plants in the Scandinavian region together with a large number of other irradiation plants all over the world have through the Agency and in collaboration with Risø, performed a series of
tests of microbiological efficiency as recommended by IAEA. These comparisons of inactivation curves for standard preparations of microorganisms have to a high degree (in combination with a solution of such dosimetry problems that have been identified through the investigations) helped to create the same quality of radiation sterilized medical equipment in the plants participating. With a few exceptions, fairly large discrepancies between the inactivation curves of the various plants have been experienced during the first steps of the investigations, but in all cases it has been possible to obtain good reproducibility and reasonable agreement in a short time (Emborg et al., 1972).

4.2. Reference standards

Standardized preparations of highly radiation-resistant microorganisms are applied as reference standards in selecting the minimum requirements for the microbiological efficiency of the radiation sterilization of medical products. Based on a reasonable knowledge of the initial contamination quantitatively and qualitatively, the minimum requirements for a given product or for a group of products may be expressed either as a minimum dose in megabars or as a minimum inactivation factor for a given standard preparation of microorganisms.

According to the IAEA's Recommended Code of Practice for Radiation Sterilization of Medical Products (section 3.2, note 1) due regard to both the average initial count and the maximum count per unit must be taken when selecting the dose for a given product. It is further prescribed (section 3.2, note 2) that whenever the type and sensitivity of the most resistant organisms contained in the products are not known, reference should be made to the inactivation factors obtained for the most resistant organism under environmental conditions maximizing its resistance, such as is the case for the biological reference standard. This procedure is analogous to the procedures involved in selecting time and temperature relations for heat sterilization.

In the radiation sterilization of medical equipment information regarding the type and sensitivity of the most resistant organisms contained in the product will in most cases be insufficient as basis for the choice of dose, even when the initial count of the product is well controlled. The reason is that the variety in radiation resistance among the various organisms occurring as incidental contaminations on most types of medical equipment is very large and that the composition of random contamination changes from one unit to another and from one time to another. The sporadic occurrence of a moderate number of organisms of a highly resistant strain must in most cases be considered possible, even when the hygienic conditions during production are good. Sporadic occurrence of an organism that is not only highly resistant but also pathogenic will of course represent a certain risk.

The health authorities should consequently define minimum requirements for sterilization from the inactivation of the pathogenic strain of bacteria with the highest known resistance towards the given sterilization method more often than they do today. The health authorities could request, for example, that the given sterilization procedure should yield an inactivation factor equal to or larger than $10^6$, $10^8$ or $10^{12}$ for an approved preparation of a particular test strain. This principle is well
known from the choice of dose in sterilization of food commodities (Schmidt, 1963), where the D-12 concept is based on a requirement for an inactivation factor of $10^{12}$ for the most resistant strains of *Clostridium botulinum*.

The microbiological conditions in radiation sterilization of medical products may, however, not be directly comparable with the conditions in the radiation sterilization of food. For most medical products being radiation sterilized today there is no possibility for microbiological growth prior to or subsequent to the irradiation. The contaminants are passive; their number may even decrease with time if the packaging is tight. In addition, the number of viable units per unit medical product is usually very low compared to the numbers in foodstuffs. Furthermore it is a basic condition today for lege artis radiation sterilization of medical products (according to the IAEA's Recommended Code of Practice for Radiation Sterilization of Medical Products, section 3) that the initial count is controlled.

It therefore seems reasonable to apply an inactivation factor lower than $10^{12}$, but this of course presumes that the reference preparation has a resistance that is representative for the worst possible contamination on the equipment.

### 4.3. Biological dosimeters

It may be useful for the controlling authority to be able to make an occasional check on the doses during routine irradiations. Such a control may be performed, for example, with Perspex dosimeters but they may also be performed by using test pieces with spores of *Bacillus sphaericus* strain CjA.

The correlation between the absorbed dose and the number of viable spores in the irradiated test piece is sufficiently good for such a practical control and test pieces may be produced in small dimensions so that the dose may be controlled at practically any location in most medical equipment.

Test pieces with approximately $10^8$ spores of *B. sphaericus* strain CjA per test piece may be applied for the control of doses up to approximately 6.0 Mrad.

### 4.4. Biological indicators

In most cases there will be no need to apply biological indicators in the daily routine control of radiation sterilization. Physico-chemical dosimetry combined with the daily control of the initial contamination should provide adequate information for continuous control. The biological standard preparations are used only after major repairs or modifications to the plant.

However, if a company, an irradiation plant or the appropriate health authority wants a continuous microbiological control, biological indicators for any given dose levels up to about 6 Mrad may be produced on the basis of mutants of *Bacillus cereus*. Ordinary strains of this spore-forming species have only moderate radiation resistance but the spores of selected mutants may be very resistant. Inactivation curves for such spores in the dry condition are shown in Fig. 23. The curves are
FIG. 23. Inactivation curves for several radiation-induced mutants of a *Bacillus cereus* strain (C 1/1) and an inactivation curve for a radiation-induced mutant of a *B. sphaericus* strain (C 1B).

bent like those of *Str. faecium*. The storage life of test pieces of *Bacillus cereus* is reasonably long. Therefore, the strains are suitable for a biological indicator to be used in a 'go-no go' test (Christensen, 1970b).

Biological indicators for control of radiation sterilization based on spores of *B. subtilis var. niger* (*B. globigii*, NTCC 9372) or *B. pumilus* strain E 601 may be used for doses up to about 2.5 Mrad, provided the initial number of spores per test piece is about $10^8$ and that the resistance of the spores is optimal.

*Str. faecium* strain A 2 1 should not be used as biological indicators in the daily routine control of sterilization procedures for medical equipment. The strain is potentially pathogenic and furthermore the *Enterococci* are not spore-forming and the storage lifetime for test pieces of this kind is rather short.

5. THE CHOICE OF DOSE FOR RADIATION STERILIZATION OF DISPOSABLE MEDICAL EQUIPMENT

The doses used for radiation sterilization of medical products in different countries vary not only from one type of product to another, which is understandable because of the variation in contamination prior to sterilization in different products, but also where the products are of the same sort (e.g. syringes) and the available data demonstrates that the microbial contamination prior to sterilization is very nearly the same in number of viable units per item and in types of microorganisms.

The reason for the differences in the choice of doses for medical equipment is that the criteria for sterility vary from one country to another.
and not infrequently even from one company or irradiation plant to another within the same country.

The lowest absorbed doses that have been used for medical equipment have often been based on conventional sterility testing on irradiated samples only. An evaluation of the doses needed for sterilization based upon a combination of conventional sterility testing and examination of the radiation resistance of various microorganisms is commonly used, according to the prescriptions given for control of sterilization and sterilized products in the various pharmacopoeias. However, the data obtained from the conventional examination of irradiated samples of a given product can only demonstrate gross contamination and therefore a choice of dose based on an evaluation of the microbial contamination prior to irradiation combined with an evaluation of the radiation resistance of the most resistant organisms in the relevant environment can be recommended.

The industrial use of radiation sterilization of medical products was introduced by Ethicon in the United States of America in 1956. Based on data on the radiation sensitivity of a wide variety of microorganisms, it was concluded that at a level of contamination less than $10^3$ per item doses of irradiation considerably less than 2.5 Mrad would effectively sterilize (United States Pharmacopeia, 1970). To allow a safety factor, an average dose of 2.5 Mrad was recommended.

If the basis for the choice of dose for radiation sterilization is sterility testing on the irradiated products combined with an evaluation on the radiation resistance of the majority of the microorganisms in biological contamination, all available data demonstrate that an average dose of 2.5 Mrad is sufficient in most cases. However, against a background of the level of hygiene in a modern hospital conventional sterility tests are entirely inadequate as a basis of the control of sterilized products. Such a test employing reasonable sampling detects only gross contamination (Council of the Pharmaceutical Society of Great Britain, 1963).

It is recommended that the microbiological efficiency of a given new sterilization procedure shall be compared to the efficiency of a first-class autoclaving procedure by means of the effects on suitable standardized preparations of highly resistant microorganisms (Christensen et al., 1967a). On this basis an average dose of 2.5 Mrad is insufficient as the sterilizing dose for medical equipment in the dry state.

(1) Other sterilization methods are evaluated in terms of the efficiency at the location in the equipment where this efficiency is lowest. Consequently the same should be the case with radiation sterilization. The minimum dose and not the average dose is a measure of the lowest inactivation effect in the equipment (Christensen et al., 1967b).

(2) The majority of bacteria and fungi isolated from unsterilized medical equipment and from dust and dirt have a radiation resistance low enough to justify the application of a dose of 2.5 Mrad if they are the most significant factor for the end result of the sterilization procedure. However, if a large inactivation factor is desired, i.e. when only a small risk of surviving organisms can be tolerated after a sterilization procedure, the 99% of the total number of microorganisms with low resistance are without significance (Christensen, 1970a). The inactivation curve for a mixed population of microorganisms from airborne dust from a factory will proceed steeply at the beginning and less steeply later on (Fig. 21). In other words, the first logsteps of the inactivation factor are inexpensive, in terms
of dose, in comparison with the later steps and the number and type of the most resistant organisms will determine the sterilizing influence needed to fulfil a given standard.

Based upon information about the initial contamination on the medical equipment produced for radiation sterilization in Denmark and the inactivation curve for a standard preparation of Streptococcus faecium strain A01 irradiated at Risø as a reference standard, the National Health Service of Denmark recommend a minimum dose of 3.5 Mrad if the average initial count is below 50, and 4.5 Mrad if the average count is more than 50 but below 500. If the count is more than 500 but below 5000, a minimum dose of 5.0 Mrad is recommended. So far recommendations for initial counts higher than 5000 have not been needed (Christensen et al., 1968). These relations between initial counts and doses are only recommended for medical equipment irradiated in the dry state. It is a condition for lege artis radiation sterilization that for any item the initial count shall be as low as good manufacturing conditions can produce.

Compared with the recommendations for autoclaving the doses recommended for radiation sterilization are moderate, if the requirements are expressed as inactivation factors for the test strains in the standardized preparations. Figure 24 compares the inactivation curves for a few strains in saturated steam at 120°C with the inactivation curves for some strains irradiated in the dry state. The doses of 4.5 Mrad and 15 minutes at 120°C are the same length on the axis for the inactivating influence. The reason for the acceptance of the doses of 3.5 and 4.5 Mrad is the quantitative and qualitative control on the biological contamination prior to sterilization and the superior physical control of the minimum efficiency in radiation sterilization.
CHAPTER 12

LEGAL ASPECTS

1. CODE OF PRACTICE

The need for legislation and control procedures for radiation sterilization was first felt in the early 1960s when the first commercial gamma-radiation plants began to come into service. During the previous few years accelerator plants had been used for sterilization; control of these plants was left in the hands of the operating companies who worked within the framework of their own experience of other sterilizing processes and of the existing legislation covering sterile products, especially suture materials.

With the commencement of large-scale industrial sterilization from 1962 onwards it was realised that standards would have to be set both nationally and internationally to ensure that the process was carried out properly and in such a way that the inherent reliability of the process could be maintained and also documented.

The first industrial gamma-radiation plants designed specifically for medical sterilization were set up in the United Kingdom and it was in that country that the first draft for an international code of practice originated (Glasson, 1965; Ley et al., 1967). At the same time Christensen, Holm and co-workers in Denmark, using their experience with the accelerator plant at Risø, had formulated certain ideas on the process (Christensen et al., 1967a), which were later incorporated into the Code of Practice. Accelerator experience and microbiological knowledge from USA and from many other countries were also drawn upon when the International Atomic Energy Agency called together a Panel in 1966 to consider a Code of Practice for Radio-sterilization of Medical Products (Ley et al., 1967; Christensen et al., 1967a). The Panel appointed a working party to write the Code, under the chairmanship of Professor L. O. Kallings of Sweden. The result of their work was presented to the IAEA Symposium on Radiosterilization of Medical Products in Budapest in June 1967 (IAEA, 1967; Kallings, 1967).

It should be emphasized that this is a Recommended Code of Practice and is intended as a guide or reference document to be used in drawing up national legislation. Its use should help to clarify questions arising when radiation-sterilized medical products are transferred from one country to another. It cannot be said that any country has so far incorporated the Code of Practice in its entirety into any form of national legislation. On the other hand, many countries recognize the usefulness of the Code and have adopted substantial parts into their regulations governing sterility control.

Many of the recommendations of the Code of Practice apply equally well to other methods of sterilization and it is desirable that any national legislation should provide for uniform conditions, whether the sterilizing agent is heat, a chemical substance or ionizing radiation.

The content of the Code is dealt with more fully in other sections of this Manual, but briefly its recommendations can be summarized as follows:
(1) Products to be sterilized by radiation should be manufactured under hygienic conditions and under the supervision of qualified persons.

(2) The products should be packed in a manner that will maintain sterility up to the time of use, and should be adequately labelled. Sterile and non-sterile goods should be strictly separated.

(3) The degree of bacterial contamination before sterilizing should be known so that the sterilizing process can be selected to give the required margin of safety.

Note that the above three recommendations can be applied equally to all methods of sterilization. The following points apply particularly to radiation:

(4) The radiation plant should be tested when first brought into use, to determine its microbiological efficiency. In other words it should be 'calibrated', using biological reference standards that have known radiation resistance.

(5) As mentioned above under (3), the radiation dose should be chosen to give the desired inactivation factor and safety margin. This dose should be checked by appropriate dosimetry measurements.

(6) The plant should be so designed that in the event of a mechanical or electrical breakdown the products do not receive an incorrect dose.

(7) The plant should conform to the safety and health requirements of the country of operation, and the energy level of the radiation source should be kept at a level such that no hazard due to induced radioactivity can reach either the user of the product or the patient.

2. NATIONAL LEGISLATION

It is clear from the foregoing that legislation for the correct operation of radiation plants cannot be discussed without reference to allied legislation on other methods of sterilization and on the products themselves.

The introduction of industrial radiation sterilization of single-use products into a country nearly always brings with it a fresh wave of thinking on the subject of sterility legislation. This is not because the process inherently requires any more stringent or complicated regulations for proper control than the older methods; on the contrary, the control system may in some ways be simpler and more direct. However, the advent of a sterilizing process that arrives complete with its own Code of Practice and mathematical concept of safety margin, or probability on non-sterility, tends to promote a re-examination of the methods of control that are specified in existing regulations; often there is a realisation that the older regulations are inadequate to provide the necessary protection for the patient.

It must always be kept in mind that the ultimate object of all sterility legislation is the protection of the patient from avoidable infection, and the administration and enforcement of such legislation should always be strongly oriented toward this objective.

Amongst the countries that are already operating radiation-sterilization plants there are many differences in the regulations covering sterile products and in the means by which these regulations are enforced. It is not proposed here to discuss in detail the regulations of individual countries, but to try to classify the different forms of legislation.
There seem to be three broad classifications of the different types of legislation. A summary of these three types is shown in Table XX.

Legislation in this context is taken to include regulations enforceable under government purchasing contracts and recommendations that are expected to become law within the next few years. Also note that more than one form of regulations may be used in any one country, for instance Type I for catgut and other sutures and Type II for plastic disposable products. Some information on the legislation in various countries was given at the IAEA Panel Meeting in 1966 (see IAEA Technical Reports Series No. 72); this is probably now out of date. No more recent survey of legislation has been compiled.

Type I

The earliest and simplest type of legislation can be summarized in the phrase, "the product shall be sterile". It is probable that this phrase is, in fact, used by all writers of sterility legislation, but some regulations go no further. They merely require the manufacturer or supplier to "prove" that the product is sterile by sterility testing. A single sample or a few samples taken from a batch of products cannot indicate the true microbiological condition of the batch — only a knowledge of its history can tell this. The limited value of sterility testing as a means of control has been pointed out by many authors (Christensen et al., 1969b; Ley and Tallentire, 1965; Tattersall, 1961). A reasonable degree of sampling can detect only gross contamination, or serious failure of the sterilizing process, and can never give the high degree of safety assurance that should be required by modern sterility regulations.

We can therefore regard this simple form of legislation, which says merely that the product shall be sterile, as hardly adequate to ensure a high standard of sterility.

Type II

A more advanced type of legislation may very well demand that the product shall be sterile, but also goes on to give some instructions on how this should be achieved. For instance, the required radiation dose may be stated in megarads or the "dose" of steam may be expressed in terms of temperature and time. In those countries that already have considerable experience of modern sterility control (including radiation) these sterilizing instructions are often accompanied by some degree of control of manufacturing hygiene, including factory inspection and pre-sterilization contamination tests. There is no doubt that under these circumstances a high standard of safety is ensured and, provided the administration and enforcement is adequate, the risk of infection to the patient is very small indeed.

Type III

An even more sophisticated system has been adopted in certain countries, notably in the Scandinavian area. Here the required "safety margin" or probability of non-sterility is defined numerically. This means that the
<table>
<thead>
<tr>
<th></th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Definition of sterility</td>
<td>&quot;Product to be sterile&quot;</td>
<td>Safety margin or inactivation factor specified numerically. Dose chosen according to pre-sterile count.</td>
</tr>
<tr>
<td>3.</td>
<td>Pre-sterilizing bacterial count</td>
<td>-</td>
<td>Required.</td>
</tr>
<tr>
<td>4.</td>
<td>Sterility testing</td>
<td>Used to prove sterility.</td>
<td>Not required.</td>
</tr>
<tr>
<td>5.</td>
<td>Bacterial test pieces</td>
<td>Sometimes required.</td>
<td>Optional, but recommended.</td>
</tr>
</tbody>
</table>
sterilizing dose, whether heat, chemical or radiation, must be chosen to give the required safety margin, taking into account the pre-sterilization bacterial count and assuming that all the bacteria are of a type with high resistance to the particular sterilizing method in use. If, for instance, the required safety margin is $10^6$, that is, a probability of one non-sterile item occurring in one million, and if it is assumed that all the organisms present are equal in radiation sensitivity to the reference strain of Streptococcus faecium A$_2$, then a dose of 3.5 Mrad can be used, provided the number of organisms on the product does not exceed $10^2$. These figures can easily be read from the inactivation curves for the reference organism. Note that small differences can be seen in the indicated doses for gamma and electron irradiation.

A corresponding line of thought is applied to the other methods of sterilizing, both chemical and heat, with prescribed test organisms in each case. All are required to show the same safety margin.

Choice of dose

The Code of Practice does not specify the radiation dose that is to be used, the choice being left to the health authorities of the country concerned. In the case of Type II legislation it is usual to specify the dose. For instance, 2.5 Mrad may be given as the normal dose, with higher doses for certain specific types of product. The Type II regulations do not usually mention any particular requirement for "safety margin", i.e. the difference in log cycles between the inactivation factor and the pre-sterilization count. However, it is possible to conclude that a value of about $10^5$ is acceptable, assuming pre-sterilization counts of less than 100 and St. faecium as the reference organism.

The Type III regulations allow for adjustment of the radiation dose according to the pre-sterilization contamination level and a typical table of values is the following, given by the Danish authorities in an instruction to manufacturers (Farmaceutisk Tidende, 1968).

<table>
<thead>
<tr>
<th>Number of Colonies</th>
<th>Dose (Mrad)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Under 50 per product</td>
<td>3.5</td>
</tr>
<tr>
<td>Between 50 and 500 per product</td>
<td>4.5</td>
</tr>
<tr>
<td>Between 500 and 5000 per product</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The philosophy behind the Type III legislation is clearly stated by Christensen, Kallings and Fystro in an article published simultaneously in the medical journals of Denmark, Sweden and Norway (Christensen et al., 1969b).

3. ENFORCEMENT AND APPLICATION OF REGULATIONS

The word "regulations" in this Chapter should be read in the broadest sense to include not only absolute requirements of the law, but also recommendations that may in due course become legally enforceable but are at the moment more in the nature of a voluntary discipline. In most countries Type I regulations are already written into the legal code and in some others Types II and III are either legally enforceable or are likely to be so in the near future.
The two principal aspects of the application of sterility regulations may be considered under the headings preventive inspection and allocation of responsibility.

**Preventive inspection**

Preventive inspection under Type I regulations can be carried out by any competent microbiological laboratory. Its main function is to detect any serious malfunction of the sterilizing system. As mentioned earlier, it does not give a true picture of the bacteriological state of the batch of products under examination.

For both Type II and Type III regulations a more comprehensive form of inspection is required to ensure that manufacturing hygiene is good, that methods of test of the hygienic conditions are adequate and that the correct dose has been given. Associated with these may be tests for efficacy of packaging and function of the product.

Such a system of inspection must be based on co-operation between the authorities and the manufacturers concerned. To keep the cost of inspection within reasonable limits the authorities must rely to some extent on the integrity of the manufacturers, who would be expected to keep complete and reliable records of all quality control tests, bacteriological tests and dosimetry tests. These records must be available for inspection by the authorities at any time and all premises and equipment must be open for inspection. On their part the inspectors of the health authority must display the greatest discretion, especially when moving from the factory of one manufacturer to that of a potential competitor. If the obligations on both sides are honoured, a very satisfactory working relationship can be established between the inspecting authorities and the manufacturers, leading to progressive development within the industry.

The advice to prospective writers of legislation, given by van Winkle at the IAEA panel meeting in 1966 (Van Winkle, 1967) might well be quoted here, and can equally apply to inspection and enforcement of the regulations:

(a) The legislation must afford proper protection to the consumer, i.e. it must ensure that the irradiated product is safe and efficacious for the purpose intended.

(b) The legislation must not inhibit future research and development in the field that may be of benefit to the consumer or the economy.

**Allocation of responsibility**

The allocation of responsibility and the associated punitive measures for failure vary very much between different countries and are to a great extent tied up to the different forms of legal practice and the common law of the countries concerned. It is not therefore possible to discuss these matters in much detail here, but a few general observations may be of some value to the reader.

In the event of death or injury caused by non-sterility of a medical product, it would in most cases be necessary for the person or persons responsible for the sterilizing process to show that the process had been carried out in accordance with generally accepted scientific principles and in compliance with the laws of the country concerned.
In a legal enquiry into such an incident it would first be necessary to establish that the incident had in fact been caused by the product in question. It is very difficult, or in most cases impossible, to establish with certainty that a particular item was non-sterile before use. Once the pack has been opened the item is potentially non-sterile and after use, even if it is still available for examination, it cannot be checked for sterility. It may be possible, for example by chemical analysis, to prove that it had been subjected at some time to a sterilizing process, but this does not indicate whether or not it was sterile just before use. In a few cases it may be possible to produce evidence that the package was open or damaged before use and so doubt could be thrown on the sterility of the product. However, in most cases the court must examine the evidence on whether the sterilizing process was properly carried out or not.

The person in charge of the sterilizing process must therefore be in a position to provide documentary evidence that the process in general was properly carried out and the batch in question was properly sterilized. This documentation should be in the form of process records, quality control records and dosimetry readings, together with general evidence of good hygienic standards during manufacture. Even where the applicable legislation is of Type I, it would probably still be necessary to produce full records concerning the particular batch and not merely records of sterility tests carried out on a few samples from the batch.

In the event of failure, responsibility may have to be allocated amongst the persons or organizations involved in manufacture, packaging, sterilizing, transport, distribution and use of the product. Morally, more than one may be responsible, but legally it may be necessary to place the responsibility on one particular link in the chain. Which link is chosen may depend on the formulation of national laws.

Even the most efficient and well-run radiation sterilizing plant may at some time find itself in legal difficulties in connection with an incident over which it had no control and the following short discussion of an actual case history may perhaps provide some guidance. Obviously sound legal advice should be taken on all such occasions.

Case history

A young man, in good health, was given a normal prophylactic inoculation against a tropical disease. He died some time after as a result of a general septic condition. A legal enquiry was held to establish the circumstances leading to death.

A scientific representative of the manufacturers of the single-use syringe and needle used for the injection gave evidence of methods of packaging, radiation sterilization and quality control of the product. His evidence included records of the samples taken from the packaging line on the day of manufacture, of the systems used to ensure that sterile and non-sterile goods could not become mixed, and of the radiation dosimetry readings for the period in question. The object of this evidence was to establish that the syringes were manufactured under good conditions and proper control.

A representative of the manufacturers of the vaccine then gave similar evidence regarding the conditions of manufacture and sterilizing of the ampoule of vaccine that was used.
The nurse who opened the syringe packet said that so far as she could see the package was undamaged before opening. The doctor and the nurse gave evidence of handling procedures, skin preparation etc.

A public health bacteriologist reported the presence of pathogenic organisms, similar to those that caused death, on the floor and bench surfaces in the clinic where the injection was given and also in nasal secretions of the doctor and one of the nurses present.

Other evidence was given regarding treatment of the patient after he became ill.

The conclusions reached by the Court of Enquiry were (a) that death was due to "misadventure"; (b) that the bacteriological condition of the clinic was unsatisfactory; and (c) that the single-use syringe and needle and the vaccine were specifically excluded from any responsibility for the infection.

The above illustrates a relatively simple case where the radiation-sterilized syringe might have been held responsible for infection had it not been possible to show evidence of adequate control of packaging, sterilization and other processes.

Note again, that it was not possible, and never is possible, to prove that the syringe in question was sterile.

**Contract sterilization**

The situation where a radiation-sterilization plant processes goods on a contract basis for other manufacturers may bring some special problems of allocation of responsibility. It is not intended here to discuss the direct responsibilities between buyer and seller, which can be regulated by common law and by the normal terms of the contract between the parties. The effect of a sterility failure on a third party may be more serious and the radiation plant may find itself under criticism for faults for which it is not directly responsible.

The two types of fault that are most likely to arise are (a) failure or inadequacy of packaging, and (b) radiation damage to the material of the product.

It could be argued that a radiation plant should never accept goods for sterilization unless it is satisfied that they are packed in such a way that sterility will be maintained. While this is a good general principle and should be applied as far as practicable, it must be remembered that the only way the radiation plant can be sure of the standard of packaging is to penetrate deeply into the customer's quality control system and in fact to station a quality control inspector on the customer's premises—a procedure that can hardly be regarded as practicable. It would seem better, therefore, to check the standard of packaging as far as reasonably feasible, but at the same time to protect the radiation plant, in the legal sense, by a suitable clause in the contract or terms of business.

It is well known that some materials are liable to radiation damage. While a radiation plant can try to steer its customers away from unsuitable materials, it cannot accept responsibility if such materials are used in products. If, for example, the customer is advised to use only a radiation-resistant grade of polypropylene, but nevertheless continues with an ordinary grade, the results can be disastrous and far-reaching. It is difficult for
the radiation plant to protect itself against the undeserved ill-will that results, but it can at least protect itself in the legal sense by a disclaimer clause in the contract, and by always giving advice of this kind in writing — or in published form.

IRRADIATION OF PHARMACEUTICAL PRODUCTS

In all the foregoing it has been assumed that the products being sterilized by radiation are devices or utensils or surgical material, mainly of a single-use character, but also including a small proportion of multiple-use articles. There is, also, the technical possibility of sterilizing some pharmaceutical products by radiation (Radiation Sterilization of Pharmaceutical and Bioproducts, 1969; Galatzeanu et al., 1967; Hangay et al., 1967; Haraszti et al., 1967; Holland et al., 1967; Hortobágyi et al., 1967). Since such materials are liable to undergo chemical change during irradiation they can only be put into use after extensive chemical and clinical trials.

It is usual, therefore, for health authorities to take a very cautious view on such irradiated pharmaceuticals. Legislation in most countries is negative, either in the form of a total ban on irradiated pharmaceuticals, or a requirement that an irradiated drug must be regarded as a completely new drug and submitted to the same scrutiny as if it had been newly discovered.
CHAPTER 13
SAFETY RULES

Massive radiation installations are susceptible to engineering principles that ensure complete safety. When cobalt-60 is used it is doubly encapsulated in stainless steel envelopes, which are helium filled to prevent corrosion. The heat generated by a $^{60}\text{Co}$ source is well defined and cannot increase. The total energy dissipated by 68 000 curies of $^{60}\text{Co}$ is 1 kilowatt and there is no possibility of this power level being exceeded. Careful sealing ensures that there is no contamination hazard.

It is essential that the safety system in force at an irradiation plant should positively preclude accidental irradiation of personnel, even for a fraction of a second. The dose rate in the region of a gamma-radiation source is about 100 000 to 500 000 rads per hour, and near the beam of an electron accelerator the dose rate may be much higher. The radiation dose that produces a 50% probability of death in humans is about 500 rads and the total permissible dose for radiation workers is about 3 rads per thirteen-week period. It is, therefore, clear that no chances of accidental radiation are permissible.

A fundamental feature of safety systems of this kind is that they should fail safe, i.e., if anything goes wrong, such as a failure of electrical supply, the result is to increase safety rather than to decrease it.

The fundamental safety interlock is between the source of radiation and all the entrances to the radiation chamber. Combined with this interlock there must be a routine that requires the cell to be inspected to make sure that nobody is left inside when the last door to the irradiation cell is closed. This inspection routine must not depend on the interest or willingness of the operator to carry it out, but must be such that the plant cannot operate unless the inspection has been made.

The interlock between the door closures and the source itself can be made electrical in the case of an electron accelerator, but the fail-safe principle must be incorporated. In gamma-radiation plants the source frame is sufficiently large for there to be a robust mechanical interlock between its position and the closure of the main entrance door. In the case of gamma-radiation plant in which there is a direct cable connection between the source frame and the hydraulic cylinder that lifts the source, it is possible to locate the cylinder next to the entrance door in such a way that there is a positive interlock between the door and the cylinder. In some other cases the interlock can be achieved by using an electrical transformer that is made up in two halves that can only work if the two halves are close to one another. One half of the transformer can then be mounted on top of the shielding plug and the other at the mouth of the shielding pit so that the transformer can only work when the source is in the safe position. The output of the split transformer can then be used to actuate a solenoid that allows the door to be opened.

To ensure that inspection is carried out, the main entrance to the irradiation cell should have a lightweight personnel gate fitted on the inside and the latch of the gate should contain an electrical switch that can only be made during a limited time after another switch has been operated.
This second switch should be located at the most remote point in the irradiation chamber. The routine then requires that the operator enters the cell, actuates the switch in the most remote place and in walking to and from the switch he cannot fail to inspect the cell. He has a limited time in which to return to the personnel gate and to close it, thereby preventing anyone accidentally entering the cell. If the personnel gate is subsequently opened before the main shielding door is closed or if the operator does not close the door within the short period (e.g., 20 seconds), the whole routine must be repeated. The main switch for an electron accelerator or the control mechanism for raising a gamma-radiation source can then be made dependent on the personnel door switch and the main shielding door being closed.

If the inspection produced by the above routine leaves areas of the irradiation cell unchecked, it is necessary to equip them also with switches at the remote point and the system arranged so that both switches must be operated during inspection.

The labyrinth entrances and exits for the product are almost always impassable because they are full of conveyer equipment and packages, but as a further precaution additional switches can be fitted to the entrances to the two labyrinths and the inspection routine modified to incorporate them. A further safety measure to protect accidental entry via the labyrinths is to fit pressure plates on the floor at the entrances, the plates being wide enough to make it reasonably impossible to step over them. The pressure plates again must be incorporated into the total safety system on a fail-safe basis.

As a separate measure, an alarm wire must run round the inside of the irradiation chamber so that in the almost impossible situation of someone being trapped in the cell, an alarm could be given to the outside, which at the same time switches off the electron accelerator or prevents the radioactive source from being exposed and returns it to a safe position.

1. TRANSPORT AND LOADING OF $^{60}$Co

The form of transport container used for $^{60}$Co depends on the type of source storage in the installation. Where a water pond is used for safe storage it is usual to use several small transport containers so that the doorway into the cell need not be large on this account. The small transport containers are lowered to the bottom of the water storage and the sources removed using long-handled tools. In transferring the radioactive material from the transport container into the source frame it is essential that it should not be brought too close to the surface of the water. This is an obvious precaution, but one which is difficult to legislate.

In a system widely used in the UK and Europe the safe storage of the source is in a dry pit and the cobalt is loaded into the source frame from one of the outside faces of the irradiation chamber (see Fig. 25). In this case the transport container handles up to 100,000 curies.

The dry loading system requires an interlock between the door of the irradiation cell and the plug that prevents radiation escaping from the exit of the transport container. The transport container itself is bolted to the loading face of the irradiation cell and there is only a small and transient
FIG. 25. Method of loading $^{60}$Co from the outside of the shielding cell using a special transport flask, which is bolted to the outside of the cell and thereby actuating safety interlocks. The rotating magazine in the flask brings each $^{60}$Co rod in turn in line with the loading tubes.

rise in radiation level as the source rods are pushed from the transport container down the loading tube towards the source frame.

In addition to all the safety precautions prescribed, it is strongly recommended that whenever a radiation chamber is being opened the operator should hold a hand monitor close to the point from which radiation would escape first, i.e. at the edge of the shielding door. The operator should then observe the hand monitor during the whole of the opening procedure and during the first entrance into the irradiation cell.

At about yearly intervals some check on the integrity of the radiation sources should be made. In the case of water storage careful analysis of the ion exchange column material for radioactivity or the water itself should be assayed. For a dry storage installation the source should be lowered into its pit and the cooling air analysed for airborne radioactive material.

2. LOG-BOOK AND STANDING ORDERS

A log-book should be kept of all occurrences that have any connection with the plant. A special section should be set aside for details of replacement of magnetrons, klystrons or modulator valves in the case of electrical machines and the cobalt loading in the case of a gamma-irradiation plant. Another section should be set aside for all details of breakdown and maintenance.

A set of standing orders for the plant operation should be on view in one or more places near the plant or electrical machine. The orders should cover at least the following subjects:

(1) The identification of the competent person in charge of the plant and how to get in touch with him for any emergency.
(2) Instructions about the wearing of film badges. Even those operators who are not required to enter the irradiation chamber should carry film badges should any question of their having received an allegedly harmful dose ever arise.

(3) Definition of persons who are authorized to enter various parts of the building containing the radiation plant, also those persons who are authorized to carry out maintenance. Reference should always be made to the need for the competent person in charge of the plant to be notified whenever any maintenance is to be carried out.

(4) The conditions under which the product conveyor can be operated during maintenance work should be laid down; it is usual to insist that a separate operator should stand by the emergency stop control during any time during which the conveyor is operated for maintenance purposes.

(5) Any special precautions required during cobalt loadings should be stated.

(6) Instructions covering fire emergencies should be laid down and details of routine maintenance necessary on fire-fighting equipment.

(7) Instructions are desirable to the effect that nothing is to be irradiated other than products agreed by the plant manager.

(8) Instructions are recommended on the type of clothing that the workers on the plant should wear.

(9) Full instructions are vital for the correct procedure in opening the irradiation chamber and closing it.

(a) A portable radiation monitor must be provided and checked on battery test before use.

(b) Only when (a) has been carried out may the opening procedure be started. When the electrical machine has been switched off or the radiation source lowered into a safe position, the door to the cell should opened slightly and the portable radiation monitor held next to the small opening. The monitor must be observed as the door continues to be opened and if a reading of more than 0.75 mrad/h is shown, the door must be immediately closed and the plant manager informed.

(c) Once the cell door has been opened, at least five minutes must be allowed to elapse and then the extraction fan indicator light should indicate that it is operating before the cell is entered.

(d) If the monitor is taken into the cell, it must be held in the hand until the person carrying it leaves the cell.

(e) In closing the irradiation chamber one operator must press all the interlock switches.

(f) All personnel must leave the cell immediately the warning sound is given indicating that the cell is about to be closed.

(g) The operator must enter the irradiation cell and operate the safety switch or switches and in so doing check that no one is inside.

(h) Apart from the operator who is carrying out the search, no other person shall go inside the cell while the warning sound is being given.

(i) When the operator has completed his search he must close the cell gate and leave the cell and only then may the plug door be closed.

(j) In the event of any deviation from this sequence, the plant manager must be informed immediately.
CHAPTER 14

THE ECONOMICS OF RADIOSTERILIZATION

An installation for radio sterilization using either electrons or gamma radiation can only become competitive economically when operated on a large scale because the minimum capital investment is usually in excess of US $250,000. There have been many theoretical comparisons of the economics of electron machines and gamma-radiation plants, which tend to indicate that for a power level of 12 kilowatts (about 800,000 curies of cobalt) the two systems are similar. Below this power rating cobalt appears cheaper and above the turnover point the electron process is cheaper in terms of weight of product treated. However, there are many practical points that invalidate the above conclusion and make the turnover point more than 50% higher and even that is open to debate.

Both types of installation require a fixed investment in civil and mechanical engineering on which interest must be paid and the capital sum amortized. The electrical machine cost includes several components with a limited life such as klystrons in the linear accelerator or the accelerating tube in a Van der Graaff or other d.c. machine. In a cobalt plant only sufficient $^{60}$Co need be purchased to serve the throughput that is envisaged in the immediate future, but more can be added as the throughput increases. In the case of an electron machine the investment cannot be similarly matched to the throughput but can only be increased by setting up a second or third machine, and only at particular times will the power rating of the machine be just right for the throughput required. Some economy can be effected in operating an electron accelerator by matching the hours per day of operation to the throughput and, in this way, savings in labour and in the cost of expendable components can be achieved.

The cost of the skilled labour component in manning an electron machine is four to five times that of manning an accelerator on account of the complexity of the electrical circuits, which require highly trained personnel for operation and maintenance. There is, in fact, a difficulty in securing staff for electrical machine maintenance because the type of person who is suitable is not usually satisfied with routine work and expects to be able to spend some time on research and development.

Perhaps the biggest factor that could influence the comparison of the cost of operating the two types of radiation plant is the reliability of the facilities. The incidence of breakdown and the amount of maintenance is about ten hours higher in the case of electron machines and unexpected gaps in production cannot usually be tolerated when the product is being sold in a competitive market. Gamma-radiation installations are well known for their reliability. The reliability of the radioisotope source is 100% and the only part of the plant at all susceptible to breakdown is the product conveyor, which, on account of the slow movement, poses few problems of unreliability. The cost of a standby electron machine adds more than 60% to the cost of irradiation and in comparison it can be argued that the economic advantage of the gamma process extends over an unlimited range of throughput.
When the throughput is small and never likely to grow to a level needing more than 100,000 curies of $^{60}$Co it is worth examining the economics of a plant processing batch after batch of product. The capital cost of the shield and mechanical parts together is less than half that of an automatic continuously operating plant. The cobalt cost is a little higher because it is less efficiently utilized and time is lost loading, unloading and moving the product half way through the treatment in order to make the minimum to maximum dose ratio tolerable. There is much greater dependence on the responsibility and devotion to duty of the operating staff of a batch plant and therefore higher rates of pay are expected.

The costing of $^{60}$Co is far from simple. The initial capital investment in the source requires amortization and interest, also the decaying radioactivity must be replaced at the rate of 12½% per annum. If the source is maintained at a fixed level of activity, it can be argued that amortization is unnecessary because the asset remains at full strength. The value of a fixed number of curies is, however, not certain, especially as the user would not be in a position to sell a source advantageously should he wish to do so. The selling of a partly used cobalt source is further complicated by the range of specific activity (curies per gram) that develops in the source frame over years of operation. The initial load of cobalt decays to half-value in five and a quarter years and to a quarter of the initial value in a further five and a quarter years. Subsequent loadings will have high specific activity and at any time after about ten years' operation only about half the cobalt will be saleable at a price comparable with the purchase price. It is therefore common practice to amortize the radioactive source value down to 50% of its initial cost in a period of ten years. From a taxation point of view it is often best to regard cobalt as an expendable fuel and to categorize the initial cost in this way.

The source frame in which the $^{60}$Co is mounted should be designed with sufficient scope to achieve the maximum rating of the plant with cobalt distributed in specific activity over the range from the initial value to less than a quarter of the initial value. Less room than this may result in useful cobalt having to be removed from the source frame in order to make space for the fresh replacement. There are some suppliers of $^{60}$Co who are prepared to credit the plant with any cobalt that must be removed when the source frame becomes full, but it is important to make sure that the conditions that are laid down in order to qualify for this credit scheme are fully established on the occasion of the initial purchase.

In working out the running cost of a particular radiosterilization operation the following items and factors must be included:

(a) Amortization and interest on all capital items, i.e. the facility, the building in which it is housed, the product handling and storage equipment, spare parts etc.
(b) Organizational, supervisory and operating staff
(c) Overheads
(d) Replacement of cobalt or electron accelerator parts
(e) Plant maintenance
(f) Power, light and heating
(g) Product packaging materials.
PART III
RADIATION STERILIZATION OF MEDICAL SUPPLIES AND MATERIALS
INTRODUCTION

The widespread industrial use of radiosterilization is the result of pioneering effort by scientists and engineers in governmental agencies, universities and industry throughout the world. However, this work would have been fruitless if radiosterilization did not fill a basic need. Indeed, the rapid development of an entirely new market for disposable single-use medical products would have been impossible without this new technique of sterilization.

Sterilization by dry heat or moist heat (autoclaving) represents the two most reliable and generally accepted techniques of sterilization. Both methods require uniform exposure of the products to high temperatures and, in the case of autoclaving, moisture as well.

Following the second world war the use of ethylene oxide for sterilization gained slow acceptance. While this process can be carried out at much lower temperatures, its control is more difficult. Temperature, moisture levels and gas concentration must be adequately controlled throughout the materials being sterilized. This requirement limits the use of ethylene oxide sterilization to materials and, in fact, packages that are sufficiently permeable to both moisture and the sterilant. An additional problem can result from the absorption and retention of the relatively toxic ethylene oxide or some of its reaction products, e.g. β-chloroethanol or ethylene glycol; prolonged exposure to vacuum or well-ventilated storage may be required to dissipate these compounds and render the products toxicologically safe.

The beginnings of radiosterilization go back to the late 1940s and early 1950s. The availability of various types of electron accelerators stimulated significant exploratory research on the applicability of ionizing radiation to the destruction of some types of microorganisms and to the sterilization of foods, pharmaceuticals and medical products.

The use of electron accelerators and isotope radiation sources for industrial sterilization processes was pioneered by several corporations. As a result of the successful demonstration of safety and reliability of both types of radiation facilities, there has been a substantial increase in the world-wide use of these systems. There are now at least 22 $^{60}$Co source and 5 accelerator plants devoted to industrial sterilization of medical products, while many others are operated experimentally or for food irradiation.

Because of the greater, penetrating power of gamma-emitting radio-isotopes compared to electron radiation from accelerators, the majority of industrial sources employ $^{60}$Co or $^{137}$Cs. High energy electron accelerators are also used for sterilizing medical products, but their major application is in industrial processing.

As with other methods, radiosterilization is not universally applicable. Materials may be adversely affected to an extent that could prohibit its use. However, radiosterilization has many advantages over the more conventional methods of sterilization.

(1) It is the first and only continuous process for sterilization
(2) It is easy to control and extremely reliable
(3) Because of the great penetrability of gamma rays, prepackaged, hermetically sealed products, possibly in their shipping containers, can be conveniently sterilized.

(4) Because radiation does not rely on heat convection or permeation of moisture or gas, there is complete freedom in the choice of packaging materials.

(5) Practically no heat is produced during sterilization and the chemical effect of radiation is low.

Because radiosterilization is more expensive than heat or gas sterilization, it should be used only when the various advantages compensate for the added cost.

One of the greatest benefits of radiosterilization is the facile production of reliably sterilized single-use medical products. Such products have, to a large degree, replaced the need for sterilization of syringes, needles, catheters, tubes and a variety of other equipment in the hospital. Repeated sterilization of these items is not only time-consuming for overburdened hospital personnel, but deterioration resulting from continual cleaning and exposure to heat can make sterilization unreliable and the products less fit for use.

When heat sterilization is not applicable because of material or design limitations chemical disinfection is common in hospitals. Before the relatively recent development of effective and otherwise unobjectionable chemical sterilizing agents (buffered glutaraldehyde) this generally ineffective practice had been a source of large-scale, hospital-acquired, cross-contaminating infections requiring substantial additional medical treatment.
CHAPTER 15

SUTURES

While the first commercially sterilized product was an eye ointment, the honour of establishing radiosterilization on an industrial scale fell to surgical sutures. Since 1956 the majority of sutures in the United States of America have been radiosterilized. This has also been true throughout the world since the early 1960s.

There are many reasons why sutures were well suited to be the first large-volume commercially radiosterilized product. They presented a unique opportunity for innovation in a highly competitive industry that used small numbers of basic materials in large numbers of products. The existing methods of sterilization were less than satisfactory because of their adverse effects on product quality. Moreover, the choice of packaging materials was restricted because of the heat-sterilization process then in common use. Since the manufacture of sutures involves a relatively high labour cost and the product has a high value to volume ratio, the cost of sterilization is not an appreciable factor in the total cost picture. Radiosterilization made it possible for the suture industry to advance its packaging from the 'glass age' to the 'plastic age'. This represented a significant consumer advantage because the quality of both the product and its package was improved.

Sutures are thread-like materials that can be in the form of monofilaments or twisted or braided multifilaments and are used to join body tissues or ligate blood vessels or other organs. Sutures have been classed into two major groups: absorbable and non-absorbable.

1. ABSORBABLE SUTURES

Absorbable sutures are biodegradable. They are attacked by certain cellular elements and tissue fluid, leading to eventual absorption or disappearance of the material from the tissues. Non-absorbable sutures are not biodegradable, although they may also be attacked by cellular elements and tissue fluid and, depending upon their chemical nature and particularly their wettability, they may undergo degenerative changes in physical properties. However, whether fragmented or unchanged, they remain in the body tissues for long periods of time and usually cause a fibrous tissue capsule to develop around them.

Catgut, the main absorbable suture on the market, consists essentially of collagen and is derived from sheep or goat intestinal submucosa or bovine intestinal serosa. Beef tendon provides a convenient source of collagen for another absorbable suture, namely the reconstituted collagen suture. Other collagenous sutures, known also as 'biosutures', are derived from tail tendons of various animals. Thus, there are several reports on the successful use of kangaroo tail tendon as absorbable sutures and rat tail tendon has been used to a limited extent in ophthalmic surgery (Vannas and Larmi, 1959).
Among the synthetic absorbables Dayer et al. (1969) have described the use of sutures based on polyvinyl alcohol and Fritsch (1967) suggested the use of rapidly absorbing polyvinyl butyral. More recently synthetic absorbable sutures based on polymers of glycolic acid and lactic acid have been reported.

For more than fifteen centuries animal tendons have been employed in surgery to provide support for healing wounds but it was not until Lister's time that catgut sutures, derived from intestines as stated above, acquired sufficient surgical reliability for universal usage. One of the main problems, of course, was sterility. Following washing, the intestines are slit longitudinally into ribbons and cleaned mechanically and chemically until an almost pure collagen ribbon results. The ribbons are then treated with formaldehyde or a combination of formaldehyde and basic chromium sulphate solutions to provide wet strength and the desired rate of biodegradability. The tanned ribbons are twisted into strings and dried under controlled tension. The resulting raw strings are sorted according to size and polished manually or mechanically to the desired diameter.

In the preparation of reconstituted collagen sutures frozen bovine deep flexor tendons are sliced into thin sections, which are then swollen in acidic mixtures of methanol and water to form a viscous gel or dispersion. After homogenization and filtration, the pure collagen dispersion is extruded through spinnerettes into a coagulant such as acetone containing adequate ammonia to neutralize the swelling acid. The 'de-swollen' collagen forms thin continuous filaments, which can be chemically treated to impart sufficient wet strength and proper rates of biodegradability, and twisted into a final round string.

1.1. Sterilization of absorbable sutures

Hudemann (1967) gives a good historical review of the various processes that had been used for catgut sterilization at one time or another. Aqueous phenol solutions, hydrogen peroxide, halogens and ethyl bromide solutions are a few of the chemical agents that have been used for this purpose. In 1962 Heicken and Bellinger (1962) compared the sterility of domestic German catgut with that of imported catgut. On average, 23.5% of German catgut was non-sterile with most suppliers in the 2 to 7% range and one as high as 93%. In the case of imported catgut the average non-sterile ratio was 25.4%. Eight of 18 suppliers produced sterile catgut; the others contributed to the 25.4% average non-sterility figure. One supplier provided only non-sterile catgut. It is obvious that most catgut sterilization methods have been ineffective and that the only processes that survive the test of time are heat, ethylene oxide and radiosterilization.

Heat sterilization was the most reliable sterilization process until the early 1950s. It is a difficult process because the moisture content of catgut or collagen sutures must be reduced from an equilibrium value of 8-15% to less than 1% before sterilizing temperatures are applied. Only after the moisture content has been reduced to less than 1% through a long and carefully controlled drying cycle can collagenous sutures be safely exposed to sterilizing temperatures of 145 to 160°C for several hours. This treatment causes an irreversible shrinkage of about 5% in length and a 10-15% loss of strength. Another consequence of exposure to high temperature is collagen degradation, which cause the suture to become
more rapidly biodegradable; the higher the sterilization temperature, the more rapid the absorption process becomes.

Heat-sterilized sutures are relatively stiff and brittle and unsuitable for surgical use unless the lost water is restored to increase pliability. Because water is an effective swelling agent for collagen, its use alone would result in oversized, weakened sutures. Therefore, a mixture of alcohol (ethanol or propanol-2) and water (5-15%) is incorporated in the suture package to restore pliability and control the degree of swelling. The addition of this sterile 'tubing fluid' requires aseptic filling and sealing conditions and must be carried out with utmost care and good control to maintain the sterility of the already sterilized suture. Because of the high temperatures involved, only glass tubes have been found suitable for packaging heat-sterilized catgut.

In the ethylene oxide sterilization process the sutures may optionally be exposed to a prior heat treatment. This is followed by the addition of an aqueous alcohol solution, generally 90% isopropyl or ethyl alcohol (tubing fluid) containing 0.25-1% ethylene oxide to the package. Chemical sterilization takes place in the hermetically sealed package and the need for aseptic handling is eliminated.

Radiosterilization further simplifies the process because, following the addition of aqueous alcohol solution, the sutures are sealed in the package and exposed to radiation. In both of these processes, plastic films, laminated metal foils or paper may be used for packaging because sterilization takes place close to room temperature.

Following very thorough investigations by Koh et al. (1956a; 1956b), Bridges et al. (1956) and Pepper et al. (1956) the radiosensitivity of a large number of different types of microorganisms was classified and 2.5 Mrad was established as the radiation dose that provides sterility safety equal to that of previous methods. These results have since been confirmed experimentally by many groups the world over as well as by the excellent record of sterility maintained in commercial operations over the past fourteen years.

British Patent No. 942,374 (American Cynamid Co., 1963) describes a method of sterilization of both collagen and silk sutures by exposure to a dose of 2.5 Mrad of gamma rays. Sutures have been radiosterilized in Scotland since 1962.

Meszaros et al. (1965) discussed the superiority of radiosterilization of sutures over all other methods. They suggest a 3.5 Mrad dose, although the highly radioresistant spores of Clostridium histolyticum can be killed in vitro using 2.1 Mrad. Bartha et al. (1969) examined the natural contamination of gut samples and devised model experiments by intentionally contaminating sheep intestines with aerobic spore-bearing bacilli. These intestines were then twisted into strings and dried. The spores were twice as resistant when dried inside the sutures than on paper discs and the authors concluded that for a $10^6$ safety factor the sterilizing dose should be 3.5 Mrad.

Van Winkle et al. (1967) reported data showing that, as a result of proper cleaning and chemical treatment with formaldehyde, the microbial level of non-sterilized catgut is relatively low, namely $10^2$ microorganisms per gram or less. The relevance of Bartha's model is therefore questionable.

Majid et al. (1965) found that a dose of 2.5 Mrad of gamma irradiation is adequate for the sterilization of locally manufactured (Pakistan) catgut.
Control catgut showed heavy contamination \((10^{11})\) per strand by five different microorganisms. After exposure to 1 Mrad the count was reduced to less than 100 and at 1.5 Mrad to less than 10. The unusually high contamination of the non-sterilized control in contrast to the American results may be due to differences in cleaning and chemical processing.

Schnell (1968) also found that 2.5 Mrad is entirely satisfactory for sterilization of catgut. Sato et al. (1967) determined sterilizing doses using gut and paper contaminated with \(10^6 - 10^7\) spores of \(B.\ subtilis\) PC I 219 and found that 2.5 Mrad sterilized all samples.

1.2. Effects of radiosterilization on the physical and chemical properties of collagenous sutures

Structurally, collagen consists of three polypeptide chains bound together in the form of a triple helix, primarily through intramolecular hydrogen bonds. This unit, termed 'tropocollagen', has a molecular weight of about 300 000. Tropocollagen molecules are about 14 Å wide and 2800 Å long and are arranged within microfibrils, which in turn are organized into fibres. The parallel organization of the long rod-like molecules is to a large degree responsible for the excellent tensile strength, high modulus of elasticity, extensibility, birefringence and ability to shrink (melt) at high temperatures. In its 'native' or undenatured form collagen is resistant to attack by non-specific proteases but is labile to digestion by collagenases. However, following chemical or heat denaturation, collagen is easily attacked by most of the non-specific proteases.

The effects of radiation on collagen have been extensively studied and reported in many excellent papers. It is extremely important that the source of the collagen, its water content and the presence or absence of oxygen during irradiation are well defined and controlled if the results are to be comparable with other work.

When dry collagen is irradiated under the electron beam, a two-peak EPR spectrum of stable free radicals is produced (Kuntz and White, 1961) which disappears on wetting. Stress-strain analysis shows an excess of bond breaks over crosslinks. An increasing dose also results in an increased carbonyl content. When irradiated wet there is a progressive increase in crosslinks between 2.5 and 10 Mrad and no stable free radicals are formed.

Losses in amino acid content appear only above 20 Mrad. A total of only 20% of glycine, alanine, proline, hydroxyproline, arginine and glutamic acid, which together account for 75% of the composition of collagen, is lost at 160 Mrad. Non-hydrolytic peptide chain scission is thought to be the main effect of radiation (Cassel, 1959).

Crystalline areas of collagen become disordered at a lower dose than those of silk fibroin or keratin. Dry samples (freeze-dried) irradiated at 50 Mrad show a noticeable loss of crystallinity under the electron microscope, while wet samples exhibit non-fibrous structures as well as a marked loss of crystallinity. Also, the dry samples show swelling and gradual dissolution when immersed in water. Wet samples do not shrink or dissolve even in boiling water because of appreciable crosslinking.

Most affected by irradiation are the basic and acidic amino acids and those with cyclic structures. Least affected are leucine, isoleucine,
valine, serine and threonine. At 50 Mrad there is an apparent increase in amide nitrogen and a 6-12% loss in amino nitrogen and ammonia.

The amino acid chromatogram of dry irradiated samples shows 10% of the residues as unidentified peaks, while 20% of the residues are new after wet irradiation (more deamination). The breakage of carbon-nitrogen bonds plays an important role in this type of degradation (Bowes et al., 1960; Bowes and Moss, 1962).

The solubility of collagen increases progressively with dose when collagen is irradiated dry or with ambient moisture, but decreases when collagen is irradiated wet or in the presence of excess moisture. This seems to confirm that crosslinking occurs as the result of indirect radiation effects. Irradiation-induced crosslinking does not occur when the sample is frozen. Stress-strain analysis indicates that samples irradiated dry undergo scission of natural crosslinks or of the protein backbone since the molecular weight between crosslinks increases rapidly with dose. Samples irradiated in saline show a gradual increase of 1 to 3 thermally stable crosslinks per tropo-collagen unit at doses increasing to 45 Mrad (Bailey et al., 1964).

Tendon collagen loses 63% of its tensile strength when irradiated at doses of 10-50 Mrad. Wet fibres are less sensitive to the effects of irradiation than dry because interchain reactions can take place and crosslinks may be formed during irradiation. The presence of cysteine in the hydrated fibre during irradiation causes an increased radiosensitivity of the fibre, probably by preventing the formation of new crosslinks.

The introduction of covalent crosslinks by formaldehyde treatment or chrome tannage prior to irradiation reduces the radiosensitivity of collagen fibres (Braams, 1961; Oneson et al., to be published). Glutaraldehyde-treated collagen is more resistant to radiation than the untreated control; also the damage is less in the wet state than dry (Cox and Grant, 1968).

The effects of gamma radiation are different on soluble and insoluble collagen fibres in the dose range of 10 to 30 Mrad. Kangaroo tail tendon was taken as a model for insoluble collagen, while rat tail tendon was used as the soluble collagen (Ramanathan et al., 1965). Electron microscopy, X-ray diffraction, optical birefringence and thermal and mechanical stability measurements seemed to show that soluble collagen is more radioresistant than insoluble collagen.

US Patent No. 3,451,394, issued to Bechtol and Artandi (1969), relates to the control of the biological absorption characteristics of collagenous sutures and sponges by the use of dry radiation within the dose range of 5 to 25 Mrad. Absorbability increases with dose.

In summary, collagen irradiated dry is degraded by the direct action of radiation, resulting in random scission of the polypeptide chain. It is known, however, that some energy may be transferred along the chain to preferential sites. As the result of this degradation collagen becomes more easily attacked by non-specific proteases, its shrinkage temperature is lowered (Bailey et al., 1962), there is a loss of crystallinity and its mechanical properties are also degraded. The damage is generally dose-related. However, even at high doses, there is little destruction of the amino acids.

Irradiation in the wet state is less damaging because concurrent crosslinking tends to counteract the scission process. The presence of
radical scavengers increases radiation damage by preventing the formation of these crosslinks. The introduction of covalent crosslinks prior to irradiation increases the radiation resistance of collagen. This is particularly important in the case of radio-sterilization of sutures because most catgut and collagen sutures are treated with formaldehyde and basic chromium salts prior to irradiation.

The fact that catgut or collagen sutures suffer minimal radiation damage in the range of 2.5 to 5 Mrad can be explained on this basis (Schnell, 1968; Oneson et al., to be published). In addition, collagenous sutures contain about 20% water because they are packaged in an aqueous alcohol solution. The presence of water and alcohol together contribute to the protection of the product by enhancing crosslinking and by absorbing excess radiation energy.

1.3. Animal and clinical experience

In the early 1950s extensive experimental work was carried out on a variety of animals to compare heat-sterilized and radio-sterilized (electron beam) catgut. The results have conclusively proved that the radio-sterilized product has better tensile strength retention characteristics during the first several weeks of implantation and causes no more tissue reaction than the heat-sterilized product. Absorption characteristics are comparable. It is important to realize that the biodegradability of catgut or collagen sutures is determined by four factors: (1) origin of material (animal species); (2) chemical processing conditions (tanning); (3) presence or absence of heat treatment; and (4) method of sterilization. Since most commercial sutures are treated differently in some or all of these categories, it is difficult to predict in vivo characteristics without knowledge of the entire manufacturing process.

Following extensive animal work, thousands of controlled clinical cases were carried out with completely satisfactory results and it was established conclusively that radio-sterilized sutures are both safe and effective. Commercialization of the irradiation sterilization of sutures began in 1956. Both the process and the product were reviewed by Artandi (1964) and Van Winkle et al. (1967) in great detail. Fitzgerald (1957) reported on 150 cases of episiotomy repair with both plain and chromic catgut that had been heat or radio-sterilized. He found greater strength, less tissue reaction and greater stimulation of fibrosis in the healing tissues when radio-sterilized sutures had been used.

Narat (1957) first compared heat and radio-sterilized catgut in rabbits and found no difference. He then used both types in 76 patients and, although he found no difference in handling or in tissue reaction, the radio-sterilized material proved to be stronger. Postlethwait (1959) compared heat and radio-sterilized catgut sutures and their effect on wound healing. Miller et al. (1964) reported favourable results in several hundred operations with radio-sterilized collagen sutures. Just-Viera et al. (1965) compared radio-sterilized catgut and collagen sutures in dogs. There was a slight separation of fibres of the collagen suture during healing; however, healing proceeded equally well in both cases. In a study involving 705 episiotomies de Alvarez (1965) found that radio-sterilized catgut and reconstituted collagen sutures were comparable and that both are satisfactory suture materials.
Gaskin and Childers (1963) reported an unfavourable tissue reaction to radiosterilized sutures used in cataract, plastic and eye muscle surgery. They observed both sterile abscess and granuloma formation, which, in their opinion, was due to slow and variable absorption of the sutures. In contrast, Brumback and McPherson (1967) found that radiosterilized sutures show desirable properties in ophthalmic surgery (keratoplasty) in terms of excellent handling characteristics, minimal tissue reaction and low antigenicity. Regan and Dunnington (1966) experienced similar favourable results using 6-0 plain collagen sutures in cataract incisions.

Kreshon et al. (1966) reported the successful use of collagen sutures in 400 cases of cataract operation with smooth suture surface, minimal tissue reaction and predictable absorption being the main advantages. McPherson and Young (1965) described 150 cases of cataract surgery with radiosterilized plain and chromic collagen sutures. There was little tissue reaction and the sutures performed satisfactorily.

Thoennes (1964) compared the physical properties of heat, ethylene oxide and electron beam sterilized catgut sutures together with their tensile strength retention in rabbits. He found that ethylene oxide-sterilized catgut was initially strongest and remained consistently higher during the 21-day implantation period. The shrinkage temperatures of this material was also highest, indicating very little degradation. Unfortunately, no absorption results are reported, perhaps indicating a high resistance to absorption for the ethylene oxide-sterilized sample. Because all three experimental groups of sutures were prepared from a particular brand of catgut, the results are meaningful only for that specific material.

Meszaros et al. (1965) reported that radiosterilized catgut shows better absorption characteristics, less tissue reaction and shorter periods of wound healing when compared with that sterilized by the classical method. Hufnagel et al. (1965) studied heat and ethylene oxide-sterilized and radiosterilized chromic catgut sutures in rabbits. They found that radiosterilized sutures absorbed more slowly than the heat and ethylene oxide-sterilized catgut, but suggested that the same source of raw catgut should have been used for both sterilization methods to permit better comparison. Perhaps heat and ethylene oxide-sterilized catgut should have been compared with a heat and radiosterilized gut to measure the effect of sterilization on the absorption properties of the suture. It is known that heat degradation causes catgut to be more easily biodegradable.

Addison and Jennings (1965) compared heat and radiosterilized catgut in an attempt to confirm the findings of Gaskin and Childers (1963). A study of fascial closures in 20 dogs up to 8 weeks postoperatively showed no difference in tissue reaction or absorption characteristics. This was followed by a double-blind study on 46 consecutive clinical cases. In 17 cases there was more tissue reaction with heat-sterilized catgut; in 5 cases the opposite was observed, while in all other cases the two sutures were equivalent. These investigators concluded that factors other than sterilization must have been responsible for the findings of Gaskin and Childers.

Reynolds (1968) tested radiosterilized collagen sutures in canine intestinal anastomoses and found that there was more tissue reaction and fibrous tissue formation around the collagen suture compared with ethylene oxide-sterilized catgut. However, the source of the sutures used was not mentioned and some of the experimental techniques appear open.
to question. Adler et al. (1967) compared radiosterilized catgut and collagen sutures in canine colon anastomoses. According to their findings, collagen was more uniform in physical and biological properties. Both sutures were entirely satisfactory and there was no significant difference in the strength of the anastomoses between three days and eight weeks postoperatively. Histologically, collagen exhibited a less prolonged inflammatory tissue reaction and absorbed more rapidly.

Schnell (1968) conducted comparative studies of ethylene oxide-sterilized and radiosterilized catgut and found no differences after in vivo implantation. Dittrich (1968) also found that ethylene oxide-sterilized and radiosterilized catgut behave very similarly as rabbit muscle implants.

2. NON-ABSORBABLE SUTURES

Non-absorbable sutures include the natural polymers: silk, cotton, linen, ramie, hemp; and many synthetic polymers: polyamides, polyesters, polyolefins, polyurethanes and polymeric fluorocarbons. Metals such as stainless steel, tantalum, silver, aluminium and others have also been used as surgical sutures. Until the late thirties and early forties the surgical use of non-absorbable sutures was confined to natural polymers and metal wire, but their use goes far back in written history.

2.1. Sterilization of non-absorbable sutures

The absorption of ionizing radiation in polymers produces ions and excited molecules. The ions may undergo charge neutralization and produce additional excited molecules. The excited species may then be subjected to bond rupture with the production of free radicals.

It is generally thought that the interaction of radiation with matter is a random process. However, it is known that energy may be transferred along polymer chains in a process that could result in preferential bond breaks. Chemical structure, crystallinity, environment and temperature may influence the fate of free radicals. Chemical structure determines to a very great degree whether crosslinking or chain scission will be the result of radiation. Most likely both processes proceed concurrently, although at different rates, and the predominant rate ultimately determines the final result.

As with catgut, the radiosterilization of non-absorbable sutures began in the mid-fifties. Prior to that time substantial experimental work was carried out to study the effects of radiation on the physical, chemical and biological properties of various suture materials that are packaged dry and can be effectively sterilized at the 2.5 Mrad dose level.

Cellulose fibres

Of the cellulose polymers, cotton is the most widely used, linen is popular in Europe and ramie and hemp are rarely used. These fibres are processed in staple form and, following cleaning and bleaching, are combed and spun into continuous filaments. Several filaments are combined by twisting into a compact yarn. In the final finishing operation a starch glaze may be applied to produce a smooth yarn. The strength of the yarn
is determined by the quality of cotton fibre used and the construction of the yarn (twist, number of plies, compactness, etc.). Cellulose is the only suture material that gains about 10% in tensile strength on wetting; all other sutures either remain unchanged or lose strength when wet. Water most likely acts as a plasticizing agent in the rigid, highly crystalline cellulose fibres.

Linen is stronger than cotton because its fibres are longer and even more highly crystalline. British Patent No. 942,374 (American Cynamid Co., 1963) describes radiosterilization of linen sutures with satisfactory results. Cotton and linen lose about 10-15% tensile strength on irradiation and do not regain it on wetting. For this reason cotton and linen sutures have never been radiosterilized in the United States of America; in this case, ethylene oxide-sterilization produces a product with superior tensile strength properties. However, in Great Britain and some European countries cotton and linen sutures are radiosterilized because the products are still entirely satisfactory for these markets.

Blouin and Arthur (1964) studied the feasibility of radiosterilization of cotton sutures under various conditions of processing, moisture content and gaseous atmosphere. The main problems are radiation-induced oxidative degradation of cellulose, causing chain cleavage and strength loss, and the production of stable free radicals, which may cause further degradation during storage. They found that the addition of water (2.7 times the weight of cotton) exerted a protective action and the strength loss did not exceed 15-20% even after 6 months' aging. The irradiation atmosphere played an insignificant role. Chemical analysis revealed that the predominant effect is formation of carbonyl groups, but chain cleavage also occurs. Arthur et al. (1965) were able to protect cotton from radiation degradation by the substitution of benzoyl groups on the cellulose molecule.

Pan et al. (1959) also investigated the radiation-induced degradation of cellulose fibres. Their findings are similar to that of the previous authors but they determined, in addition, that neither mercerization nor acetylation protects cellulose fibres from radiation damage. Kinoshita et al. (1963) sterilized absorbent cotton and gauze at 2 Mrad without damaging the essential qualities of the products.

Arthur et al. (1965) extensively studied the effects of radiation on cellulose and found that the initiation of oxidative degradation was the primary consequence. The degradative processes include dehydrogenation, destruction of anhydroglucose units resulting in liberation of CO and CO$_2$ and cellulosic chain cleavage.

Silk

Silk is extruded by the silkworm during the cocoon building phase. Two filaments of about 1.5 denier fibroin protein are extruded simultaneously and adhered with another protein extrudate called sericin. The filaments are somewhat triangular in cross-section, very highly oriented and possess great tensile strength. The double filament of silk is unwound from the cocoon and combined with sufficient numbers of other filaments to form a yarn of the desired denier.

In the manufacture of silk sutures the filaments are twisted, 'degummed' under mildly alkaline conditions to remove the sericin and dyed if a
The yarn may then be processed into twisted or braided sutures. Twisted sutures, generally stronger because of decreased interfibre shear, may unravel or show broken filaments but they cannot be made as compact as braided sutures. In the braiding process, 4, 8, 12 or 16 ends of yarn are interlocked 40-50 times per inch. A core yarn around which the ends are braided may be used. The resulting suture is heat-stretched to set the braided structure, after which it may be impregnated with beeswax, silicone derivatives or other materials to fill the interstices.

Silk is by far the most popular non-absorbable suture. Its handling and tying qualities are outstanding and set the standard for many surgeons. Silk is also used without removal of the sericin (virgin silk) as extremely fine ophthalmic sutures. Human hair (keratin) has been used occasionally in surgical procedures.

Silk fibroin is quite radiation-resistant. A 2.5-Mrad dose causes about 5-6% loss of tensile strength. Interestingly, white silk is more radiation-sensitive than black silk. Black silk is generally dyed with wood extracts (Brazil wood), which contain mainly multifunctional aromatic phenols. An acidic potassium dichromate solution is employed as mordant in the dyeing process. As a result of this treatment, numerous covalent crosslinks are introduced into the fibroin through the action of phenolic compounds and chromium salts. The silk becomes stabilized and more radioresistant. Even 10 to 15-Mrad doses cause only relatively little loss in strength. British Patent No. 942,374 (American Cynamid Co., 1963) describes radiosterilization of silk with good results. Both Horibe and Kikuchi (1968) and Sato and Ito (1968) report little effect on strength and elongation of silk sterilized at 2.5 Mrad.

Metals

Many metals including silver, tantalum, titanium and aluminium have been used experimentally as sutures (Wu et al., 1967; 1968) but the single most important metallic surgical suture is stainless steel (Type 316 L). Its main advantages are inertness in biological tissues and high strength, which does not diminish on tying as it does for all other suture materials. When permanent reinforcement is required, stainless steel is frequently used. One disadvantage is that stainless steel fatigues on repeated bending and in some applications it will eventually fragment. A second problem is related to difficulty in handling and tying (kinking).

Metals are not altered by radiosterilization because all the energy is harmlessly dissipated.

Synthetics

Almost as soon as a new synthetic fibre is developed surgeons are anxious to evaluate it in their quest for the ideal suture material.

Polylefins. Since the late 1950s monofilaments of both polyethylene (high density) and isotactic polypropylene have been used in surgery. High density polyethylene possesses many desirable properties such as good strength, inertness and superior retention of tensile strength on long-term implantation. However, proper melt extrusion of this material is extremely...
difficult because of the tendency toward melt-fracture. This problem has limited the use of polyethylene as a suture material.

Polypropylene has all the good characteristics of high density polyethylene without the extrusion difficulties. In many ways it is an ideal suture material because of its inertness in tissue and because it retains its original tensile strength many years post implantation. It can be extruded as monofilament or multifilament. In the latter case polypropylene is braided into suitably sized sutures.

Polyethylene shows little damage on irradiation. Dehydrogenation and some crosslinking take place during irradiation at 2.5 Mrad but with no noticeable effect on the physical properties of the sutures. Polypropylene, on the other hand, is very radiosensitive and sutures show excessive loss of strength (about 20%) at 2.5 Mrad. In this case random cleavage of the molecule occurs. Horibe and Kikuchi (1968) observed a decrease in tensile strength and elongation at a dose of 0.5 Mrad. It is possible to reduce radiation damage significantly by the incorporation of protective agents in the polymer. At present, however, all polypropylene sutures are sterilized by ethylene oxide.

Polyamides, particularly nylon 66 (polymer of hexamethylene diamine and adipic acid) and nylon 6 (polycaprolactam), were among the first synthetic fibres to be used as surgical sutures. They possess many advantages over natural fibres including low cost, facile production of mono- and multifilaments in a variety of sizes and generally lower tissue reactivity. Ballantine and Shinohara (1961) observed free radical formation upon irradiation of nylon 6 filaments. Little or no change in strength and elongation of nylon sutures irradiated at 2.5 Mrad is reported by others (Horibe and Kikuchi, 1968; Sato and Ito, 1968).

Polyesters, particularly ethylene glycol terephthalate polymers, have gained good acceptance in surgery since their introduction in the fifties. Polyesters have two advantages over the nylons: because they do not imibe appreciable water or tissue fluid, they are less reactive than nylon; and they retain their original tensile strength for many years. Cotton, silk and nylon are gradually degraded in the tissues because they absorb fluids to various degrees. Polyesters are especially useful in cardiovascular applications where permanent strength is of paramount importance. Because it is more rigid than nylon, polyester monofilament is useful only in very fine suture sizes; in general applications it is necessary to employ twisted or braided sutures to overcome the inherent stiffness of polyester.

This material exhibits very good resistance to radiation. The presence of aromatic ring structures acts as a protective agent by absorbing a large part of the radiation energy. Little if any effect on strength and elongation by irradiation doses of up to 2.5 Mrad has been reported (Horibe and Kikuchi, 1968; Sato and Ito, 1968).

Segmented polyurethanes. Spandex type filaments have been employed experimentally in surgery since the sixties (Wagner et al., 1966). These materials are elastomeric (rubber-like) and are very inert in tissues but their strength is relatively low. They can be radiosterilized without deleterious effect on tensile strength.
Fluorocarbons. Various types of polymers similar to Teflon have been evaluated as experimental sutures. While they are very inert, their tensile strength properties are much lower than those of other suture materials.

Polytetrafluoroethylene (Teflon) and fluoroethylpropylene (FEP) monofilaments have been used experimentally. They are very radiosensitive, the main effect being the scission of carbon-carbon bonds in the backbone of the polymer. Sato and Ito (1968) reported a significant decrease in the tensile strength of braided Teflon sutures even at doses less than 1 Mrad, while Horibe and Kikuchi (1968) found that tetrafluoroethylene sutures lost both tensile strength and elongation at 0.5 Mrad levels of irradiation.

2.2. Animal and clinical experience

About one-half of all sutures used in surgery are of the non-absorbable type, with silk being the most important. The selection of a suture material is based upon both objective and subjective criteria.

Among the objective criteria the following are important: strength, inertness, long-term retention of strength, ease of handling, knot security. Other factors being equal, monofilament fibres are generally more difficult to handle (stiffness) and do not have as good knot-holding properties as braided sutures. On the other hand, they are easier to remove (plastic and retention sutures), do not swell (Homsy et al., 1968) and are less likely to harbour infection because they have no interstices. Alexander et al. (1967) report that monofilament sutures withstand contamination better than multifilament sutures prepared from the same material. Among the subjective criteria, smoothness of texture, colour, past experience of the surgeon and other similar factors may be mentioned.

Cotton, linen and silk lose strength, fragment over long periods of time and cause greater tissue reaction than metals or synthetics. Nylon absorbs water and is gradually degraded, but causes less tissue reaction than cotton, linen or silk. All other non-absorbable sutures, as well as stainless steel, are very inert and retain their strength for many years.

Before any suture material can be used in actual surgery exhaustive chemical, physical and biological studies must be conducted to establish both 'safety' and 'efficacy'. Animal work includes acute, subacute and chronic toxicity investigations in several species, as well as carcinogenicity evaluations. All colour additives are studied separately for safety. With most sutures many years of clinical experience in humans, including controlled studies with follow-up, biopsies and autopsies, continually confirm their safety and reliability.

Dittrich (1968) showed good tissue compatibility of polyester (Mersilene) sutures in rabbit muscle. Schnell (1968) compared ethylene oxide- and gamma-sterilized polyester sutures in animals and found both satisfactory. Narat et al. (1956) found Dacron sutures to be stronger than cotton or silk; there was no loss of strength on radiosterilization. In 89 surgical cases no untoward effects, extrusions or granulomas were observed. Latimer and Werr (1961) used Dacron in 500 patients without any incidence of tissue irritation, granuloma or extrusion and considered it superior to cotton and silk. Schumacker and Mandelbaum (1961) performed 747 operations with electron beam-sterilized Dacron sutures. Wound healing
was eminently satisfactory, the sutures were easy to handle, strong, non-reactive and readily removable from tissue. They consider Dacron the best suture material.

Wagner et al. (1966) reported excellent results with segmented polyurethane sutures. They were almost inert in tissue and elasticity, tying and snugging were satisfactory.

McPherson et al. (1970) found polyamide sutures almost completely inert in the rabbit cornea and much less reactive than silk. Postlethwait et al. (1959) compared catgut, silk, cotton, ramie, wire, nylon, nylon-bonded nylon multifilament (Nymo), Dacron and Teflon sutures in animals. The synthetic sutures, particularly Teflon, caused little reaction. Dettinger and Bowers (1957) report that a comparative study on synthetic fibres proved Orlon, Dacron and monofilament nylon to be far less irritating to tissues than cotton or silk. Moore and Aronson (1969) found that 8-0 virgin silk caused a more severe inflammatory reaction in corneal graft procedures than 10-0 monofilament nylon.

Phelan (1959), Usher (1961) and Koontz and Kimberly (1963) reported favourable results on the use of linear polyethylene sutures (Marlex), particularly in infected wounds. Usher et al. (1962) discussed the successful clinical use of polypropylene monofilament sutures for closing contaminated wounds. Miller and Kimmel (1967) participated in the clinical evaluation of monofilament polypropylene (Prolene) sutures and found the most striking advantage to be the ability of the wound to withstand infection. The suture retained its mechanical properties in contaminated wounds and had good knot-holding ability. Postlethwait (1970) reported a two-year animal study in which silk, cotton, nylon, polypropylene (Prolene), Dacron (Mersilene) and Teflon-coated Dacron sutures were compared. Nylon eventually lost 30%, cotton 40% and silk all of its tensile strength, while the others were unaffected. Silk and cotton showed the greatest tissue reaction. Nylon was the least reactive, Prolene and Mersilene being second best.

Catchpole and Winn (1960) compared nylon, polyethylene, polypropylene, Teflon, Terylene (Dacron), Orlon, linen and silk in dogs in a 12-month study. Both linen and silk lost strength rapidly, nylon more slowly. The remaining suture materials retained their original strength. Polypropylene showed the least tissue reaction. Lilly et al. (1968) implanted a variety of suture materials in the oral tissues of dogs and found monofilament steel and nylon the least reactive. Plain and chromic catgut were next, while silk, cotton and linen were the most reactive. Jordan et al. (1966) tested various non-absorbable sutures in vascular replacement procedures. Silk was unsatisfactory because of premature loss of strength. Monofilament polyethylene and polypropylene were strong and inert but had poor knot-holding properties. Dacron (Mersilene) was good but tended to saw tissue. Teflon-coated Dacron was satisfactory.

Ever since the introduction of commercial radiosterilization of suture materials in 1956 both absorbable and non-absorbable sutures have been used in surgery with excellent results. In the United States of America alone about 15 million surgical procedures are at present performed annually and in the majority of these radiosterilized sutures are employed. Our total, world-wide experience exceeds one billion radio-sterilized sutures of all types. There have been very few products that
can equal these suture materials not only in terms of numbers but also in outstanding performance.

A better product, more convenient usage and greater safety have all resulted from a unique, imaginative and peaceful use of atomic energy.
CHAPTER 16

PLASTIC AND RUBBER
INSTRUMENTS AND APPARATUS

Radiation-induced changes in plastics may be manifold: appearance, chemical, physical and mechanical properties. Changes in appearance may be temporary or permanent (colour change, bubbles).

Chemical changes include hydrogen extraction, dehydrochlorination, double-bond formation, scission, crosslinking, oxidative degradation, polymerization, depolymerization and gas formation (decarboxylation or deamination). Among the physical changes are effects on viscosity, solubility, conductivity, free radical formation, crystallinity and fluorescence. Mechanical properties that may change are: tensile strength, elastic modulus, hardness, elongation, flexibility, plastic flow and others.

It is a very fortunate fact for both the health and radiation industries that most of the plastics and other materials commonly used in the manufacture of disposable items are not significantly affected in their physical, chemical and mechanical properties at radiation doses sufficient for sterilization.

Crawford has published a detailed list of materials suitable for radiosterilization. Other currently used materials have been added to this list to make it more complete:

Thermoplastics

Polystyrene – both general purpose and high impact (rubber modified)
Styrene – acrylonitrile copolymers
Acrylonitrile – butadiene-styrene copolymer
Polyethylene – both low and high density
Ethylene vinylacetate copolymer
Ethylene acrylic copolymer
Polymethylpentene
Polyamides (nylon 6, 66, 10)$^2$
Polyesters (Mylar, Dacron, Terylene, Melinex)
Polycarbonate (Lexan)
Polyurethane
Polyvinylchloride$^2$
Polymethylmethacrylate (Perspex, Diakon, Plexiglas
Polyoxymethylene (acetal resins, Delrin)$^2$
Ionomers (Surlyn A)
Polypropylene (specially stabilized)$^2$
Cellulose esters
Polyphenylene oxide
Polydimethylsiloxane rubbers
Other thermoplastic rubbers

$^1$ May undergo slight colour change and/or degradation; however, the use of pigments may mask the colour and proper formulation should protect the materials sufficiently.
Thermosetting resins

- Epoxy
- Phenolics
- Melamine
- Urea
- Unsaturated polyesters

Rubbers

- Natural latex (also vulcanized)
- Segment polyurethane (Spandex)
- Butadiene-styrene
- Neoprene
- Butyl

Adhesives

- Various latex-rubber, vinyl and acrylic types

Other materials

- Wool, paper, metals, cellulose, glass

The only material that cannot be radiosterilized without excessive degradation is polytetrafluoroethylene (Teflon).

This list should provide the manufacturers of single-use items with an excellent selection of materials.

Over the last two decades the availability of disposable items has revolutionized health care. In addition to eliminating costly labour in cleaning, resterilization and maintenance, they provide both greater safety by eliminating the risk of cross-infection and greater patient comfort and mobility.

Most disposable hospital products were first used as individual items. However, before long the idea of combining these items into trays, packs or kits for specific procedures was conceived. This innovation resulted in further labour-saving because it eliminated assembly and sterilization at the hospital and represented a great convenience to the hospital staff and physician.

The variety of items (shape, size, materials) has presented new problems in sterilization because the method of choice had to be safe and effective for all items. Radiosterilization has proved to be extremely valuable for these new concepts by permitting the use of a very wide choice of plastics and packaging materials. The list of disposable items is indeed very long and limited only by the imagination of designers. The host of medical supplies and materials can be conveniently categorized into three main groups:

1. Items used in conjunction with the delivery of medical care, but not on patients
2. Items used in direct contact with the body for examination or in surgery
3. Temporary or permanent implants and devices.
(1) Air filters, face masks, non-woven gowns, trousers, boot covers, specimen collecting bags, brushes, containers for vaccines and pharmaceuticals, colostomy bags, disposable pipettes, glass droppers, Petri dishes, blood-agar-containing Petri dishes, packaging materials, tubing, connectors, test sets for urine analysis, tubes for ointments, test tubes, baby feed bottles, starch powder and cream glove lubricants.

(2) Adhesive tapes, artificial eyelashes, airways, cotton gauze sponges, burn bandages, drapes, dressings of various types, dressing packs, fenestrated surgical drapes, balloon-type splints, eye pads, examination gloves (latex, vinyl, polyethylene, ethylene-acrylic copolymers), drains of various types, catheters (intravenous, latex balloon, Robinson, urethral, suction, peritoneal dialysis, cardiovascular, umbilical, artery), catheter collars, cannulae, electrode leads, dental drills, foam sponges, impregnated cotton buds, procedure packs, hand towels, scalpel blades, scalpels, forceps, scissors, haemostats, lancets for blood samples, mucus extractors, syringes, needles, blood collecting, spinal and dental needles, retractors, fibre-optics, surgical trays, umbilical cord clamps, operation kits, preparation razors, sanitary napkins, swabs, suction tubes, speculums, endotracheal tubes, tracheostomy tubes, staplers, staples (single and cartridges), clips, suture removal sets, containers for kidney transplants, artificial kidney tube sets, and cartridges, haemodialysis sets, transillumination sets, oxygen tents, disposable oxygenators.

(3) Arteriovenous shunts, peritoneal dialysis sets, solution administration sets, heart valves, mammary prostheses, aortic and peripheral vascular prostheses, pacemaker parts, hydrocephalic valves, contraceptive implants, artificial corneas, bone replacements, metal pins, screws, hip joints, plastic finger joints, dental implants, haemostatic sponges, urethral valves, dentures, artificial eyeballs, artificial testicles, formed implants for plastic surgery repair, artificial tracheas, aortic balloon pumps, other temporary heart assist devices, artificial hearts, artificial knee-caps.

It is extremely important that the effects of radiosterilization on the physical and mechanical properties of these items are carefully checked out immediately after sterilization and after appropriate aging periods if the reliability of the products is to be ensured.

Products that are used to contain specimens of any kind or fluids and solids used in testing, medication or infusion must be even more carefully tested for potential deleterious effects of radiosterilization on the contents of the packages. Radiation-induced changes in plastics (hydrochloric acid liberation, coloured by-products, plasticizers, stabilizers) may result in the extraction of harmful products into the contents of the containers or changes in the stability of the contents.

Of all disposable medical products, hypodermic syringes and needles represent the single largest volume of items. Within less than ten years the use of glass syringes has almost completely disappeared except for the operating room and in special cases. At first, styrene was the preferred material because of its low cost and easy mouldability. However, it was found to be incompatible with several drug products and polypropylene soon replaced it.
In the USA at present approximately 500 million injections are given annually with disposable plastic syringes and needles. The worldwide figure should amount to about 1500 million.

Disposable plastic gloves, latex examination gloves and surgeons gloves are probably the second largest product in volume. In the USA alone several hundred million of these gloves are used. Radiosterilization is competitive in cost with gas sterilization, just as in the case of plastic syringes, and does not present the problems of penetration and residual gas.

Implants may be exposed to constant motion while immersed in complex body fluids, conditions which may lead to mechanical failure. The original parts that are being replaced by plastic implants are made of living tissue and are capable of withstanding unbelievably prolonged and hard use. A leaflet of a heart valve bends back and forth about forty million times per year. There are no known materials that have a flex-life of this order over long periods of time.

Any plastic material considered for implants must meet some very critical requirements (O'Leary et al., 1965):

1. Chemically and physically inert and thermally stable
2. Dimensionally stable
3. Non-carcinogenic, non-toxic
4. Non-allergenic
5. Capable of sterilization
6. Resistant to mechanical strain
7. Capable of fabrication
8. Have complete tissue acceptability for prolonged periods of time.

Many attempts have been made by people in governmental agencies to assess the total value of radiosterilized products. If the figure amounted to several hundred millions of dollars, the costs of this new process and its development were felt to be justified. However, it would be even better if the contributions of radiosterilization could be expressed in terms of human values. Available statistics show that 10% of all surgical patients acquire infections during their stay and 1% die as a result. If, through the greater patient safety achieved by reliably sterilized products, this figure is reduced even by only a fraction, the saving of lives and the reduction in hospitalization time, as well as in suffering, would certainly represent a far greater contribution than the monetary value of the products.

Acknowledgements

Personal communications from the following companies are gratefully acknowledged: Conservatome, France; Johnson & Johnson Limited, Great Britain; Radona, Sweden; Smith & Nephew, Great Britain; Tasman Vaccine Laboratory Limited, New Zealand; Willy Rüsch, Federal Republic of Germany.
CHAPTER 17

EQUIPMENT FOR PRESERVATION OF BLOOD AND BLOOD TRANSFUSION

1. INTRODUCTION

It is a routine procedure in blood banks to store citrated donor blood up to three weeks at 2-6°C before transfusion. Blood fractions and other products intended for intravenous infusion are often stored under similar conditions. Blood is an excellent growth substrate for many microorganisms, even at these low temperatures, and so one viable microorganism left in contact with the blood may lead to a heavily contaminated product that can cause fatal accidents if used.

Blood bank equipment is regarded as a special group of medical devices in many countries, where specifications for the products and guidelines for the control of the quality at all steps in the production provide a frame for ensuring the maximum safety in the use of this highly critical class of equipment (Council of Europe, 1967; Pharmacopeia Nordica, 1964; United States Pharmacopeia, 1970).

Sterility is only one of the many aspects that must be considered in the production and use of these products, albeit a very important one.

2. STERILIZATION STANDARDS FOR BLOOD BANK EQUIPMENT

Production and sterilization of blood bank equipment and equipment for intravenous infusions must be carried out under circumstances that make it possible to demonstrate that there is a very low probability of the occurrence of surviving microorganisms on any given item. This probability must at least be as low as that experienced with equipment sterilized lege artis in hospitals: $10^{-6}$, i.e. less than one out of one million items may contain viable microorganisms after the sterilization (see Part II, Chapter 11.2).

The following discussion will have the sterilization standards in the Scandinavian hospitals as background, but most of the guidelines will also be valid if it is felt that an even higher sterilization standard is desirable for this critical class of devices. A request for a higher standard will fortify the arguments for application of sterilization methods with a well-defined relationship between controllable physical parameters and the microbicidal effect in all parts of the equipment, as well as stress the importance of maintaining a good production hygiene and efficient protection against recontamination after sterilization.

3. CHOICE OF STERILIZATION METHOD

Blood transfusion has been carried out routinely for more than 50 years. The equipment applied originally consisted mainly of glass
and rubber parts and such equipment is still in common use in many places. The equipment was normally sterilized by autoclaving at the hospital.

The use of disposable plastic equipment was introduced in the 1950s. It was demonstrated that blood stored in plastic bags for 21 days had at least as high quality as blood stored in glass bottles (Dudley et al., 1958; Walter et al., 1964; Revol et al., 1964; Dern et al., 1966). It was appreciated that the flexibility of the bags decreased the risk of air embolism, that the development of closed systems minimized the problems from microbial contaminations and that fewer problems with pyrogens were encountered when using disposable devices (Reissigl, 1963; Gibson II, 1964; Guess et al., 1967; Spielmann, 1967).

The sterilization methods normally used for these industrially produced materials are autoclaving, radiation sterilization and sterilization by means of some toxic gases.

3.1. Equipment design

In the design of equipment to be used under circumstances where it is crucial that sterility is maintained it is necessary to consider, among others, the following questions:

(a) Is it easy for the user to handle the device according to its purpose without breaking the sterility?
(b) Is it possible to ensure with reasonable safety that the sterilizing agent penetrates to all parts of the equipment?
(c) Does the packaging ensure with a reasonable margin of safety that sterility is maintained throughout the time from sterilization to use?

The design of a device will often be a compromise between considerations of these three aspects, especially if it is intended that the product shall be sterilized by autoclaving or toxic gases, because it is then necessary to ensure that considerations to points (a) and (c) do not lead to the introduction of diffusion barriers, which may cause the sterilization to fail. Figure 26 presents examples of diffusion barriers often found in blood bank equipment. Many blood transfusion assemblies have a far more complicated design. Besides the diffusion barriers, which can be detected by careful study of design details, microbarriers such as crystals and organic matter around the bacteria can also cause problems for autoclaving and gas-sterilization procedures.

If the equipment is to be radiation sterilized, it will in most cases be possible to avoid compromises in the design resulting from sterilization considerations. The penetrating nature of ionizing radiation makes it easy to ensure that all parts of the equipment receive the minimum radiation dose. The physical dose control system will reveal the rare cases where the equipment is so bulky that parts are shielded from the irradiation.

3.2. Mechanical properties

The safety of blood transfusion depends heavily on the mechanical reliability of the devices applied. It is well known that radiation can cause
changes in the mechanical properties of plastic materials. Some materials are softened, others become brittle. Aging phenomena occur in some cases so that radiation damage develops slowly over a period of some months after irradiation. It is therefore necessary to store control samples of radiation-sterilized equipment for at least one year to accumulate experience and for documentation. Furthermore, the routine examination of devices irradiated with 3-5 times the sterilization dose (15-25 Mrad) is strongly recommended. The radiation-induced changes will increase with increasing dose and aging is accelerated so that a wider margin of safety is provided by the inspection of heavily overradiated devices.

Experience with this sort of control at Risø has shown that most materials in normal use pass this test. If a material fails to pass the test, it will in most cases be possible to suggest other materials that can be safely applied.

3.3. Toxicological aspects

The US Pharmacopeia and the Code of Federal Regulations recommend that water extracts of transfusion and infusion assemblies should be tested
<table>
<thead>
<tr>
<th>Batch size N</th>
<th>$\frac{4}{10}\sqrt{N}$</th>
<th>Total number</th>
<th>Classification according to level of contamination</th>
<th>Average count</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td>0</td>
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</tr>
<tr>
<td>3000</td>
<td>22</td>
<td>104</td>
<td>35</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1051</td>
<td>0</td>
<td>94</td>
</tr>
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<td>55</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>702</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>2500</td>
<td>20</td>
<td>19</td>
<td>14</td>
<td>5</td>
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<td>6</td>
</tr>
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<td>30</td>
<td>16</td>
<td>2</td>
</tr>
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<td></td>
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<td>0</td>
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</tr>
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<td>3</td>
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</tr>
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<td>35</td>
<td>16</td>
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<td></td>
<td></td>
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<td>29</td>
</tr>
<tr>
<td>Total 18610</td>
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<td>297</td>
<td>139</td>
<td>109</td>
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<tr>
<td></td>
<td></td>
<td>3740</td>
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<td>176</td>
</tr>
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<td>Irradiated units</td>
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<td>55</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19</td>
<td>0</td>
<td>11</td>
</tr>
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</table>
routinely for pyrogenity and undue toxicity and that the plastic materials used must be carefully tested by several extraction techniques and implantation tests. In the European agreement on the Exchange of Therapeutic Substances of Human Origin it is required that tests should be carried out for pyrogenity, haemolytic effects and in vivo survival of red cells. Chemical tests are also specified.

All these tests should be undertaken with sterilized items. Undesirable reactions caused by sterilization treatments have been reported on several occasions (Kjåster et al., 1959; Hirose et al., 1963; Guess, 1970). Some of the reactions observed could, however, be attributed to residues of the toxic gas used for sterilization rather than to changes in the material itself.

Undesirable reactions caused by radiation will also be easier to detect if items irradiated with 15-25 Mrad are used as controls, thereby giving a wider safety margin.

PVC, the material most commonly used in the production of transfusion assemblies, is a quite unattractive material from a toxicological point of view, because it contains a wide range of various compounds that may leak into fluids in contact with it (Guess et al., 1967; Jaeger and Rubin, 1970a). The risk of sterilization-induced toxicity is increased with the chemical complexity of the material. Materials that are more biocompatible than PVC are available, but are also more expensive (Brady, 1968; Hastings and Harmison, 1969).

The toxicological reactions of plastics reported from the medical practice are, however, few compared with the very large number of items used (Gullbring, 1964). The overall frequency of complications (including mild allergic reactions) after blood transfusions is around 1% (Spielmann, 1967) and most probably is only a minor part of the complications seen in connection with blood transfusions caused by the equipment applied.

4. INITIAL COUNTS - STERILIZATION DOSE

The minimum radiation dose necessary to reach the desired level of sterilization (e.g. $10^{-6}$) will depend on the number and type of microorganisms on the items to be sterilized, i.e. the initial microbial count.

The initial microbial count is determined by evaluation of the average count of a number of items taken at random from the production. Initial counts taken over longer periods may show that the overall hygienic standard of a production is high, but this gives no absolute guarantee against a sudden increase in counts due to some unforeseen event. The choice of sterilization dose must therefore be based partly on the average initial count and partly on the possibility of detecting an increase in the occurrence of single items with counts considerably higher than average.

The possibility of detecting a sudden occurrence of heavily contaminated items in a production run is closely related to the number of items taken for control of initial counts, the sample size. Similar sampling problems are already known from traditional sterility testing. Some examples of initial counts from a routine control may illustrate this (Christensen et al., 1968).

Table XXI shows initial counts from six different batches of packed disposable sets for venesection of blood donors sent to the Accelerator
<table>
<thead>
<tr>
<th>Batch size</th>
<th>( \sqrt[10]{x} )</th>
<th>Total number</th>
<th>Classification according to level of contamination</th>
<th>Average count</th>
</tr>
</thead>
<tbody>
<tr>
<td>3000</td>
<td>22</td>
<td>20</td>
<td>9 10 1 0 0 0 0 0 0</td>
<td>0.9</td>
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<tr>
<td>3000</td>
<td>22</td>
<td>21</td>
<td>7 10 3 1 0 0 0 0 0</td>
<td>2.8</td>
</tr>
<tr>
<td>3000</td>
<td>22</td>
<td>21</td>
<td>7 11 2 0 1 0 0 0 0</td>
<td>3.5</td>
</tr>
<tr>
<td>3000</td>
<td>22</td>
<td>21</td>
<td>7 11 3 0 0 0 0 1 0</td>
<td>42</td>
</tr>
<tr>
<td>3000</td>
<td>22</td>
<td>21</td>
<td>5 11 4 0 0 0 0 0 0</td>
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</tr>
<tr>
<td>15000</td>
<td>49</td>
<td>104</td>
<td>35 53 13 1 1 0 1 0</td>
<td>10</td>
</tr>
</tbody>
</table>

TABLE XXII. DIVISION OF COUNTS FROM BATCH No.1 IN TABLE XXI IN 5 GROUPS
Treated as counts of 5 individual batched with 3000 units each.
Department at Risø for radiation sterilization. The size of the batches and the number of units taken from each batch is also shown. The sample size for each batch according to the $(4/10)\sqrt{N}$ rule suggested in WHO Technical Report Series No.200 (WHO, 1960) is shown for comparison.

The average count for all six batches is around 10, but the average count for the single batches varies from about 1 to more than 50. The fraction of donor sets showing no growth varied from 20-75% and the fraction of donor sets with counts less than 4 (including no growth) varied from 50-100%. 75% of the observed colonies were isolated from only 2.5% of the donor sets. The highest observed counts were around $10^3$.

The blank value of the bacteriological technique was 0.3.

Some of the variations observed may be a consequence of the fact that the production of these donor sets included several manual steps. It may, however, be mentioned here that some handling of blood bank equipment may be difficult to avoid in certain phases of the production, especially during quality control procedures (e.g. checks for airtightness).

We know from control of initial counts from hypodermic syringes of an automated production that average counts can be lower, but also here a major part of the microorganisms is isolated from less than 10% of the items.

Some of the variations in average count between the different batches of donor sets can be attributed to the sampling. In the first batch in Table XXI 104 units were taken out for control. This sample size is 5 times bigger than the sample size for the batch according to the $(4/10)\sqrt{N}$ rule. An evaluation of the influence of sample size on average count is given in Table XXII. The donor sets from the first batch were arbitrarily divided for the purpose into 5 groups and the counts from the sets in each group were then tabulated in the same way as in Table XXI, as if they represented the counts of 5 batches of the same size as batch No.1, 3000 units. It will be seen that the average count of the groups varies from about 1 to more than 40, a variation rather similar to that encountered in the practical control results in Table XXI.

If the sample taken for control of initial contamination consists of 20 units, a sudden increase in the occurrence of items with high contamination will be detected with 0.9 probability when about 10% of the items in the production are contaminated. It is necessary to take a sample consisting of 200 units if it is desired to have a 0.9 probability of detecting a frequency of occurrence of highly contaminated items as low as 1% (Lawrence and Block, 1968).

It is clear from the discussion above that a reasonable evaluation of the initial microbial contamination cannot exclude the possibility that a significant part of the equipment is contaminated with a high and unknown number of microorganisms. The minimum inactivation effect of the sterilization treatment for blood bank equipment must therefore also ensure against the possibility of rare accumulations of radiation-resistant germs on single items. A minimum inactivation factor of $10^8$ for radiation sterilization of blood bank devices is suggested in the IAEA's Recommended Code of Practice for Radiosterilization of Medical Products and this inactivation factor seems to give the necessary safety if the average initial contamination can be demonstrated to be low (e.g. counts below 50). If the sterilization standard is based on radiation-inactivation curves of reference standard preparations with \textit{Str. faecium} strain A21.
(see Part II, Chapter 10, sections 4 and 5), a minimum dose of 4.5 Mrad should be used for radiation sterilization of this class of equipment. Higher contamination levels need higher minimum doses, but it must be emphasized that higher doses should not be compensation for bad hygiene. 5 Mrad corresponds to an inactivation factor of approximately $10^{12}$ for Str. faecium.

Every possible effort should of course be taken to locate the sources of contamination when highly contaminated items are discovered. Although safely sterilized, high initial microbial counts will increase the risk of adverse reactions due to pyrogens, which are but little destroyed by sterilization (Sykes, 1965). The variation in initial counts may also reflect variation in the risk of contamination with dust particles, another potential hazard (Köster et al., 1959). An impression of the level of mechanical contamination may also be gained by microscopic inspection of membrane filters from the microbiological control of initial counts.

5. CONCLUSION

Blood bank equipment, devices for intravenous infusion and other items, where the consequences of insufficient sterilization can be fatal, must be produced under carefully controlled hygienic conditions and sterilized in a way that ensures that less than one item out of 1 million can contain microorganisms that have survived the sterilization process. In many cases radiation sterilization will be the only sterilization method available that can meet this demand. The minimum radiation dose for sterilization of this class of equipment is 4.5 Mrad.

Aspects other than sterility, such as toxicological quality and mechanical reliability, must also be carefully considered in the production and use of these devices.
CHAPTER 18

NON-BIOLOGICAL MATERIALS FOR IMPLANTATION AND FOR USE IN CONTACT WITH CIRCULATING BLOOD

In the choice of materials for use in medicine and surgery various criteria have to be satisfied: the physical and mechanical properties must be adequate after sterilization, the material should retain its physical and chemical stability during use, the material should not cause physical damage to the patient's tissues and it should be non-toxic.

In the case of disposable equipment that is manufactured and used on a large scale there is another requirement – that the cost should be kept as low as possible. Very frequently a material that is not necessarily the best technically, but whose performance is adequate, is chosen because it is less costly. For materials to be used as permanent or temporary implants, or in contact with circulating blood, technical requirements are more stringent and cost becomes a less important consideration. The design requirements are diverse so that, whereas for hypodermic syringes these requirements can be outlined fairly precisely, it may be better, because of the variable and changing needs for implanted devices, to outline the general principles involved in the choice of material and its sterilization as a guide for those who have to make these decisions.

For any device the primary objective is to benefit the patient and to do that it must perform its function adequately. There are two extremes to be avoided. One is obvious and one not so obvious. In the first instance care is needed in the choice of materials and the handling of equipment. It is not enough to decide to use PVC, or nylon, or polypropylene. There are many grades of PVC and some inexpensive plasticizers are even incompatible with the PVC so that they migrate to the surface. This would not be desirable for contact with blood. PVC also needs a stabilizer, which is the most expensive ingredient. When a grade containing too little stabilizer is irradiated the resultant degradation alters the physical characteristics of the material. This grade might be perfectly adequate for a hose to water the lawn but it is not suitable for contact with the patient's tissues or blood. Again, the word nylon covers a number of polyamides, those most generally available being 66, 610, 6, 11 nylon and various copolymers. Even though there are variations in melting point and degree of crystallinity, there is a considerable overlap as far as their physical properties are concerned and for some uses the choice of a particular nylon would not be critical. But nylons all contain a -CO·NH- group that can be hydrolysed. 6 nylon is more susceptible to hydrolysis than the others, while its breakdown products produce a greater tissue reaction, so whereas 6 and 66 nylon can be used interchangeably in the manufacture of clothing, they are not comparable in the manufacture of some items of surgical equipment. There are many other examples where a knowledge of both the properties of the polymers and of the reactions with the tissues are needed if a satisfactory choice of material and design is to be made.

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At the other extreme there is the need to avoid being overmeticulous. One type of precaution to avoid was exemplified when AERE first came into being and the disposal of effluent into the Thames was considered. Various published tolerance levels for isotopes were discussed, one of which was a tenth of the others because someone had decided to be on the safe side and divide the realistic level by ten. A member of a committee then suggested that this figure probably represented the most careful work so if it was divided by ten as an added precaution .... and the result was below the natural activity of the river. Toxicity considerations lend themselves to this type of error. Another trap to be avoided is to base recommendations or action on unverified speculation. One speculation has been that all tissue reaction is due to toxicity and to suggest a 'white list' of polymers and plastics, such as would be possible, to some extent, if they were drugs. But the major cause of tissue reaction is a mechanical abrasion of the tissues so that whether or not a particular polymer was placed on such a 'white list' would depend on the physical conditions of the test and the surface finish of the particular devices. It would be unrelated to other possible uses.

1. PHYSICAL AND MECHANICAL PROPERTIES AFTER STERILIZATION

The two most reliable methods of sterilization, which can be used for a wide range of materials, are the autoclave and irradiation. However, each can modify the properties of some materials so as to render them unacceptable. Thus, autoclave temperatures are above the melting point of polyethylene, while PTFE is seriously degraded by sterilizing doses of radiation. Here there is no choice – polyethylene must be irradiated and PTFE must be autoclaved. For other materials, such as polyethylene terephthalate (Terylene or Dacron), either method is satisfactory and the choice rests on such matters as convenience or cost. For complex devices, where saturation with steam would create problems, it is as well to choose materials and components that will withstand radiation.

So far as radiation sterilization of implants is concerned there is now no problem over the choice of radiation dose. During over ten years experience of all kinds of product, with a total that by now is of the order of thousands of millions of items, 2.5 Mrad has been found to be a satisfactory sterilizing dose (Part III, Introduction; Ley and Tallentire, 1965; Ley et al., 1967). For products manufactured in a sterile condition and not subsequently handled precautionary doses down to 1.0 Mrad are adequate. Implant devices tend to be handled more during their preparation and assembly than do disposable items manufactured on a large scale. They also require 100% inspection. This increases the risk of contamination prior to sterilization and it might be suggested that the irradiation dose should be raised as a precaution, but preparation under reasonably hygienic conditions is the better alternative. During this type of inspection and handling the main source of bacteria is the mouth. Experience in one tissue culture laboratory may be of interest in this connection. Over a period of almost ten years with no air conditioning and minimal precautions (no draughts, the surroundings washed down with
a bactericidal agent and instruments sterilized (Rigal, 1961)) there was no contamination of cultures provided that the operator breathed through his nose, kept his mouth shut and did not talk while handling the cultures. In discussing the effect of irradiation on polymers, therefore, it will be assumed that the radiation dose involved is 2.5 Mrad.

The effect of radiation on polymeric materials is to rupture chemical bonds. Low molecular weight fragments such as H₂ or HCl may be released as gas, the bonds may reform, or new bonds may form where the free ends are suitably positioned. Fairly stable free radicals can be formed and slow chain reactions initiated. Some bonds, particularly to oxygen and the halogens, tend to rupture irreversibly, while other groups, e.g. -C≡N or an aromatic ring, confer stability on their immediate neighbourhood. Generally stresses within the structure are released. Results may be modified by the surrounding atmosphere. Water if present may react with free ends of the molecular chains to prevent repolymerization and oxygen may take part in the initiation of oxidative chain reactions. All these events are influenced by local conditions at the site where energy is released. The presence of chemical groups or crystalline areas, which mechanically stabilize the molecular chain, can inhibit radiation degradation even at very high doses (Little, 1957).

The situation is, therefore, that some polymeric compounds are unstable to radiation because bonds break and substantial amounts of low molecular weight fragments are released. One example is PTFE and another is butyl rubber, which becomes fluid at comparatively low doses. Many more polymers, particularly when irradiated in the range of 1 to 5 Mrad, are stabilized. This is because internal stresses are released and cross-links formed. This stability is particularly noticeable as far as resistance to heat is concerned. For some polymers, e.g. polyethylene, the tendency to stress cracking is reduced.

During the irradiation of partly crystalline polymers the effect in ordered and disordered regions is frequently very different, even in some cases resulting in stabilization of the ordered regions and degradation of the disordered regions. The overall effect of irradiation for this group of polymers is thus very sensitive to the mould temperature or other conditions of preparation. Preparative methods leading to a highly ordered structure can result in a device whose properties are enhanced by an irradiation dose of 2.5 Mrad, whereas in products with regions of low crystallinity or ordering and with irregularly arranged molecules degradation reactions can dominate. This can occur in mouldings from the same initial batch of polymer. Polypropylene is an example of a polymer whose subsequent properties are very sensitive to the conditions of preparation.

Sometimes the atmosphere in which the irradiation takes place is important, particularly if oxidative chain reactions can be initiated. Absorbed water can also affect the results. An example of this is in the preparation and sterilization of collagen sutures.

One further point may be mentioned. The effect of irradiation sometimes leads to a product assuming a yellow or brown tint. Depending on the composition of the plastic, this colour can indicate either that some conjugated double bonds have formed – this often enhancing the physical properties – or that undesired degradation has taken place in the plastic. In the preparation of plastic disposable products colouring matter is sometimes added with the intention of masking these colour changes. So
far as implants are concerned, where the best possible performance is required, this practice is not recommended.

2. RETENTION OF PHYSICAL AND CHEMICAL STABILITY

Five separate categories of product have to be considered: resorbable implants; permanent implants; permanent implants in contact with circulating blood; and equipment or devices temporarily in contact with the tissues or with circulating blood.

Resorbable implants

Resorbable implants need to lose their stability at a predictable rate and also to degrade or break up into harmless fragments. The majority of resorbable implants in use are sutures, which have been considered in detail in a previous chapter.

Permanent implants

Of the many plastic materials that are now available only a few are suitable for permanent implantation. Low molecular weight compounds tend to either migrate or be leached out so that mixtures of ingredients, such as PVCs or natural rubbers, which depend on these to maintain their physical properties, can be eliminated immediately. Compounds liable to oxidative degradation, unless genuinely non-migratory anti-oxidants are incorporated, are also unsuitable. The most widely used polymers are medical grades of silicone, prepared under especially clean conditions. (The autoclave has been found to be better than irradiation for silicone rubbers.) Others that have been used successfully for certain purposes are PTFE, polyethylene, polypropylene epoxy-resins (these have been used to encapsulate electronic components, and are frequently covered with silicone), methacrylate, and some high-temperature and oxidation-resistant polymers. These all have a reasonable stability and the choice of grade is determined by:

(a) The proportion and degree of ordering of the non-crystalline regions after the device has been fabricated

(b) Their ability to form a surface with suitable properties (see next section)

(c) Their resistance to stress cracking.

After sterilization in 70% ethyl alcohol containing 0.5 hibitane (3 min) methyl methacrylate has been used, apparently successfully, for a number of years in sites involving stress, and then over a short period of time devices (e.g. prosthetic femoral heads) have broken up, cracked or shattered. Butyl and isobutyl methacrylate are less liable to failure from this cause. For some polymer types irradiation of the order of 2.5 Mrad improves the resistance to stress cracking. With the methacrylates, however, containing a high proportion of -C-O- links, it increases the probability of cracking and embrittlement.
Metals are also used as implant materials. They are subject to failure by stress corrosion, by electrolytic degradation, and by saline corrosion so that for metallic implants care has to be taken to choose corrosion-resistant materials. For such uses as nails, screws and plates to fix fractures, or joint replacement, certain grades of steel have been found to be adequate, while cobalt-chromium-molybdenum alloys have also been used. In sites where there is no applied stress, e.g. replacement of areas of the skull, titanium has been used. Conditions are more critical for wires inserted in the body and for electrical circuits and electrodes alloys of platinum with rhodium or irridium have been used. None of these metals are affected by irradiation.

Some 'permanent' implants, for example the fabric used to anchor devices such as the artificial breast or as vascular replacements, have their desired effect because they are impregnated and surrounded by fibrous tissue. This induced fibrous tissue then serves the main biological function so that a loss of physical strength of the original material can be tolerated.

Implants in contact with circulating blood

When implants are in contact with circulating blood requirements are more stringent than for other types of implant. Blood is a very good oxidizing and hydrolysing agent so that plastics containing chemical groups capable of being hydrolysed cannot be used. The nylon, which when used as a suture material loses its strength only slowly, loses almost all its tensile strength in a few weeks when in contact with blood (Harrison and Adler, 1956).

Another problem arises from the fact that patients who have conditions that necessitate the insertion of plastic devices into their circulatory system are frequently under stress, both physical and emotional. This gives rise to an increase in the circulating lipids. When there is turbulence in addition these lipids are deposited on the device (Pierie et al., 1968; Gibbons et al., 1969). Many plastics absorb lipids into their non-crystalline regions. When the blood lipids are absorbed properties such as wear resistance and impact strength diminish so that it would be unwise to use this type of polymer in a heart valve or other device with moving parts. Radiation, with its preferential degradation of non-crystalline regions of the polymer, could increase the amount of lipid uptake. For permanent implants in contact with circulating blood, therefore, the autoclave is to be preferred, unless positive evidence is available that the polymeric material under consideration does not absorb lipids after radiation sterilization.

Temporary implants

The criteria concerning the retention of physical and chemical stability for implants that are temporarily in contact with the tissues or blood are not so rigid as for permanent implants, but the same general principles hold. In particular, care must be taken that they do not contain necessary components that might migrate or be leached out. Even when used for only a few hours, heart-lung tubing should not contain migratory plasticizers or stomach tubing any component that can be attacked or dissolved by hydrochloric acid.
3. PHYSICAL DAMAGE TO THE TISSUES

Abrasive damage to the tissues is probably the most important cause of adverse tissue reactions to polymeric and other materials used in contact with the tissues (Little, 1968) but so far has been the least recognized. The reactions observed have often been mistaken for toxic or allergic reactions. Abrasive damage can occur with either temporary or permanent implants and is a function of the hardness of the surface of the material, its roughness — the proportion and type of hard edges and corners — and its movement relative to the tissues. Here there are three main categories of product to be considered: those in contact with the skin and epithelial surfaces, those in contact with other tissues, and those in contact with the blood. The two former differ in the extent of the damage caused by given degrees of hardness, roughness and movement, while the problems caused in circulating blood are somewhat different.

Contact with tissues

One method of assessing the relative importance of the different causative factors on the reaction of the tissues with different surface types is to implant specimens of a suitable size and shape subcutaneously in the guinea-pig. During the first week or so while wound healing is taking place the picture is somewhat confused, but by the end of the second week cell activity surrounding the implant is primarily the result of the reaction of that implant with the tissue. Should toxic chemicals be present in the implanted material, then small round darkly staining cells are seen. A resorbable implant, even if slowly resorbed, tends to have multinucleated phagocytic cells on its surface, and fragments removed are conveyed away from the site in the cytoplasm of macrophages. Dead tissue caused by movement of the implant or toxic materials acting at the surface of the implant is similarly removed. Should it be known that the material is non-toxic, then one would suspect surface contaminants.

During wound healing thin-walled blood vessels are found close to the surface of implanted materials. When the abrasive characteristics of the surface are such that the vessel walls are snagged then local bleeding and a further wound healing reaction takes place. The more liable these vessels are to snagging, the greater the vascular and fibrous reaction round the material, and the thicker the wall of the ensuing fibrous capsule. This type of abrasion implies at least some movement of the implant relative to its surroundings. The surface of a soft silicone rubber, even with a little movement, rarely damages the cells and vessels. Harder surfaces, if very smooth, also cause minimal damage, but when rough they will break adjacent vessels. Thus a polyethylene implant for plastic surgery causes very little reaction, but if it is machined by the surgeon to alter its shape, there may be an unacceptably prolific reaction. Hard filler particles protruding from the surface also cause this type of damage and first led to its recognition (Little and Parkhouse, 1962).

Sometimes, with an awkwardly shaped implant, movement of the whole implant relative to the tissues can cause trauma with repeated wound healing. For rats, but not for other species such as the guinea-pig, this has been reported as causing a fibrosarcoma (Oppenheimer et al., 1948; 1956). These reports have given rise to a fear that plastics might
be carcinogenic, but the supposition would seem to be unjustifiable. In the first place the experimental animal involved was the rat, which is abnormally liable to sarcoma formation, and secondly, the carcinogenic mechanism involved was not a chemical one but the effect of repeated trauma and tissue repair. For any prosthetic device that would itself cause concern long before the malignant change was imminent.

**Contact with blood**

Observation on clotting mechanisms suggest that as far as contact with blood is concerned there is always a possibility of clot formation when a solid surface is in contact with the blood, as opposed to a fluid surface or a gel. These observations are consistent with the suggestion that clotting and embolus formation are minimal when the surfaces are smooth and are encouraged by rough surfaces.

4. **TOXICITY**

Although the majority of adverse reactions to materials and devices are due to their physical state, and sometimes to their lack of physical stability, there are occasions when toxic chemicals may be present. This is rare for implanted polymers because, with the limitations outlined in the section on physical and chemical stability, those likely to have toxic components are rejected on other grounds. A few possibilities remain. One is that a catalyst or accelerator is retained in sufficient quantity to show toxic effects. This is unlikely, but possible. One fact that needs to be remembered is that there is a toxicity threshold concentration for each material and below this threshold it is non-toxic, even when larger quantities are harmful. Again, it is possible that a breakdown product may have toxic properties. This is a problem that is relevant to resorbable implants. Surface contaminants are another possible cause of toxic reactions.

There remains the possibility that irradiation of a plastic might itself give rise to toxic products since irradiation does affect these materials chemically. The effect of irradiation on PTFE is to produce low molecular weight fluorides, which have been observed to kill surrounding tissues, but PTFE is one of the materials for which radiation sterilization is never recommended. Few other polymers have toxic breakdown products. Pellets of 66 nylon, for example, which had been irradiated in BEPO for 6 weeks until all the crystalline areas had degraded, showed no sign of any toxic reaction when implanted subcutaneously in a guinea-pig.

Restereilization with irradiation after sterilization with ethylene oxide is undesirable. Should ethylene oxide or its reaction products have been retained in the article, their irradiation reaction products are likely to be even more toxic than the ethylene oxide itself.

In this account of the use of non-biological materials for implantation the aim has been to give an outline of the general principles involved in the choice of a material and its sterilization. The possible applications are increasing and few definite conclusions can yet be reached. It is apparent,
however, that technical criteria are the most important and cost or convenience have to take second place. This is in contrast to the approach that has sometimes dominated for disposable items. Usually the nature of the material or technical factors concerned with its use determine which method of sterilization is best. Thus, silicone rubbers retain their properties better after sterilizing with the autoclave. For PTFE there is no choice. Similarly, for other materials affected by heat or steam radiation sterilization is needed. When the various technical factors are evenly balanced, then sterilization by radiation soon after manufacture, with a lower risk of subsequent contamination, could be a deciding factor.
CHAPTER 19
MEDICINES AND PHARMACEUTICAL
BASE MATERIALS

The successful use of ionizing radiation for sterilizing medical supplies and equipment has given considerable impetus to the application of this method for the sterilization of pharmaceuticals (IAEA, 1967a), particularly where conventional methods have proved inadequate. A difficulty, however, is that certain undesirable chemical and physical changes may accompany treatment with a sterilizing dose of irradiation. Indeed, the development of the subject has been inhibited because so little definite information is available about the extent and nature of the chemical changes induced by irradiation of organic materials used in the pharmaceutical industry. Attention has mainly been focussed on loss of potency, colour changes, acid formation, or the production of ultra-violet absorbing species which accompany irradiation, and undoubtedly this superficial attitude has been detrimental to the subject.

1. ANTIBIOTICS AND OTHER DRUGS

1.1. Solid-state systems

Generally, pharmaceuticals are considerably more stable in the dry solid state to ionizing radiations than in any other form of molecular aggregation and only small losses in activity are found under these conditions. Table XXIII presents the data on irradiation of dry solid samples. Streptomycin and its derivatives were studied by Pochapinskii et al. (1961). Streptomycin and dihydrostreptomycin sulphates, a calcium chloride-streptomycin complex, dihydrostreptomycin p-amino salicylate and streptopenicillin (streptomycin and dihydrostreptomycin sulphates with the sodium, potassium and procaine salts of benzyl penicillin) were each submitted to a dose of 2.5 Mrad. Only for freeze-dried streptomycin and dihydrostreptomycin could a change in activity be observed, although 'dulling' of the crystals was evident. Changes in colour were also reported for several other drugs at this dose level (Controulis et al., 1954; Horne, 1958; Association of the British Pharmaceutical Industry, 1960) and sometimes these were accompanied by changes in pH of solutions prepared from the irradiated drugs (Grainger, 1957).

Practical interest, however, often centres on ointments containing the drugs and other vehicular materials added either for consistency or palatability. There is little doubt, however, that even such ointments can be successfully sterilized by ionizing radiations without loss of potency. We cite the following, which have been successfully treated in this manner.

(i) Ophthalmic ointments containing tetracycline hydrochloride, oxytetracycline, chlortetracycline (dose 2.0 Mrad) (Pochapinskii, 1962).
TABLE XXIII. LOSS OF POTENCY OF SOLID PHARMACEUTICALS ON IRRADIATION

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (Mrad)</th>
<th>Loss of potency (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlortetracycline</td>
<td>1.79 - 10</td>
<td>0</td>
<td>Controulis et al., 1954; Holland et al., 1967</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>1.79 - 10</td>
<td>0</td>
<td>Controulis et al., 1954; Holland et al., 1967; Grainger and Hutchinson, 1957</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1.79</td>
<td>0</td>
<td>Controulis et al., 1954</td>
</tr>
<tr>
<td>Tetracycline hydrochloride</td>
<td>8</td>
<td>0</td>
<td>Grainger and Hutchinson, 1957</td>
</tr>
<tr>
<td>Streptomycin hydrochloride</td>
<td>2.5</td>
<td>0</td>
<td>Horne, 1958; Association of the British Pharmaceutical Industry, 1960</td>
</tr>
<tr>
<td>Sodium benzyl penicillin</td>
<td>2.5</td>
<td>0</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Phenoxymethyl penicillin</td>
<td>2.5</td>
<td>0</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Benzathine penicillin</td>
<td>2.5</td>
<td>0</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td>2.5</td>
<td>0</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Potassium benzyl penicillin</td>
<td>1.79</td>
<td>0</td>
<td>Controulis et al., 1954</td>
</tr>
<tr>
<td>Polymyxin sulphate</td>
<td>2.5 and 25.0</td>
<td>0</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Polymyxin</td>
<td>up to 8.0</td>
<td>0</td>
<td>Grainger and Hutchinson, 1957</td>
</tr>
<tr>
<td>Colimycin</td>
<td>up to 8.0</td>
<td>0</td>
<td>Grainger and Hutchinson, 1957</td>
</tr>
<tr>
<td>Nystatin</td>
<td>up to 8.0</td>
<td>0</td>
<td>Grainger and Hutchinson, 1957</td>
</tr>
<tr>
<td>Mycerin</td>
<td>up to 8.0</td>
<td>0</td>
<td>Grainger and Hutchinson, 1957</td>
</tr>
<tr>
<td>Sulphapyridine</td>
<td>2.5 and 25.0</td>
<td>0</td>
<td>Horne, 1958</td>
</tr>
<tr>
<td>Sulphathiazole</td>
<td>2.5 and 25.0</td>
<td>0</td>
<td>Horne, 1958</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>2.5</td>
<td>3</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Streptomycin sulphate</td>
<td>25.0</td>
<td>5</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Dihydrostreptomycin</td>
<td>25.0</td>
<td>5</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Neomycin sulphate</td>
<td>2.5</td>
<td>4</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Sodium benzyl penicillin</td>
<td>25.0</td>
<td>3</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Benzathine penicillin</td>
<td>25.0</td>
<td>3</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Phenoxymethyl penicillin</td>
<td>25.0</td>
<td>3</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Zinc bacitracin</td>
<td>2.5</td>
<td>7.1</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Zinc bacitracin</td>
<td>25.0</td>
<td>26.7</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
</tbody>
</table>
(ii) Ointment containing chloramphenicol and hydrocortisone acetate (no loss of antibiotic activity after 5.0 Mrad) (Hangay et al., 1967a).

(iii) Ointment of neomycin and hydrocortisone (Wills, 1963).

(iv) Ointment containing polymyxin sulphate (2.0 Mrad) (Pochapinskii, 1962).

(v) An ointment of polymyxin sulphate and zinc bacitracin in soft paraffin showed no decomposition of the drug even at 25.0 Mrad. However, gas formation from the paraffin caused distension of tubes even at 2.5 Mrad and extrusion of ointment occurred at doses in excess of 8.0 Mrad (Horne, 1958; Association of the British Pharmaceutical Industry, 1960).

A general point should, therefore, be made here. While the drugs when irradiated alone or in a normal vehicle may themselves be stable to the sterilizing dose, there are frequently undesirable effects found as a result of chemical changes induced in the vehicle. Those expecting radiation treatment to be a panacea applicable to all states and mixtures have consequently been disappointed. In our view the best value will be obtained from the method if the optimum conditions are sought. We shall be continually making this point throughout this Chapter.

1.2. Aqueous solutions

Pharmaceuticals are generally more susceptible to irradiation in aqueous solution than in the solid state. This is because reactive species and molecular products are produced by radiolysis of water, which induces chemical changes in almost any type of organic compound:

\[
\text{H}_2\text{O} \longrightarrow \text{H}^+, \text{e}_\text{aq}, \text{OH}^-, \text{H}_2, \text{H}_2\text{O}_2
\]

While it is beyond the scope of this Chapter to consider the radiation chemistry of aqueous solutions, it must be pointed out that certain well-defined principles are now established in this field. The current practice in almost all studies undertaken on aqueous solutions of pharmaceuticals and medicines does not take account of these principles. Solutions are irradiated without control of solute concentration, degree of oxygenation, presence or absence of radical scavengers, buffers, etc., with the result that sometimes there is little reproducibility or correlation between individual investigators. We wish to draw attention to this serious deficiency and urge that future studies be carried out under the more controlled conditions demanded by the development of radiation chemistry. Only in this way will it be possible to correlate individual investigations and extrapolate findings to any particular conditions that might be necessary in practice. The development of pulse radiolysis (Matheson and Dorfman, 1969) too allows the reactivity of the individual products of water radiolysis to be determined accurately with any particular solute so that the element of guesswork, so marked up to this time, can be considerably eliminated.

The susceptibility of aqueous solutions of drugs is clearly illustrated by the complete inactivation of aqueous solutions of oxytetracycline and chlortetracycline by 0.3 Mrad (Holland et al., 1967). Degradation could even be observed with doses as low as 10 krad.
Another point of principle of particular relevance to solution irradiation deserves mention here. Whereas dose is a completely satisfactory method of measuring the radiation absorbed by the sample per unit weight or volume (eV \cdot g^{-1} or eV \cdot ml^{-1}), the total chemical change found in aqueous solution, it must be understood, will depend on energy input to the total solution (dose \times volume or weight = eV). Within the limits of dose rate (eV \cdot ml^{-1} \cdot min^{-1}) used in this work, no variations in yield are generally observed with this parameter. Yet frequently investigators have expected the same amount of chemical change whether the sample of unit weight was irradiated at a particular dose rate in 1 ml or 1000 ml of water. The confusion could be eliminated if all product yields or extents of decomposition were expressed as G-values (yield of product formed or material decomposed in molecules per 100 eV energy input), using the total energy input to the solution and stating the concentration. The present practice of expressing percentage decompositions occurring after a certain dose should definitely be discouraged.

A particular example of an aqueous system that has been extensively studied is sulphacetamide. Single dose units of ophthalmic ointment containing 6% sulphacetamide and eye drops containing 10% sulphacetamide are now being commercially marketed in the United Kingdom following sterilization by \(\gamma\)-irradiation (Chemist and Druggist, 1968; Phillips et al., 1971). Here a summary is given of the findings with aqueous sulphacetamide irradiation as an indication of the type of information that is required if the radiation sterilization method is to find reliable usage in the pharmaceutical field.

Pulse radiolysis and steady-state experiments demonstrate that hydrated electrons (\(e_{aq}\)) and hydroxyl radicals (OH) are mainly responsible for sulphacetamide degradation on \(\gamma\)-irradiation between \(10^{-4}\) and 1.2M solute concentration. Accurate rate constants for the reaction of these species with sodium sulphacetamide were \(k_2 = 4.1 \times 10^9 M^{-1} \cdot s^{-1}\) for the reaction with \(e_{aq}\) and \(k_\text{OH} = 4.7 \times 10^9 M^{-1} \cdot s^{-1}\) for the reaction with OH radicals. Reaction with each of these individual species leads to specific chemical products. Reaction with \(e_{aq}\) yields sulphanilic acid and an additional unidentified product. Hydroxyl radicals give monomeric and dimeric phenolic derivatives of sulphacetamide. Suitable \(e_{aq}\) and OH scavengers can be added to the reactive mixture that selectively remove these products and prevent degradation. G(-sulphacetamide), which in radiation chemistry is defined as the number of sulphacetamide molecules destroyed per 100 eV energy input, reaches a maximum of 4.8 in aqueous solution at the highest concentration studied, namely 1.2M, but G(-sulphacetamide) is < 0.01 for the solid. The practical implications are that sterilization of sulphacetamide by 2.5 Mrad in the solid state offers no difficulty since no significant degradation occurs. For the concentrated solutions normally used in eye preparations (20 and 30%) the decomposition by a dose as large as 5 Mrad is extremely small and compares satisfactorily with alternative conventional procedures. Since the precise amounts and nature of degradation products are now known, it is possible to state with confidence that the method of radiation sterilization by 2.5 Mrad is both reliable and safe. Finally the success of adding suitable radical scavengers to prevent degradation of the solute points to the practicability of adding comparable scavengers, for example, bicarbonate or other additives, that can be tolerated in pharmaceutical preparations and thus of reducing decomposition even further if this proves necessary. Table XXIV indicates the extent of the decomposition as -G values under a variety of conditions.
TABLE XXIV. G-VALUES FOR SULPHACETAMIDE DISAPPEARANCE AND SULPHANILIC ACID FORMATION ON γ-IRRADIATION

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Solute (M)</th>
<th>Scavenger (M)</th>
<th>-G</th>
<th>G(sulphanilic acid)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Argon 10^-3</td>
<td>-</td>
<td>3.6</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Argon 10^-2</td>
<td>4 x 10^-1 a</td>
<td>2.2</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td>Argon 10^-3</td>
<td>2 x 10^-1 b</td>
<td>-</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>N2O 10^-2</td>
<td>-</td>
<td>2.9</td>
<td>~0.1</td>
<td></td>
</tr>
<tr>
<td>O2 10^-4</td>
<td>-</td>
<td>2.5</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>O2 10^-3</td>
<td>-</td>
<td>2.7</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>O2 10^-2</td>
<td>-</td>
<td>4.1</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>O2 10^-1</td>
<td>-</td>
<td>4.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>O2 1.2</td>
<td>-</td>
<td>4.8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Air Solid</td>
<td>&lt; 0.01</td>
<td>&lt; 0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Ethanol.  
b Acetone.

Another system extensively studied at the Department of Chemistry and Applied Chemistry, University of Salford, is penicillin. The G-values for disappearance of penicillin G (10^-5M) in argon and N2O saturated solutions at pH 7.0 are 5.9 and 6.5 respectively. Since no loss of biological activity is observed after γ- radiolysis of sodium benzyl penicillin in the solid state, it would appear that the high -G values obtained are a result of a chain mechanism. Second-order rate constants for the reaction with eaq and OH are 2.7 x 10^9 M^-1.sec^-1 and 3.4 x 10^8 M^-1.sec^-1 respectively.

The nature of certain of the radiolysis products formed by attack of eaq and OH on 10^-5M sodium benzyl penicillin are shown in Table XXV. Hydrolysis of γ-irradiated sodium benzyl penicillin yields o-, m- and p-hydroxy phenyl acetic acid. Penilloic, penilloicoic acids and the penichlloyl dimer are probably formed by initial proton abstraction by OH from the β-lactam ring. Penilloic acid, on the other hand, appears to be formed by direct attack of eaq on the β-lactam carbonyl group. The hydrated electron also reacts directly with the >S of the thiazolidine ring to give rise to thiol groups. Attack by OH on the amide side chain group appears to yield phenyl acetic acid and 6-amino penicillanic acid. Addition of OH radicals to the phenyl ring yields o-, m- and p-hydroxylated benzyl penicillins in a ratio of 2:1:1.

Since this Manual is intended mainly as a working document for potential users of the radiation sterilization method, we again stress here the desirability of obtaining quantitative data of this sort on the system requiring radiation treatment. Susceptibility to radiation damage is not an absolute quantity, a type of magic number that applies under all conditions. Degradation depends on dose, solute concentration, atmosphere, scavengers present, etc. Since these are directly related to the reactivity of the solute to water radiolysis products, each factor is capable of independent and rational evaluation. Thus, even if a material under the required commercial
2. VITAMINS

Colovos and Churchill (1957) irradiated sealed dry samples of two multivitamin preparations to a dose of 1.86 Mrad and stored them for four years at 25°C before analysis. The effects of irradiation on one such preparation are shown in Table XXVI. The amounts of the individual vitamins present do not change significantly after irradiation.

Dry dilution of thiamine HCl in calcium carbonate and calcium biphosphate were irradiated and the main breakdown products were found to be thiamine disulphide and thiochrome in the calcium carbonate mixture and 4-methyl 5-β-hydroxy ethyl thiazole and thiochrome in the calcium biphosphate mixture (Takahashi, 1965).

Pyridoxine shows the typical behaviour that we have already noted. It is stable in the solid state but highly susceptible to radiation damage in aqueous solution when aldehydes, phenols and carboxylic acids were formed (Galatzeanu and Antoni, 1967). In 0.1N HCl solution the degradation by
TABLE XXVI. EFFECTS OF IONIZING RADIATION ON A MULTIVITAMIN PREPARATION (Colovos and Churchill, 1957)

<table>
<thead>
<tr>
<th>Components present</th>
<th>Weight Before irradiation (mg)</th>
<th>Weight After irradiation (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiamine</td>
<td>7.5</td>
<td>7.3</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>9.4</td>
<td>9.2</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Calcium pantothenate</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>248.0</td>
<td>250.0</td>
</tr>
</tbody>
</table>

2 Mrad was extensive but was less in $10^{-3}$ M sodium hydroxide and $10^{-1}$ M sodium chloride. In ethanolic solution there is no significant decomposition. In addition, it was found that the addition of ascorbic acid to pyridoxine stabilized the compound, possibly due to the formation of a hydrogen-bonded complex (Galatzeanu and Antoni, 1966). In any event, the observations substantiate our claim that the added materials profoundly influence the course of degradation. All the materials added in this instance are known to react with certain of the products of water radiolysis. For example, there is a facile reaction $e_{aq}^- + H^+ \rightarrow H$ and when acid and NaCl are present OH radicals are scavenged by the reaction

$$H^+ + OH + Cl^- \rightarrow OH^- + Cl$$

However, it would have been desirable to have more detailed information to evaluate the practical value of the results fully.

Early work on the radiation stability of vitamin B₁₂ was carried out in connection with the production of radioactively labelled cyanocobalamin, the labelling atom being one of a number of cobalt isotopes. Instability of such compounds as cyanocobalamin-$^{60}$Co was investigated by Mollin (1955) and Herzmann and Hennig (1962). Suitable storage conditions were recommended by Smith (1959). The effects of $\gamma$-irradiation on aqueous solutions of the vitamin were studied by Sjöstedt and Ericson (1962), who irradiated cyanocobalamin in various buffer solutions and demonstrated the existence of at least five breakdown fragments that retained biological activity. The doses used by these workers were rather high (up to 3.0 Mrad) and no conclusion can be drawn from these results as to the initial mechanism of radiolytic breakdown. Herzmann and Hennig (1962) showed that the effect of very large concentrations of ethanol reduced the amount of cyanocobalamin destroyed and this is almost certainly the result of complete scavenging of the radical species that are responsible for the breakdown of the cyanocobalamin. No conclusions were drawn by these workers about which of the radical species were responsible for breakdown, and it is not clear to what extent these results refer to oxygenated or deoxygenated solutions.

In an attempt to elucidate the mechanism of initial breakdown, it is necessary to use smaller doses of radiation and recent work is summarized...
### TABLE XXVII. EFFECT OF IONIZING RADIATION ON CYANOCOBALAMIN

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Dose (Mrad)</th>
<th>Vitamin remaining (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mg/ml in 0.05 M acetate buffer</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 3</td>
<td>3.0</td>
<td>20</td>
<td>Sjöstedt and Ericson, 1962</td>
</tr>
<tr>
<td>pH 5</td>
<td>3.0</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>pH 7</td>
<td>3.0</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0.1 wt/vol(%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>10</td>
<td>Horne, 1958; Association of the British Pharmaceutical Industry, 1960</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>52 ppm degassed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 1</td>
<td>$6.8 \times 10^{-2}$</td>
<td>69.7</td>
<td></td>
</tr>
<tr>
<td>pH 7</td>
<td>$6.8 \times 10^{-2}$</td>
<td>73.1</td>
<td></td>
</tr>
<tr>
<td>pH 10</td>
<td>$6.8 \times 10^{-2}$</td>
<td>75.0</td>
<td></td>
</tr>
<tr>
<td>52 ppm oxygen gassed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 1</td>
<td>$6.8 \times 10^{-2}$</td>
<td>78.3</td>
<td>Blackburn, Cox and Phillips, unpublished</td>
</tr>
<tr>
<td>pH 7</td>
<td>$6.8 \times 10^{-2}$</td>
<td>82.7</td>
<td></td>
</tr>
<tr>
<td>pH 10</td>
<td>$6.8 \times 10^{-2}$</td>
<td>84.6</td>
<td></td>
</tr>
<tr>
<td>52 ppm N₂O gassed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 1</td>
<td>$6.8 \times 10^{-2}$</td>
<td>68.3</td>
<td></td>
</tr>
<tr>
<td>pH 7</td>
<td>$6.8 \times 10^{-2}$</td>
<td>66.7</td>
<td></td>
</tr>
<tr>
<td>pH 10</td>
<td>$6.8 \times 10^{-2}$</td>
<td>70.3</td>
<td></td>
</tr>
<tr>
<td>52 ppm degassed + tert butanol (10⁻² M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 1</td>
<td>$6.8 \times 10^{-2}$</td>
<td>74.2</td>
<td></td>
</tr>
<tr>
<td>pH 7</td>
<td>$6.8 \times 10^{-2}$</td>
<td>78.0</td>
<td></td>
</tr>
<tr>
<td>pH 10</td>
<td>$6.8 \times 10^{-2}$</td>
<td>76.9</td>
<td></td>
</tr>
<tr>
<td>Degassed solid</td>
<td>90</td>
<td>90</td>
<td></td>
</tr>
</tbody>
</table>

In degassed solutions cyanocobalamin is least stable in acid (pH1) conditions, being characterized by a G-value for disappearance of about 1.5. In neutral and alkaline conditions the G-value falls to about 1.25. The effect of oxygen is to lower the radiosensitivity of cyanocobalamin by 20-30% and the effects of t-butanol at a concentration of 500 μM/litre is comparable. Nitrous oxide increases the radiosensitivity. There is a marked concentration dependence at vitamin concentrations below $2.9 \times 10^{-4}$ M, but at this concentration the G-value for disappearance of cyanocobalamin approaches that of the total yield of reducing species. These workers have noticed four breakdown products, three of which contain cobalt. It appears from this work that oxidizing species (OH, H₂O₂) have little or no role in the radiation degradation of the cyanocobalamin, whereas the primary reducing radicals (H atoms and e⁻) lead to reduction of the Co³⁺ in the central corrin ring. Reduction of Co³⁺ to Co²⁺ diminishes the intensity of absorption of the
absorption peaks at 278, 361 and 550 nm and post-irradiation oxidation partially restores these peaks. Comparison with a synthetic standard of vitamin B12r has shown that it is this compound that is responsible for the post-irradiation oxidation effect, and no doubt the apparent protective effect of oxygen during irradiation results from continuous re-oxidation of the reduced radiolytic products. Spectral shifts show that hydroxocobalamin is formed as a result of this reduction-oxidation step. Cobalt (as Co²⁺) is not produced at low doses and may be excluded as an initial product. Preliminary results for hydroxocobalamin solution suggest that this compound is rather less sensitive to radiation; incidentally it is now favoured in preference to cyanocobalamin for pharmaceutical use.

Solid-state irradiation of cyanocobalamin in the absence of oxygen also results in the formation of a reduced form. Preliminary G-values obtained are \( G(-\text{cyanocobalamin}) = 1.0 \), \( G(+\text{reduced form}) = 0.6 \) and \( G(+\text{inorganic cobalt}) = 0.1 \).

An aqueous solution of p-aminobenzoic acid (0.3%) was irradiated to doses of 2 and 4 Mrad under conditions of both oxygen and nitrogen saturation. The main radiolysis products were found to be p-nitrobenzoic acid in oxygen and 4-amino 3-hydroxybenzoic acid in nitrogen (Marriott, 1963).

Ascorbic acid in the dry state is stable to 2.5 Mrad, but in dilute aqueous solution (20 mg/ml) it is destroyed by \( \gamma \)-radiolysis (Association of the British Pharmaceutical Industry, 1960) and X-radiolysis (Proctor and Goldblith, 1949). Concentrated solutions (280 mg/ml) were irradiated to 1.9 Mrad and showed little loss of potency (Controulis et al., 1954) and the amount of ascorbic acid destroyed for a given dose was similar to that in the dilute sample. This behaviour is unusual and does not accord with the findings for other carbohydrate systems discussed later.

Niacin was found to be more stable than riboflavin or ascorbic acid to X-rays. Riboflavin was stabilized by the addition of oxalic acid (Proctor and Goldblith, 1949). Solid methyl linoleate in lactose was degraded by irradiation to a conjugation type hyperoxide (Takahashi and Ishihara, 1965).

The order of radiation stability of the fat soluble vitamins was found to decrease in the order vitamin K, cholecalciferol, vitamin A, vitamin E (Knapp and Tappel, 1961). Calciferol and ergosterol are stable between 6-18 Mrad in the absence of air, but the presence of oxygen causes 15% breakdown in the latter (Fazakerley, 1960). A tocopherol irradiated to 2.0 Mrad as a 0.01% solution in methylmyristate underwent 90% destruction in vacuo and was completely decomposed in the presence of oxygen.

3. STEROIDS AND HORMONES

A number of ointment formulations containing hydrocortisone and neomycin (Wills, 1963) and one containing hydrocortisone acetate and chloramphenicol (Hangay et al., 1967a). Irradiated cortisone acetate injection was found to be unchanged chemically and toxicologically (Colovos and Churchill, 1957).

Many workers have studied the effects of ionizing radiations on pure samples of steroids and their results are given in Table XXVIII. Keller and Weiss (1950) investigated the action of X-rays on cholesterol and 3β-hydroxy-pregn-5-en-20-one in aqueous systems, and later (Keller and Weiss, 1951) on (+) oestrone-b. Their results are summarized in Table XXIX.
<table>
<thead>
<tr>
<th>Dose (Mrad)</th>
<th>Compound</th>
<th>Conditions</th>
<th>Effect</th>
<th>Activity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>Pregnenolone acetate</td>
<td>Aqueous suspension</td>
<td>Slight lowering of melting point</td>
<td>100</td>
<td>Tarpley et al., 1954</td>
</tr>
<tr>
<td>2.5</td>
<td>Deoxycorticosterone acetate</td>
<td>Solid in air</td>
<td>Slight discoloration</td>
<td>100</td>
<td>Hortobágyi et al., 1967</td>
</tr>
<tr>
<td>6 - 18.0</td>
<td>Prednisone</td>
<td>Air and in vacuo</td>
<td>Unchanged</td>
<td>100</td>
<td>Fazakerley, 1960</td>
</tr>
<tr>
<td>18.0</td>
<td>Cortisone</td>
<td>Air and in vacuo</td>
<td>Unchanged</td>
<td>100</td>
<td>Fazakerley, 1960</td>
</tr>
<tr>
<td>12.7</td>
<td>Cortisone acetate</td>
<td>Air</td>
<td>Slight lowering of melting point</td>
<td>100</td>
<td>Tarpley et al., 1964</td>
</tr>
<tr>
<td>2.5</td>
<td>Hydrocortisone</td>
<td>Air</td>
<td>Turned yellow</td>
<td>slight loss</td>
<td>Hortobágyi et al., 1967</td>
</tr>
<tr>
<td>2.5 - 5.0</td>
<td>Hydrocortisone acetate</td>
<td>Air</td>
<td>Turned yellow</td>
<td>100</td>
<td>Hortobágyi et al., 1967</td>
</tr>
<tr>
<td>1.9</td>
<td>Oestrone</td>
<td>Aqueous suspension</td>
<td>Turned yellow</td>
<td>8.1.</td>
<td>Controulis et al., 1954</td>
</tr>
<tr>
<td>3.74</td>
<td>Oestrone</td>
<td>Solution in N sodium hydroxide</td>
<td>13% yield of 2-hydroxyoestrone</td>
<td>70</td>
<td>Wheeler and Montalvo, 1965; 1967</td>
</tr>
<tr>
<td>2.5</td>
<td>Oestradiol benzoate</td>
<td>Solid in air</td>
<td>Unchanged</td>
<td>100</td>
<td>Hortobágyi et al., 1967</td>
</tr>
<tr>
<td>2.5</td>
<td>stilboestren dipropionate</td>
<td>Solid in air</td>
<td>Unchanged</td>
<td>100</td>
<td>Hortobágyi et al., 1967</td>
</tr>
<tr>
<td>2.5</td>
<td>Progesterone</td>
<td>Solid in air</td>
<td>Turned pale yellow</td>
<td>100</td>
<td>Hortobágyi et al., 1967</td>
</tr>
<tr>
<td>6 - 18.0</td>
<td>Progesterone</td>
<td>Solid in air</td>
<td>Turned pale yellow</td>
<td>100</td>
<td>Horne, 1958; Association of the British Pharmaceutical Industry, 1960</td>
</tr>
<tr>
<td>Substance</td>
<td>Conditions</td>
<td>Dose (x 10^5 R)</td>
<td>Starting material (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------------------------------------</td>
<td>-----------------</td>
<td>-----------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na cholesteryl succinate</td>
<td>0.26% aqueous solution in air</td>
<td>1.8</td>
<td>Cholestane-3β : 5α : 68 triol (19%)</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Cholesteryl acetate</td>
<td>0.17% in aqueous; 90% acetic acid in air</td>
<td>1.8</td>
<td>3β : 68 diacetoxycholestane-5α-ol (32%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3β-acetoxycholestane-5α : 68-diol (48%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.16% in aqueous; 90% acetic acid in air</td>
<td>3.6</td>
<td>Cholesteryl acetate (4.5%)</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3β-cholesta-5-en-7-one (17.5%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cholestane-3α : 5α : 68 triol (37.5%)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.5% in glacial acetic acid in vacuum</td>
<td>2.1</td>
<td>Cholesteryl acetate 3β-68-diacetoxycholestan 5α-ol (4%)</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>3-hydroxy-pregna-5-en-20-one</td>
<td>0.9% in aqueous acetic acid</td>
<td>2.7</td>
<td>68-acetoxy-3β-5α-dihydroxyallopregna-20-one (18%); 3β : 5α : 68-trihydroxyallopregna-20-one (25%)</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>+ Oestrone-b</td>
<td>0.5% in alkaline solution</td>
<td>~ 3</td>
<td>Lactone ring formed by a CO₂H group involving C₁₇ and an OH group at C₁₆ - called Westerfield's lactone</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>+ Oestrone-b</td>
<td>0.1% in 90% acetic acid</td>
<td>~ 3</td>
<td>Westerfield's lactone</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Aqueous solutions of cortisone and deoxycorticosterone (Allinson et al., 1955) have been irradiated with X-rays (200 kV). When deaerated solutions were irradiated, dehydroxylation in the 17α or in the 2-position of deoxycorticosterone occurred, together with hydrogenation of the double bond of cortisone and deoxycorticosterone. The following processes also occur with cortisone solutions: (i) complete reduction of the Δ4-3-oxo system to give 3β-hydroxy derivatives; (ii) reduction of the 3-oxo group (before reduction of the Δ4-double bond occurs); (iii) addition of one hydroxyl group and one hydrogen atom to the double bond; and (iv) elimination of a carbon atom from ring A to give A-nor-3-oxo-steroids. This was the most important single reaction. A small amount of adrenosterone, formed by elimination of the side chain of cortisone, was also isolated.

Several steroids have been irradiated in organic solvents (Coleby et al., 1954). When irradiated by large doses of X-rays (205-kV 15 mA X-rays for 15 h) cholesterol, cholest-2-ene, cholest-5-ene, and cholest-3-one in methanol suffer little change. Cholesterol in methanol gives 3β-hydroxycholest-5-en-7-one, cholest-5-en-3β-diol and cholestone-3β-5α:6β-triol. Irradiation of cholesterol in acetone and dioxan produces cholesterol α- and β-oxide (5α:6α epoxycholestan-3β-ol and 5β:6β epoxycoprostogen-3β-ol) together with cholest-5-en-3β:7β-diol. Cholesteryl acetate in acetic anhydride gives cholesteryl acetate α- and β-oxide. A mixture of cholesteryl acetate α- and β-oxide in aqueous acetic acid gives 3β-acetoxycholestone-5α:6β-diol. When cholesteryl benzoate α- and β-oxide are irradiated in methanol, acetone or dioxan, only the α form is converted into the β-oxide. Cholest-4-en-3-one in methanol gives 6β-hydroxy-cholest 6β-hydroxycholest-4-en-3-one. Hydroxylation in the 6-position presumably also occurs when progesterone is irradiated in methanol, but only the isomerization product, allpresnane-3β:6β:20-brione, could be isolated in a pure condition.

### TABLE XXX. EFFECTS OF IONIZING RADIATIONS ON INSULIN PREPARATIONS

<table>
<thead>
<tr>
<th>Dose (Mrad)</th>
<th>Preparation</th>
<th>Effect</th>
<th>Activity (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>Crystalline solid</td>
<td>Crystals dis-coloured</td>
<td>Significant</td>
<td>Horne, 1958; Association of the British Pharmaceutical Industry, 1960</td>
</tr>
<tr>
<td>2.5</td>
<td>Insulin injection B.P.</td>
<td>Discoloration, pH drop</td>
<td>2.5</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>2.5</td>
<td>Protamine zinc insulin injection</td>
<td>Discoloration, pH drop</td>
<td>10</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>25.0</td>
<td>Protamine zinc insulin injection</td>
<td>Discoloration, coagulation</td>
<td>2</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>0.1</td>
<td>Insulin, aqueous soln.</td>
<td>-</td>
<td>0</td>
<td>Nickerson et al., 1953</td>
</tr>
<tr>
<td>0.425</td>
<td>Insulin, injection USP</td>
<td>Discoloured</td>
<td>77</td>
<td>Hangay et al., 1967b; Hagasawa et al., 1957</td>
</tr>
</tbody>
</table>
Testosterone has been shown to be stable in the dose range 0.8 to 1.1 Mrad (Brasch et al., 1949; Huler, 1948; Traub et al., 1951), but Arnaud and Balestic (1962) found that under certain conditions ergosterol can be converted by γ-radiation to precalciferol or vitamin D₂.

Thyroxine shows slight loss of total iodine content after a dose of 3.0 Mrad and 2.5% loss after 5.0 Mrad (Hangay et al., 1967b). ¹³¹I-labelled thyroxine irradiated in 50% propylene glycol solution showed 50% liberation of inorganic iodine at 0.5 Mrad and nearly 100% at 1.0 Mrad (Galatzeanu, 1967).

Oxytocin is very sensitive to γ-radiolysis. After 80 krad the activity is reduced to 50% (Controulis et al., 1954), after 425 krad to 44.4% (Hagasawa et al., 1957) and after 1.9 Mrad to 0.02% (Controulis et al., 1954). Vasopressin was reduced to 42.3% activity by 425 krad (Hagasawa et al., 1957). Adrenocorticotropic hormone can be successfully sterilized by electron bombardment and is biologically effective and non-toxic in man (Colovos and Churchill, 1957). The pituitary hormones are reported to be stable to 0.8 - 1.1 Mrad (Huler, 1948; Brasch et al., 1949; Traub et al., 1951).

Radiation severely degraded insulin preparations as indicated in Table XXX. From the results it is evident that polypeptide and proteinaceous hormones are not as stable to radiation as those containing a steroid nucleus.

4. ALKALOIDS

Atropine sulphate (0.1%) irradiated to doses of 0.5 and 3 Mrad was found to retain 79.5 and 29.5% activity respectively. The percentage breakdown was reduced in more concentrated solutions and also with increasing dose rate up to a limiting rate of 0.1 Mrad/h. Aeration of the solution prior to irradiation increased the amount of degradation (Pandula et al., 1967). Aqueous solutions of atropine sulphate (0.6%) were found to be completely degraded by 2.5 Mrad (Horne, 1958; Association of the British Pharmaceutical Industry, 1960), but French investigators (Bonet-Maury and Lormand, 1959) found solutions to be stable up to 0.1 Mrad. An eye ointment containing atropine sulphate was marketed after radiation sterilization in the United Kingdom in 1968 (Chemist and Druggist, 1968) and found to have undergone only 12% breakdown after 2.5 Mrad (Trigger and Caldwell, 1968).

Physostigmine sulphate (10%) in water was found to undergo radiation degradation by an oxidative process but the rate of degradation could be reduced by addition of 0.5 wt/vol (%) sodium metabisulphite. After 2.5 Mrad it was found that 72.5% activity remained in the solution in distilled water, but 87.5% remained in the solution containing 5% sodium metabisulphite (Fletcher and Davies, 1968). Solutions of between 0.01 and 0.5% theophylline in water were irradiated to doses of 0.088 and 20.0 Mrad. The solutions changed in colour and the degradation of theophylline was found to follow zero-order kinetics (Rasero, 1966; Rasero and Skauen, 1967).

Dilute solutions of morphine hydrochloride were unchanged after 0.1 Mrad (Bonet-Maury and Lormand, 1959) and 1% solutions showed no change in pH or ultra-violet spectra after 2.5 Mrad (Pandula et al., 1967). Morphine sulphate B.P. injection turned yellow after irradiation with 2.5 and 25.0 Mrad, but retained 100% potency (Horne, 1958). No change in infra-red spectra of
morphine hydrochloride was found upon irradiation with 2.5 Mrad (Bonet-Maury et al., 1964). Solid morphine sulphate irradiated to 2.5 and 25.0 Mrad turned yellow, retaining 98% and 97% activity respectively (Horne, 1958).

Similar observations followed the irradiation of solid ergometrine maleate, which, although turning yellow, retained 97% activity after 25.0 Mrad. Ergometrine maleate injection B.P., 0.1%, sealed under nitrogen was reduced to 6% activity after 25 Mrad.

No measurable decomposition was found after irradiating solid caffeine, emetine hydrochloride, sparteine bisulphate, strychnine bisulphate to 2.5 Mrad (Bonet-Maury et al., 1964).

5. CARBOHYDRATES

The extensive uses of carbohydrate systems justifies consideration of this group from a more fundamental standpoint. The results can be applied to such varied systems as intravenous dextrose-saline to dextran used as a blood plasma substitute, heparin and the range of salts of gluconic acid and the polysaccharides that are so widely used as vehicles for all types of pharmaceutical preparations.

Oxidative degradation is the most general consequence of radiation action on carbohydrates. The course and mechanism of degradation differs according to whether ultra-violet light (Phillips, 1963a) or high-energy ionizing radiations (Phillips, 1961) are used. Here, attention will be directed to the mechanisms of degradation of carbohydrate systems on \( \gamma \)-irradiation in aqueous solution (Phillips, 1961) and in the solid state (Phillips, 1966).

When dilute aqueous solutions are irradiated with ionizing radiations the radical and molecular products are

\[
H_2O \rightarrow H_2, H_2O_2, e_{aq}^-, H, OH
\]

A mechanistic study of the \( \gamma \)-irradiation of aqueous D-sorbitol indicated that at \( 5 \times 10^{-2} M \) a degradative mechanism involving the radical species produced in the above equation cannot satisfactorily account for the course of radiolysis. Product yields are dependent on the solute concentrations in the range \( 5 \times 10^{-4} - 5 \times 10^{-2} M \) and furthermore the observed yields cannot be accounted for on the basis of accepted radical yields (Phillips and Davies, 1964b). Similar behaviour was found during \( \gamma \)-irradiation of monosaccharides (Phillips and Criddle, 1962b) and disaccharides (Phillips and Davies, 1964a). This type of variation in product yields with solute concentration would not be anticipated if chemical changes were due solely to the classical indirect action effects. Typical behaviour is shown by D-glucose, which on \( \gamma \)-irradiation in \( 5 \times 10^{-2} M \) solution at pH 7 gives \( -G(D-glucose) \) 3.5 in oxygen and in vacuo. This value is greater than \( G_p(OH) \) at pH 7 and it is, therefore, possible that in addition to \( \cdot OH \) radical abstraction, other reactive species produced by water radiolysis initiate chemical reaction. Such reactions need to compete efficiently with the fast reactions

\[
H^+ + O_2 \rightarrow HO_2
\]

\[
e_{aq}^- + O_2 \rightarrow O_2^-
\]
Therefore, to evaluate the contribution of the OH radical in relation to other processes leading to D-glucose decomposition, the effect of concentration on the chemical changes during low dose-rate cobalt-60 γ-irradiation experiments were examined in relation to pulse radiolysis studies using 4-MeV electrons (Phillips et al., 1966).

Studies on γ-irradiation of carbohydrates in the solid state have demonstrated that these molecules can promote efficient energy transfer alone and in association with other molecules. These observations have been utilized to effectively protect carbohydrates from radiation damage and to study the nature of the excited states produced during γ-irradiation of sugar crystals. There are indications that the role of hydrogen bonding in the solid-state systems and in aqueous solutions, when the carbohydrate concentration is > 5 x 10^{-2}M, may not be unconnected.

5.1. Aqueous aldohexoses

On irradiation of carbohydrates in aqueous solution considerable degradation occurs and it was necessary to develop new radioisotopic methods of analysis (Phillips and Criddle, 1962a). Yield-dose curves for the major products were obtained using carrier-dilution analysis at several dose levels and in this way primary and secondary products were distinguished. The overall consumption of hexose during irradiation in 5.5 x 10^{-2}M solution shows an initial G 3.5 in oxygen and in vacuo. The main changes, given here for D-mannose (Phillips and Criddle, 1962b), are paralleled with other hexoses (Phillips et al., 1958; Phillips, 1963c). The main processes in oxygen are:

\[
\begin{align*}
\text{D-erythrose} & \quad \text{glyoxal} & \quad \text{mannuronic acid} & \quad \text{D-mannonic acid} & \quad \text{D-arabinose} \\
\text{and glyoxal} & \quad \text{oxalic acid} & \quad \text{D-lyxose} & \quad \text{D-arabinose} & \quad \text{formaldehyde}
\end{align*}
\]

In the absence of oxygen the complication of secondary reactions involving oxygen are excluded and the degradative pattern follows a rather different path (Phillips 1963c, Phillips and Criddle, 1960):

\[
\begin{align*}
\text{D-mannose} & \quad \text{D-glucosone} & \quad \text{three-carbon aldehydic fragments} & \quad \text{two-carbon aldehydic fragments} & \quad \text{four-carbon + two-carbon aldehydic fragments}
\end{align*}
\]

The initial G-values of primary products are given in Table XXXI. A distinctive feature (not encountered in oxygen) or irradiations under vacuum is the formation of polymer at high doses. This behaviour was first reported by Stacey and co-workers (Barker et al., 1959; Bailey et al., 1961).

A striking feature is the identical initial rate of degradation of D-glucose and D-mannose in oxygen and in vacuo (G = 3.5). This indicates that the
primary abstraction processes are similar and independent of oxygen. On this basis the differing products found in oxygen and in vacuo irradiations must be attributed to differing secondary reactions of identical primary radicals. Certain products might, therefore, be expected to be the same under both conditions and any differences would be capable of rationalization by consideration of the fate of the initially formed radicals. The radiation stabilities of individual hexoses appear to be identical (Kochetkov et al., 1965).

Common processes are the formation of hexonic acids and ring scission reactions, which lead to two- and three-carbon aldehydic fragments. The presence of oxygen does not appear to have any marked effect in controlling the extent of these processes.

Oxygen influences the product arising from radical attack at C-2. Glucose is formed in vacuo but ring scission occurs in oxygen to give arabinose and formaldehyde. Both reactions occur to a comparable overall extent (G = 0.4 to 0.6). This behaviour is analogous to that of aqueous glycollic acid during irradiation. The carbon-carbon scission that occurs in oxygen is diminished in vacuo (Grant et al., 1958; Grant and Ward, 1959). However, the same primary radical may yield all the products:

- **in vacuo**
  - H - C = O
  - HO - C - OH
  - HO - C - H
  - HO - C - H

- **oxygen**
  - H - C = O
  - HO - C - OH
  - HO - C - O₂
  - HO - C - H

No uronic acid is found in vacuo and no polymer is produced in oxygen. These observations may be rationalized by considering the fate of the primary radical RCHOH, formed by hydrogen abstraction at C-6 in oxygen and in vacuo. After the initial dimerization step in vacuo further radicals may be grafted to the growing polymer chain. The radiation decomposition is first order in aldohexose concentration. On the basis of such kinetics, simple competition between initial hexose and products for the primary species formed during water radiolysis may be envisaged. From a practical point of view it serves as a useful generalization to enable calculation of hexose concentration at any particular dose.

\[
\text{RCHOH} \rightarrow \text{RCHO} + \text{HO}_2 \rightarrow \text{RCOOH} + \text{H}_2\text{O}_2
\]

\[
\text{oxygen} \quad \cdot \text{O}_2 \quad \text{uronic acid}
\]

\[
\text{RCHOH} \rightarrow \text{R} - \text{CHOH} \rightarrow \text{polymer}
\]

\[
\text{in vacuo} \quad \text{R} - \text{CHOH}
\]
TABLE XXXI. INITIAL G-VALUES OF PRIMARY PRODUCTS FROM γ-IRRADIATED ALDOHEXOSE SOLUTIONS (5 x 10⁻² M)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>D-glucose</th>
<th>D-glucose</th>
<th>D-mannose</th>
<th>D-mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vacuo</td>
<td>Oxygen</td>
<td>In vacuo</td>
<td>Oxygen</td>
</tr>
<tr>
<td>Hexose (-G)</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Hexonic acid</td>
<td>0.35</td>
<td>0.4-0.5</td>
<td>0.45</td>
<td>0.6-0.7</td>
</tr>
<tr>
<td>Hexuronic acid</td>
<td>Absent</td>
<td>0.9</td>
<td>Absent</td>
<td>0.8-1.0</td>
</tr>
<tr>
<td>Arabinose</td>
<td>$^b$</td>
<td>Absent</td>
<td>$^b$</td>
<td>Absent</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>$^b$</td>
<td>0.3</td>
<td>$^b$</td>
<td>0.3</td>
</tr>
<tr>
<td>Glucosone</td>
<td>0.4</td>
<td>Absent</td>
<td>0.5</td>
<td>Absent</td>
</tr>
<tr>
<td>Two-carbon fragments</td>
<td>0.85</td>
<td>0.8</td>
<td>0.95</td>
<td>0.7</td>
</tr>
<tr>
<td>D-erythrose</td>
<td>0.25</td>
<td>0.25</td>
<td>(0.25)</td>
<td>0.18</td>
</tr>
<tr>
<td>Three-carbon fragments</td>
<td>0.8</td>
<td>0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymer</td>
<td>$^b$</td>
<td>Absent</td>
<td>$^b$</td>
<td>Absent</td>
</tr>
</tbody>
</table>

^a Absent in significant proportions.
^b Secondary product.
^c Formed from C-1 and C-2.

Summarizing, therefore, it would appear that when aldohexose is irradiated in about 5.5 x 10⁻² M solution -G is 3.5 in oxygen and in vacuo; attack is not confined to any particular part of the molecule. The products formed in oxygen and under vacuum, respectively, demonstrate that all bonds are affected. Oxidation occurs at the extremities of the molecule and simultaneously chain-scission leads to lower aldehyde fragments.

5.2. The solid state

Indications that energy transfer processes might be important in the radiation chemistry of carbohydrates were obtained initially with D-glucose crystals. The physical state in which anhydrous α-D-glucose is irradiated with ionizing radiations profoundly influences the extent of radiation decomposition (Phillips, 1963b; Phillips and Baugh, 1963b). In particular, it was found that after freeze-drying polycrystalline anhydrous α-D-glucose from aqueous solution, the crystals that were produced were more stable to $^{60}$Co γ-radiation than the initial α-D-glucose.

Polycrystalline α- and β-D-glucose behave similarly and from the yield-dose curves initial -G is ~20 and thereafter -G for glucose decomposition decreases, until after a dose of 5.8 x 10⁻²¹ eV/g -G is ~9. For the freeze-dried form -G is initially about 7. For α-D-glucose monohydrate -G is initially about 11 and, as in the other cases studied, decreases as irradiation is continued. No differences were observed in the nature of the products produced on irradiation, although their overall yields for the various forms
TABLE XXXII. G-VALUES FOR ACID AND GAS FORMATION DURING THE IRRADIATION OF α-D-GLUCOSE

<table>
<thead>
<tr>
<th></th>
<th>Acid</th>
<th>Hydrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In vacuo</td>
<td>Air</td>
</tr>
<tr>
<td>Polycrystalline</td>
<td>13.2</td>
<td>13.3</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>3.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Monohydrate</td>
<td>5.2</td>
<td>5.0</td>
</tr>
<tr>
<td>Syrup</td>
<td>6.5</td>
<td>-</td>
</tr>
</tbody>
</table>

were examined. This may be illustrated by reference to acid yields (Table XXXII), which show the same general variation as the -G values of the individual forms of D-glucose. In contrast to acid yields, hydrogen and free radical production are not appreciably influenced by the state in which the solid is irradiated. For all forms examined G(H₂) is ~4. It is possible, therefore, that the processes that lead to acid production are different from those that give rise to hydrogen and long-lived free radicals, which may be observed by electron spin resonance.

The high-radiation decomposition evident in polycrystalline anhydrous α- and β-D-glucose indicate a mechanism of energy transport in the crystal that is facilitated by a highly ordered structure. The existence of such an exciton transport mechanism was suggested by Collinson et al. (1962; Phillips and Baugh, 1963a) to account for the high -G(Fe Cl₃) values in crystalline solid solutions in various organic solvents. To account for the observations with sugar crystals it was suggested that on γ-irradiation non-conducting excited (or exciton) states are produced by interaction of the allowed excited electronic states of the identical molecules in the aggregate. This interaction is most probably associated with the extensive three-dimensional hydrogen-bonded network in these crystals. Chemical change will, on this view, be dependent on the efficiency of energy transfer, which will be at a maximum in a perfect crystal.

On this basis, the change in crystal regularity and increased inter-molecular distance (the group may contain up to 1% water) would significantly modify the exciton spectrum of the aggregate. For the freeze-dried state no difference, compared with polycrystalline anhydrous α-D-glucose, was immediately apparent. From the X-ray powder diagrams of the two forms freeze-dried glucose was crystallographically and chemically indistinguishable from anhydrous α-D-glucose (Phillips, 1963b). However, on examination by electron microscopy using a replication technique, the surface characteristics showed that the crystals produced after freeze-drying were highly dislocated. The characteristic river pattern was indicative of a textured solid (Gilman, 1953; 1956), where the imperfection and disorder in position reach such a degree that a space lattice is no longer a useful representation. It is highly probable, therefore, that the greater degree of imperfection in the freeze-dried state is the most likely explanation for the different behaviour on irradiation (Phillips, 1963b).
CHAPTER 10

6. MISCELLANEOUS PHARMACEUTICAL SYSTEMS

Mersalyl injection B.P. irradiated to 2.5 Mrad turned yellow with a black precipitate forming; inorganic mercury was present. At 25.0 Mrad 50% of the organically bound mercury was lost. Solid mersalyl acid, on irradiation, turned brown and was incompletely soluble in water (Horne, 1958). Sodium antimony gluconate changed colour on irradiation and precipitation occurred. There was a reduction in the concentration of trivalent antimony present (Horne, 1958).

D-limonene, camphor and phenol were irradiated as 10 wt/vol (%) solutions in ethanol with 2.5 - 100 Mrad. Some polymerization occurred in the d-limonene, but phenol and camphor were unchanged.

Sodium fluorescein in 2.2% aqueous solution has been successfully sterilized by γ-radiation (Webb and Hayes, 1956; Ogg, 1963). A nominally 1% eye drop preparation marketed commercially in the United Kingdom in 1968 (Chemist and Druggist, 1968) was degraded from 1.18 to 1.0% by 2.5 Mrad (Trigger and Caldwell, 1968).

Procaine hydrochloride was unchanged in potency after irradiation in the solid state to 2.5 and 25.0 Mrad (Association of the British Pharmaceutical Industry, 1960) and in solution after irradiation to 2.5 Mrad (Hortobâgyi et al., 1967), but there were accompanying colour changes. Aqueous lidocaine solution (2%) irradiated to 2.5 Mrad appeared unchanged on the basis of ultra-violet spectral measurements although there was a drop in pH (Pandula et al., 1967). Pentobarbitone deepened in colour after the solid was irradiated to 2.5 and 25.0 Mrad; solutions of this solid were darker and odorous (Association of the British Pharmaceutical Industry, 1960).

Several compounds with anaesthetic properties have been subjected to low doses of irradiation with little or no effect on their potency. Halothane, divinyl ether, diethyl ether, fluoroxene and a halothane-ether azeotrope were unchanged, as indicated by gas chromatograph analysis after 140 krad (Perry et al., 1965). Later work confirmed that diethyl ether was stable up to 18.6 krad (Laug, 1956), but that 6.7 krad partially degraded halothane dichlorohexafluorobutene (Pennington, 1968). Thiopentone sodium as a powder, electron bombarded to 3.0 Mrad, was unchanged (Miller, 1954b) but radiation from 2.5 and 25 Mrad caused a change in the ultra-violet absorption spectrum and potency (Horne, 1958). Chlormerodrin (3 chloromercuri-2-methoxy propyl urea) (3.4 x 10^-4M) in aqueous or 0.9 wt/vol (%) sodium chloride solution underwent 45% degradation after irradiation with 1.0 Mrad. The main degradation products were found to be inorganic mercury and 3-chloromercuri-2-hydroxy propyl urea. The latter has not been formed in the saline solutions, but found in greater amounts in the presence of 0.9% benzyl alcohol (Burianek and Cifka, 1970).

7. EXCIPIENTS

The substances included in this section are not themselves therapeutic agents but are used in pharmaceutical preparations as preservatives, solvents, fillers, etc.
TABLE XXXIII. IRRADIATION OF EXCIPIENTS USED IN THE PHARMACEUTICAL INDUSTRY

<table>
<thead>
<tr>
<th>Excipient</th>
<th>Conditions</th>
<th>Dose (Mrad)</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetyl alcohol</td>
<td>Liquid</td>
<td>2.5</td>
<td>Unchanged</td>
<td>Hangay et al., 1967a</td>
</tr>
<tr>
<td>White petrolatum</td>
<td>Liquid</td>
<td>2.5</td>
<td>Discoloured, viscosity decreased</td>
<td>Hangay et al., 1967a</td>
</tr>
<tr>
<td>Liquid paraffin</td>
<td>Liquid</td>
<td>2.5</td>
<td>Discoloured</td>
<td>Hangay et al., 1967a</td>
</tr>
<tr>
<td>White soft paraffin</td>
<td>2.5 and 25.0</td>
<td>Discoloured, gas bubbles formed</td>
<td>Association of the British Pharmaceutical Industry, 1960</td>
<td></td>
</tr>
<tr>
<td>Talc</td>
<td>Solid</td>
<td>2.5 and 25</td>
<td>Increase in acid soluble matter</td>
<td>Horne, 1958; Association BPI, 1960</td>
</tr>
<tr>
<td>Octyl gallate</td>
<td>0.5% soln, in cod-liver oil</td>
<td>20.0</td>
<td>Active oxygen number and acid value increased</td>
<td>Association BPI, 1960</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.9% aqueous solution</td>
<td>2.5</td>
<td>Unchanged</td>
<td>Ogg, 1963; 1967b</td>
</tr>
</tbody>
</table>

Propylene glycol was exposed (Schuenker, 1961) to electron bombardment in air to doses of 100 Mrad and the radiolysis products (expressed as mol.%) were found to be unchanged propylene glycol 85%, acetaldehyde 0.04%, propylene acetal 1.45%, propionaldehyde plus propylene propional 0.1%, acetone 1.02%, propylene ketal 0.66%, water 10%, ethanol 0.37%, methanol 0.33%, isopropanol 0.2%, propylene formal 0.76%. Other workers considered that propylene glycol irradiated to 2.5 Mrad underwent no colour change or significant pH change (Hangay et al., 1967a) and that no toxic compounds were formed (Hickman, 1965). Less acetone and water were produced when the electron bombardment was performed on a nitrogen saturated system.

Reduction in the viscosity of sodium carboxymethyl cellulose (CMC) (0.125 - 0.5 wt/vol (%)) solutions of 48-66% accompanies exposure to 5.7 krad, and after 250 krad the viscosity of a 0.5% solution was reduced from 4500 cP to less than 500 cP. Sodium CMC in solution (2%) after a dose of 85 krad changed in its viscometric behaviour from pseudoplastic to Newtonian in character, indicating a breakdown of the gel structure (Rasero, 1966; Rasero and Skauen, 1967).

Upon irradiation in aqueous solution chlorbutanol was found to yield acetone, hydrogen peroxide and an unidentified yellow precipitate. A dose of 370 krad caused the pH of a 0.5% solution of chlorbutanol to fall from 5.78 to 2.37. It was found that the more dilute was the solution of chlorbutanol, the greater was the percentage loss for a given dose (Rasero, 1967; Rasero and Skauen, 1967).

The effect of γ-radiation on three antioxidants was studied by Chipault and Mizuno (Chipault and Mizuno, 1965). Solutions of propyl gallate (0.01%), butylate hydroxyanisole and α-tocopherol were irradiated to 2.0 Mrad in methyl myristate in vacuo, resulting in 16, 8 and 90% breakdown respectively.
In oxygen the destruction was 89, 90 and 100% respectively. Irradiated to 8.0 Mrad as a 0.1% solution in methyl linoleate, propyl gallate was 12% destroyed in vacuo and 90% destroyed in oxygenated solution.

Poly α-L-glutamic acid irradiated to 2.0 Mrad showed a marked pH decrease and inversion of L to D glutamic acid residues was postulated, although appearing most improbable. The main effect of radiation was the cleavage of the peptide bond giving rise to amide and carboxylate groups (Southern and Rhodes, 1965). Other pharmaceutically utilized excipients that have been irradiated are given in Table XXXIII.

8. MICROBIOLOGICAL ASPECTS

A detailed discussion of the effects of ionizing radiation on microorganisms is beyond the scope of this Chapter. However, it is pertinent here to consider the effects of radiation of microorganisms in the presence of pharmaceuticals. This question is being investigated in the Department of Pure and Applied Chemistry at the University of Salford, England, by Hall, Power and Sewart.

To ascertain the effect of a pharmaceutical on the action of radiation in a biological system, the spores of two organisms, one radiation sensitive, *E. coli* B, the other radiation resistant, *B. pumilis* E601, were irradiated in the presence and absence of sodium sulphacetamide (10%). With *E. coli* B it was found that under anoxic conditions survival curves were identical in the presence and absence of drug, but that when the lethal effect of the drug itself on the bacterium in the absence of irradiation was taken into account, it was evident that the sulphacetamide protected the organism from the lethal effects of radiation (Table XXXIV). This behaviour would be anticipated following the efficient scavenging of water radiolysis products by sulphacetamide as shown above.

Spores of *B. pumilis* E601, which were unaffected by the sulphonamide alone, had D10-values in an argon-saturated solution of 200 krad and in the presence of 10% sodium sulphacetamide of 240 krad. This increase in D10-value can again be attributed to the scavenging of radical species by the sulphonamide.

<table>
<thead>
<tr>
<th>TABLE XXXIV. SENSITIVITY OF <em>E. coli</em> B TO GAMMA IRRADIATION IN THE PRESENCE OF SODIUM SULPHACETAMIDE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conditions</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Argon</td>
</tr>
<tr>
<td>N2O</td>
</tr>
<tr>
<td>O2</td>
</tr>
</tbody>
</table>

^a Dose in megarads to reduce population to 10% of original value of $10^7$ organisms/ml; temperature 0°C. I in aqueous solution; II in 10% aqueous sulphacetamide; III in 10% aqueous sulphacetamide after correction for lethal effect of drug.
9. CONCLUSIONS

Radiation sterilization of pharmaceuticals is now possible and this Chapter cites examples where the method is now being employed commercially. We urge caution in the application of the method. It must not be thought as the universal method that can be used on any system when other more conventional methods have been found wanting. It is a specific method, which requires careful choice of the conditions. It can be concluded that all types of materials are generally more stable to chemical damage in the solid state than in solution. Without exception, the use of the method presents a battle between sterilization requirements and the accompanying chemical decomposition. We have sought to point out repeatedly that if the basic principles of radiation chemistry are borne in mind, then the extent of radiation damage can often be reduced to acceptable levels if suitable conditions of dose, solute concentration, atmosphere, nature of the accompanying materials, etc. are selected. If more stringent sterilization requirements are demanded, then the available methods will come under greater pressure and under these conditions radiation sterilization could offer unique advantages. We would urge, therefore, that more attention should be given to the radiation chemistry of the particular system under attention. If the products, -G values, rate constants for attack by the products of water radiolysis, etc., are known, then the optimum conditions for achieving the most satisfactory result can be selected. It may be that only a dose that can partly sterilize the material can be tolerated. However, if this presents the best result that can be attained and could appreciably reduce contamination, then the method is justifiable. No panacea can be offered to meet all systems and all conditions.
PART IV

RADIATION STERILIZATION OF
BIOLOGICAL TISSUES AND PRODUCTS
INTRODUCTION

The experimental and practical uses of irradiated biological tissues in medicine have been the subject of investigation during the past ten years in a large number of research institutes. A great deal is known about the effects of radiation on isolated tissues under laboratory conditions and a number of articles dealing with the theoretical and experimental aspects have been published. However, the practical application of this experimental work to man has been limited in its scope and few authoritative reports have yet been produced.

From a practical point of view, biological tissues used as a replacement graft must be prepared using methods that ensure sterility with little or no deterioration in the physical properties of the tissue. It is important that the tissue graft should produce minimal antigenic reaction so that it can be incorporated in the body without the grave risk of rejection by the host tissues. Some tissue grafts, such as heart valves and arteries, should be capable of normal function in the body after a transplantation operation. Others, such as bone or nerve, act as a scaffold, which is invaded and ultimately incorporated by living tissue from the host.

There is little point in producing irradiated tissue grafts if they are not as good from a functional point of view as tissue grafts prepared by other means or artificial material grafts. Many factors will influence a surgeon when deciding the best material to use. The availability of tissue graft material is of great importance and there is no doubt that in some countries this type of material is difficult or impossible to obtain. This is often due to the laws relating to the use of post-mortem material. There may be insufficient facilities for the irradiation and storage of tissue grafts and in these circumstances the use of artificial materials or grafts prepared and sterilized by other known and proven methods will be the only choice given to the surgeon. The cost of collection and preparation of irradiated grafts is generally not a significant factor, because it is the experience of most investigators in this field that the basic costs of artificial materials and subsequent sterilization is much greater than that of tissue grafts collection and preparation.

From a surgical point of view, assuming that grafts are available and that facilities exist for irradiation and storage, the most important factor is their subsequent fate after operation. This requires a long and detailed comparison with, in some instances, patients who have had grafts of artificial material or other tissue grafts inserted at operation. My own opinion is that several years of careful documented checking on all these patients, subsequent to the operation, is required.

We now have sufficient information on the clinical results using tissue grafts to draw valid comparisons and conclusions and these are mentioned in the subsequent text.

From the international medical point of view there seems no practical reason why irradiated and preserved graft material, with the co-operation of the governments concerned, should not be supplied to countries who at present have no means of producing their own. Very satisfactory clinical results have been obtained with the use of certain irradiated tissue grafts and it is possible that other irradiated body tissues will be used in future medical work.
CHAPTER 20

BIOLOGICAL MECHANISMS

Successful clinical results have been obtained using empirical trial and error methods, but to obtain uniform success in grafting or transplant operations we need to have an understanding not only of the technical factors that lead to the success of an operation but also of the underlying biological mechanisms. In this section an attempt will be made to outline the present state of knowledge of some of these biological mechanisms that come into play during the process of tissue grafting and can be used as a basis for more detailed research for special applications. The whole subject is still at such an early stage of development that we do not have the knowledge on which to base unequivocal recommended procedures.

This Manual is primarily concerned with the sterilization of medical products by the use of radiation doses in the range of 1 to 4 Mrad. These radiation doses sterilize biological tissues also, but they have other effects that may well be more important. The factors that appear to be responsible for rejection reactions in organ transplantation seem to be inactivated. Cells are killed. The physical properties of the tissues in the graft are modified. Such physical and chemical modifications affect the incorporation of the graft and its replacement by host tissue and in practice it is best to choose the optimum radiation dose so far as these tissue properties are concerned. This is not necessarily the dose that is customary for the routine sterilization of medical products in any given plant.

In the preparation and use of tissue grafts there are a number of variables that have to be considered, of varying degree of importance. These include:
- Type of irradiation, dose and dose rate
- Temperature of storage, irradiation and subsequent storage
- Time between death and irradiation, and between irradiation and use
- Handling and possible mechanical damage
- Medium in which irradiation takes place
- Environment during storage
- Stabilization versus degradation (Little, 1970a).

For any particular grafting procedure that may be contemplated the previously mentioned variables can only be found by trial and error or animal experiments. The limits for other possibilities can be set when the appropriate biological mechanisms are known. These more general considerations include the stimulating factors for tissue growth, regeneration and resorption, calcification, inflammatory reactions, and modifications for special cases such as bone, nerve and avascular tissues.

1. STIMULATION OF TISSUE GROWTH AND REGENERATION

In the growth and regeneration of connective tissues there are two separate phases to be considered. One is cell division and proliferation,
and the other the formation of intercellular matrices. For these there are a number of requirements. Energy is needed, which is provided by the oxidation of suitable chemicals, amino acids are needed for protein synthesis, and so on.

The presence of suitable carbohydrates or other material whose oxidation provides the energy for cell division and other cellular activities is one need. These oxidizable chemicals, when an abundant energy is required locally, come from the solids in a blood clot or from degrading intercellular matrices, or they are produced directly by stem cells.

In normal wound healing the fibres in a clot serve a dual purpose. They provide the necessary energy source for cell division and proliferation, and they also provide a framework over which cells can migrate into the region where new tissue is required. The first stage of repair after operation or injury is therefore clot formation. Cells proliferate from the broken ends of vessels to form new vessels, which penetrate into the clot. These new thin-walled vessels transport oxygenated blood, which releases the necessary energy from the surrounding clot. The cells that form the new vessel walls progress along the fibres in the clot. Some of them differentiate to form granulation tissue and these granulation tissue cells themselves produce low molecular weight polysaccharides that can stimulate further cell activity. Campbell et al. (1970) have described how capillary penetration follows this material in experimental nerve regeneration. When the area filled by the clot has been replaced by vessels and granulation tissue cells mature to fibroblasts and lay down collagenous scar tissue. The processes described are rapid and collagen is being laid down by the end of a week. Later the fibrous scar tissue may be resorbed or remodeled, but this is a slower process to be measured in months rather than in weeks.

Remodelling of bone is faster. An autograft is consolidated in about 12 weeks.

When a tissue is grafted into a new site its surroundings are repaired and adhesion is obtained by the normal processes of wound healing. There remains the question of incorporation and here the main energy source for cell penetration is from degrading intercellular matrices. Again there are a number of possibilities: the matrix may fail to degrade, it may calcify, it may degrade to necrotic tissue, or it may degrade to components that can provide energy for cell activity. It is this last that is desired.

Some observations by Jelinek (1909) have helped to confirm that without a breakdown of the tissue vascular proliferation, cell proliferation and inflammation do not occur. Among his patients he found two quite different clinical reactions to burns caused by heat and to electrical injuries. In the former case the heat accelerated breakdown of tissue components with consequent cellular activity. In the latter case the mechanism of the process was such that cell death occurred without any significant breakdown of the tissue components. These electrical injuries (including lightning burns) are caused by high frequency oscillating currents, which have the property of liberating energy in materials with a high dielectric constant. Consequently, when the water contained in the tissues, which has a high dielectric constant, is heated and evaporated the release of energy and consequently the temperature promptly drops, since the solid components of the tissues have a low dielectric constant. Degradation of tissue components is a comparatively slow reaction, which has not time to get going during the short period while the water is evaporating. So in the patients with electrical injury only there was little or no cell activity around the injury.
A common method of preparing heart valves for grafting is to freeze-dry them. In all the cases that have so far been reported (e.g. Missen and Roberts, 1970) there has been no instance of connective tissue cells penetrating into the tissue. The process of drying would appear to have denatured the intercellular matrices present in such a manner as to prevent breakdown to those components that stimulate cell division. Sometimes freeze-drying is used together with other treatments, such as sterilization by ethylene oxide gas or irradiation, and the consequences of the freeze-drying erroneously attributed to those treatments. Drying tissues has another disadvantage. Tissues are brittle when dry and their components crack easily. In a tissue such as the heart valve, which is continually being flexed, cracks in the dense collagen and elastic layers provide points of weakness, which can lead to rupture, while cracks in the surface allow penetration of blood, which also leads to a mechanical weakening.

A frequent outcome of the use of freeze-dried tissues for grafts, particularly of arterial replacements, is calcification (Brock, 1968). This is one of the three commonest consequences of tissue breakdown. The others are a necrotic degradation or the type of breakdown that stimulates cell activity. That there can be more than one mode of chemical breakdown of the same tissue is partly because these intercellular matrices are a mixture of several components. The main proteins are collagen and sometimes elastic tissue, but some tissues contain other non-collagenous proteins. Each protein has its associated polysaccharide, the compositions of the polysaccharides varying from the fairly unstable and chemically reactive to inert cellulose-like materials that in fossils have survived almost unchanged for periods of up to 400 million years. The third category of matrix component, which is frequently overlooked, is the group of lipids. These are closely associated with the protein and fat from adipose tissues. In general the proteins provide the mechanical stability of a tissue. The polysaccharide complexes are present as gels that maintain a flexible texture and also allow nutrients for the cells and waste products to pass to and fro. A breakdown of this gel structure is one of the prerequisites for calcification. Drying automatically destroys the gel structure, which is not reconstituted on subsequent wetting.

There remains those types of matrix degradation that lead to the formation of products that stimulate cell activity. As well as this special case of the incorporation of grafts, these tissue breakdown products provide energy for normal remodelling processes in the body.

Oxygen and carbon dioxide in the blood

The first requirement for energy production is the fuel and the second is a supply of oxygen. This in turn needs an adequate blood flow. In the early stages of wound healing and also in fracture healing, osteoarthritis, or any other condition where granulation tissue is formed, mast cells are also formed. Secretions from these provide local assistance for blood flow. Otherwise the flow of blood is partly dependent on the pumping action of the heart and partly on muscle activity. Thus, about half of the pumping action of blood through the long bones is provided by the surrounding muscles, while the action of the diaphragm assists in the return of blood to the heart through the vena cava. In choosing the site of a graft, if it is not to be in its
normal anatomical position, thought must be given to the surrounding vascular supply.

For cell division and proliferation, then, the supply of oxygen must be sufficient to supply extra energy for wound healing and tissue incorporation above that needed for ordinary tissue maintenance.

The partial pressure of carbon dioxide in the blood is also important. Carbon dioxide affects cell division in such a way that there is an optimum value of pCO\textsubscript{2} for cell division. Above or below that value mitosis is partially or completely inhibited. The effect of too high a pCO\textsubscript{2} is the more important. Carbon dioxide is one of the products of cell metabolism so that, unless there is a reasonable blood flow, a local concentration of carbon dioxide can build up that inhibits further cell division and may initiate resorption.

**Hormones and steroids**

Vessel and cell proliferation are also affected by the hormones and steroids that are present (Little, 1970b). Anabolic hormones or steroids, as well as encouraging an abundant formation of the intercellular matrix, also stimulate the movement of cell membranes that is needed for proliferation. Catabolic hormones inhibit cell division and also decrease the quantity of matrix that is formed. The intercellular matrix formed when they are present in excess is more stable and inert than normal.

Among the anabolic compounds, peptides and steroids induce different quality matrices. The proteins and polysaccharides formed in the presence of growth hormone are less stable than those formed in the presence of those anabolic steroids that are produced in greater quantity after adolescence. Wound healing and tissue turnover in subsequent remodelling are faster in children than in adults. There are also racial differences. Adult negroes, for instance, retain a high level of growth hormone throughout life, as compared with whites or Asians (Rubenstein et al., 1967), and they also retain a greater facility for wound healing and subsequent remodelling. This means that they are more susceptible to keloid scar formation.

2. **RESORPTION AND REMODELLING**

So far we have considered the essential conditions for vessel and cell proliferation and new tissue formation — either scar tissue or incorporation of a graft. But replacement of the blood clot or graft implies resorption of dead tissue. Cells responsible for tissue removal are generally called phagocytic cells, although they sometimes go by a variety of other names, e.g. histiocytes. Sometimes they act singly and at other times they coalesce to form multinucleated giant cells. They need energy, in the same way that granulation tissue and fibroblasts need energy. When tissue is resorbed degradation products act as the source of energy. The general sequence of events is that phagocytic cells enlarge and coalesce to form multinucleated cells, which then produce hydrolytic enzymes. Some debris from this hydrolysed tissue is taken up into the cytoplasm of the multinucleated cells. They have only a limited life and all the debris is eventually taken into the cytoplasm of single macrophages, which take it away from the site. Part of the tissue goes into solution and is a useful energy source. This is easily
seen in bone that is in the process of resorbing. There is usually a patch of haemopoietic tissue around each resorption site. In the vertebrae, where there is constant remodelling, the bulk of the marrow space is occupied by haemopoietic tissue. The shafts of the adult long bones, where there is virtually no remodelling, are filled with inert fatty marrow.

When tissue in the body dies it is not resorbed immediately. There is a time lag as chemical decomposition proceeds to the stage when the chemically active decomposition products are formed. For immature bone this lag may be about 2 weeks, while in the adult femur the time lag between bone death after a fracture or other incident and the vascular proliferation, which is the first stage of resorption, has been estimated to be 5 to 8 weeks. If the more chemically active organic components are extracted from bone with ethylene diamine, it may remain for 7 years or more after implantation with no sign of resorption.

In the incorporation of grafts tissue removal and remodelling is an essential part of the procedure, so that hormone irregularities that affect the action of the macrophages and multinucleated phagocytic cells influence the whole process of graft incorporation and may themselves lead to failure of the graft. Cortisone and the corticosteroids, often used as anti-inflammatory drugs (not because they remove the cause but because they hinder some of the effects of inflammation) and the cortisol that is produced naturally as a part of the body's stress reaction inhibit enlargement of phagocytic cells to active macrophages. Without these macrophages normal tissue resorption is inhibited. A partial exception is bone, which will be discussed in the next section. When phagocytic cells have enlarged to macrophages they still need to coalesce to form the large multinucleated cells that produce the hydrolytic enzymes that break dead tissue down to a manageable consistency and size. The presence of parathyroid hormone is necessary for the coalescence to take place.

3. BONE FORMATION AND REMOVAL

At the present time the tissue most widely used for grafts is bone. The result aimed at is rather different from that for most other tissues. In nerve grafts the graft is used as a channel through which host nerve tissue can grow. Heart valves are also expected to keep their shape and to function immediately with a gradual replacement by host tissue. With the exception of the skull (Campbell et al., 1970) bone grafts are not meant to either keep their shape or survive. The extra tissue is used to accelerate repair and healing, with the intention that the host bone should regain a normal function and strength.

In their repair mechanisms bone tissues show differences from other connective tissues. The initial stages are the same. There is clot formation, followed by the ingrowth of vessels, the proliferation of granulation tissue and the beginnings of the differentiation of this to fibrous tissue. But there is present in bone a complex chemical, whose composition is still uncertain, known as the osteogenic factor. It is produced by enlarging cartilage cells during the process of enchondral ossification, by osteoblasts during normal bone formation and by the somewhat different type of osteoblast that forms woven bone. The presence of this osteogenic factor is needed for cells to differentiate into osteoblasts. Figure 27 is a photograph of repair.
FIG. 27. Fibrous repair tissue (left) and woven bone (right). Cells that have been stimulated by the osteogenic factor are rounded, while fibroblasts are elongated (x 200).

FIG. 28. Vessels entering woven bone during remodelling. Removal of tissue fluids has left spaces around the osteogenic cells. Osteoblasts on the bone surface have direct cellular connections with the cells of the vessel walls (x 200).
tissue at the edge of a cavity drilled in a rabbit's skull. To the left is ordinary fibrous repair tissue and off the picture on the right is dead and degrading bone. The osteogenic factor from this has stimulated the production of osseous repair tissue, woven bone. The cells of the fibrous repair tissue are seen to be elongated (left), while the cells that have been stimulated by the osteogenic factor are rounded. Those towards the right of the photograph have laid down a calcifiable matrix and some have been incorporated as osteocytes. Bone that has been produced in this manner from granulation tissue is known as woven bone.

Normally bone tissue is formed by cells differentiating from those of the walls of sinusoid vessels in bone (Trueta, 1962) and when the woven bone produced in the initial repair process is replaced during the remodelling process the new bone is formed from osteogenic cells. Figure 28 shows three vessels entering woven bone during the first stage of remodelling. In the photograph the spaces around the vessels and cells are due to removal of tissue fluids during specimen preparation and it can be seen that the osteoblasts on the bone surface have direct cellular connections with the cells of the vessel walls.

During bone removal two separate processes may operate. In each multinucleated cells produce enzymes that hydrolyse and break down the tissue. The precursors of the two types of multinucleated cells (both referred to as osteoclasts) are quite different and a corresponding difference has been noted in the behaviour of the two types of osteoclast. Gaillard (1955, 1959) showed this in cine pictures of osteoclasts in tissue culture. One type of osteoclast stayed in the same place, while the other moved around freely. It would seem reasonable to assume that the anchored osteoclasts were formed from the osteogenic cells derived from the vessel walls, while the freely moving osteoclasts were derived from macrophages. This hypothesis is supported by experimental observations. Cortisol stimulates the formation of osteoclasts, while at the same time inhibiting the enlargement of phagocytic cells. A resistance to blood flow stimulates the formation of osteoclasts from macrophages. An example of this is shown in Fig.29. Blood flow in the cortical bone was from left to right. A saw cut, subsequently filled with a blood clot, provided the barrier. Cells in the bone on the right, deprived of their blood supply, have died but the bone tissue has not yet degenerated to the extent needed for resorption. On the left the increased pressure due to the defect in the bone has caused the formation of osteoclasts, which are resorbing the bone. This is the type of bone resorption that occurs in disuse osteoporosis and it has been found that a lack of parathyroid hormone prevents resorption during disuse (Burkhart and Jowsey, 1967). When the degree of loss of bone tissue due to disuse, cortisol and cortisol combined with disuse is compared (Little and Valderrama, 1968) it is again apparent that in the presence of cortisol bone resorption continues until all the osteogenic precursor cells are used up, while resorption due to multinucleated phagocytic cells is inhibited.

A consideration of these detailed cell activities enables the sequence of events in a bone graft to be followed. When deprived of their blood supply osteocytes die rapidly so that a bone graft can be looked on as dead tissue, whatever the method of preparation. After pieces or chips of dead bone have been placed in position there is clot formation, vessel ingrowth, granulation tissue proliferation and woven bone formation. This is followed by remodelling to normal bone. The formation of woven bone from granulation tissue is
stimulated by the osteogenic factor derived from the dead bone tissue present. Remodelling to normal bone follows after a further vascular invasion. When large pieces of cortical bone are present in the graft, resorption and re-modelling tend to be very slow because a necessary prerequisite for each is vascular invasion. On occasion this disadvantage may be offset by the structural stability supplied in the early stages of healing. During the remodelling process new bone formation needs an adequate supply of blood and oxygen. For this the blood needs to flow freely through the bone. When there is a low flow rate the formation of bone matrix is inhibited, while with an induced pressure resorption of existing bone takes place. This means that a bone graft will only be successful when an adequate blood flow can be maintained.

Polezhaev (1968) has recently described evidence that suggests that a similar specific compound can stimulate the repair of muscle tissue in the same way that the presence of the osteogenic factor is needed for the development of bone tissue.

4. INFLAMMATORY REACTIONS

It has been said that the breakdown of tissue is necessary to provide energy for cell activity, for the proliferation of vessels and granulation tissue, and for the removal of dead tissue by phagocytic cells. Another mode of tissue degradation results in calcification and yet another mode of tissue destruction results in a local inflammatory reaction. These inflammatory reactions are characterized by the passage of plasma through the
capillary walls so that there is an increase in pressure and volume of the intercellular tissue fluids. Small darkly staining cells congregate in the vicinity of the causative agent, small blood vessels dilate and there is often a vascular proliferation. Either the causative agent or damage to or blocking of blood vessels may lead to local tissue necrosis. Dead tissue and debris is removed by phagocytic cells in the normal manner.

One cause of an inflammatory reaction has already been mentioned, the heat degradation of tissues in a burn. Another chemical that causes an inflammatory reaction is released from dead bacteria. Yet another stimulant of inflammation is the presence of tissue from an individual whose tissues belong to a different tissue type. This last type of inflammatory reaction is known as the rejection reaction. The precise cause is not yet known, but a certain amount of information seems to be reasonably well established. There needs to be direct cellular contact. A porous membrane, allowing passage of tissue fluids but not cells, is sufficient to prevent the reaction. Intercellular matrix in an avascular tissue, as with fresh transplanted cartilage, is also sufficient to prevent the reaction.

For some causative agents, but not others, there is an additional reaction. Once lymphocytes have come into contact with bacteria or foreign tissue of a different tissue type changes are initiated that result in an immunological reaction.

5. EFFECTS OF IRRADIATION ON TISSUES

Irradiation has a number of effects on tissues and in choosing the optimum radiation dose they must be carefully balanced. These tissues are not homogeneous. They contain cells, collagen and perhaps elastic tissue and other beta-proteins, mucopolysaccharides and lipid complexes; nerves contain myelin, and so on.

When tissues are taken from the body the time that individual cells can survive varies. In some tissues cell death occurs within a few minutes of their being deprived of their blood supply. Osteogenic cells, for example, are dead well before the tissue is irradiated. Other cells can survive longer. So far as the direct effect on cells is concerned, early work at AERE, Harwell, showed that the mechanism can depend on both dose and dose rate (Little and Young, unpublished). The cells studied at that time were erythrocytes. When a dose of the order of $10^4$ to $10^5$ rads of X-rays was administered cells were haemolysed, the mechanism of haemolysis being the production of tears in the cell membrane. A dose of $10^6$ rads delivered sufficiently rapidly to avoid haemolysis fixed the cells and once their protein had been fixed they could no longer be haemolysed. This dose of $10^6$ rads probably represents the maximum beyond which a mammalian cell could not survive. No vascular tissue could survive beyond about $10^5$ to $10^4$ rads, because at those doses irreversible damage is done to the walls of capillary and sinusoid vessels.

In moribund tissue enzymes continue to function for a while after death or removal from the body so that autolysis may be fairly rapid in a tissue with a high enzyme content. These compounds are inactivated by irradiation doses of 1 Mrad and so further autolysis is prevented. In the choice of time between death and irradiation this is one of the factors to be taken into account. Depending on the nature of the graft, some preliminary autolysis
may be helpful in breaking down matrix constituents to compounds that will stimulate proliferation of host tissue. In the case of nerves, where the graft is intended to function as a channel through which new proliferating nerve tissue grows, such breakdown would prevent a return of function.

There is another problem that arises in the use of nerve grafts. In experiments using dogs, whether homografts or heterografts, there has been a complete return of both the sensory and motor function, but with patients there has been a return of sensory function (other conditions being satisfactory), but not of the motor function (Marmor, 1967). The difference here is that the nerves in the dogs were replaced immediately, while for patients there has usually been an interval of months between damage and replacement. This has not in itself interfered with the regeneration of nerve tissue, as shown by the return of sensation, but motor nerves terminate in an end plate where a thin membrane separates them from fibres of the muscles. In Fig. 30 the nerve ending is seen on the left and a small group of muscle fibres on the right of one such membrane. For adequate motor function to return these membranes need to remain intact.

Other chemical constituents of the tissues are inactivated or destroyed by the time a dose of 1 Mrad is reached. One is the osteogenic factor. This means that irradiated bone grafts can supply nutrients necessary for the proliferation of cells to form new bone and the necessary scaffolding for this bone to grow over, but for the granulation tissue to differentiate into bone the graft needs to be seeded with autologous bone. Comparisons of different methods of grafting have shown that recovery and consolidation of irradiated homografts is better than homografts produced by other methods, but not quite so fast as autografts (Marmor, personal communication).
usually required for consolidation of autografts is about 12 weeks and the irradiated homografts may take slightly longer.

Another constituent inactivated by 1 Mrad or more of radiation is the compound or group of compounds that lead to the rejection reaction. This means that homografts and heterografts may be used without the risk of stimulating a rejection or immune reaction. The difference between the effects produced with and without radiation may be demonstrated by implanting pieces of tendon subcutaneously. After irradiation there is an interval of two weeks or a little longer with no reaction. During this period degradation of the matrix of the tendon is initiated and by the end of the third week one may expect to find vascular proliferation around the implant, but no inflammation. At six weeks vascular channels will have penetrated into the 'graft', again without inflammatory cell reaction or fibrous capsule formation.

When pieces of unirradiated homograft or heterograft tendon are implanted subcutaneously there is no lag period. During the first and second weeks there is an inflammatory reaction, very strong in the case of the heterografts, with round darkly staining cells, multinucleated phagocytic cells present, and the beginnings of fibrous tissue deposition around the perimeter.

There remains the choice of radiation dose for any particular tissue taken as a graft. Provided that uninfected tissue is taken under surgically clean conditions, doses of 1 Mrad upwards are sufficient to ensure adequate sterilization and possible changes in antigenic properties. The optimum dose is determined by the effects of the radiation on the tissues. There are two opposing effects. One is degradation: bonds are broken, particularly hydrogen bonds and -C-O-C-links, oxidation chain reactions may be initiated, and so on. The gel structure of the tissues, depending on intact polysaccharides, is irreversibly destroyed. The formation of a new gel depends on the activity of freshly proliferating cells. This means that cartilage cannot be used for grafting after irradiation; the cells are killed, and its function depends upon the maintenance of a gel structure.

The other effect is further polymerization and cross-linking. Campbell et al. (1970), for instance, have reported that 2 Mrad cause a hardening of the calvarium. A balance of these two processes is required, which may vary from tissue to tissue. Bone remodelling needs to be fast. Nerve sheaths must remain intact until the new nerve fibres have matured. Heart valves, throughout the remodelling process, must retain their mechanical properties and function efficiently. Optimum doses have not yet been established, although present indications are that they are in the range of 1.5 to 3.5 or 4 Mrad.
CHAPTER 21

A REVIEW OF PREPARING TISSUE GRAFTS FOR SURGICAL USE WITH SPECIAL REFERENCE TO GAMMA RADIATION

This brief review of the preparation of biological tissue for grafting is primarily intended to serve as a guide for those embarking on tissue banking procedures. One of the chief considerations in preparing tissue grafts for surgery is that the methods of processing should in no way inhibit the functional role of the tissue grafts. Since the function of each graft is specific, the method of preparation should be applied accordingly and be consistent with clinical requirements.

1. SKIN

To provide the best possible chance of survival for patients with extensive burns, functional skin cover at the earliest possible date is mandatory. Only in the presence of suitable skin cover is there a body surface that can provide normal physiological functioning of heat exchange, water transfer, infection control, etc. Three types of skin are normally used for grafting; non-viable homograft skin, viable homograft skin and autograft skin.

When non-viable skin homografts are used as a biological dressing, their duration as a protective cover is rather limited. However, if used to cover areas with poor healing qualities or poor granulation, they permit improvement of these areas and so facilitate permanent acceptance of subsequent autografts.

Freeze-dried skin as a temporary biological dressing in the severely burned patient has been reported to be life-saving in many instances, and it is here that the beneficial aspect of conservation of cadaver tissue is most dramatically demonstrated (Gresham et al., 1963).

Hyatt (1960a) reported that freeze-dried skin had the most satisfactory initial take of three storage methods: freeze-dried 80%; refrigerated nutrient media 70%; and glycerol frozen 50%. The duration of 'stay' varied almost inversely: freeze-dried 21 days; refrigerated nutrient media 30 days; and glycerol frozen erratic at 14 to 52 days.

In 1966 Perry (1966) reviewed skin preservation and in 1968 (Perry 1968) he described a technique of preparing non-viable skin homografts. The skin was procured under aseptic conditions and subsequently freeze-dried. Other methods involving skin being taken under normal post-mortem conditions followed by chemical sterilization and freeze-drying have also been found suitable, although it should be appreciated that the duration of 'stay' of the grafted skin may be influenced by the technique of processing. The chief advantage of freeze-drying is that it permits large stocks of skin grafts to be stored at normal temperatures for long periods and to be despatched to anywhere in the world by conventional methods of transport. When the skin is subsequently required for grafting, it can be reconstituted and used within 20 minutes.
Viable homograft skin as a temporary functional cover is generally used when there is insufficient autograft skin available. If viable homografts are applied in 1-cm wide strips alternating with autografts then, at the time of homograft rejection, the autografts will be sufficiently established to bridge by epithelial outgrowth the area vacated by the homografts. Much larger homografts can be used to provide temporary functional cover with a view to physical removal before rejection and immediate replacement with autografts. In both instances functional skin cover is achieved much earlier than would be possible with the exclusive use of limited autografts. Viable skin homografts are generally applied, either as a direct transfer from living donor to recipient, or following skin preservation procedures. Three methods of viable skin storage are considered; normal refrigeration at 2 to 4°C, nutrient media refrigeration at 2 to 4°C, and cryo-preservation at below -100°C.

Viable homograft and autograft skin, if loosely rolled in saline-moistened gauze dressing, can retain viability for up to 10 days if stored in a normal refrigerator at 2 to 4°C (Wentscher, 1903).

Perry et al. (1957) reported that split-thickness viable homograft skin behaved quite satisfactorily in nutrient media consisting of 90% Earle's balanced salt solution and 10% by volume of pooled homologous serum. Optimum storage of skin was carried out at 4°C with a ratio of 2.4 ml of nutrient media to 1 cm² of skin cut at 0.015 inches. Under these conditions skin demonstrated viability as evidenced by cellular outgrowth in tissue culture after 6 to 8 weeks of storage.

Optimum survival of cells subjected to very low temperature has been reported by a slow-freezing rapid-thaw program, during which the cells were protected by glycerol or dimethyl sulphoxide (Barlyn et al., 1964; Polge et al., 1949). In 1968 Cochrane (1968) attempted to develop a method for long-term storage of split-thickness viable skin by cryo-preservation. A bio-freeze unit primed with liquid nitrogen was used and provided an automatic program controlled cooling rate from ambient temperature to -150°C. The viable skin was protected with 15% glycerol suspended in nutrient media and was packed into nylon film envelopes. The envelopes were placed into the freezer unit and the temperature was automatically reduced at not more than 1 degC per minute down to -30°C, not more than 4 degC per minute down to -70°C, then quickly down to -100°C and beyond. On completion of the freezing cycle, the envelopes were transferred to a liquid nitrogen storage vessel. When the skin was required for grafting the envelopes were removed from the storage vessel and immersed into a water bath maintained at 37°C. This method of processing has also been found invaluable in the preservation of lymphocytes used as a reference panel for histocompatibility typing and will no doubt be found equally useful for other tissues, particularly eye corneas. Cryo-preservation of viable skin by this method has been indicated as being up to 12 months.

2. FASCIA LATA

Fresh and preserved fascia grafts are used routinely in reconstructive surgery. A recent development is its use in cardio-thoracic surgery for heart valve replacement. Two types of fascia are normally used for grafting:
non-viable homograft fascia, preserved as grafts and autograft fascia, grafted by direct transfer.

The functional role of non-viable homograft fascia lata as a facial sling in plastic surgery is considered, since its function as a supporting structure clearly emphasizes the importance of preserving its tensile strength. Thomas and Gresham (1963) reported the tensile strength of bilateral strips of fascia taken from 25 cadavers within 30 hours of death. The mean average tensile strength of the fascia tested in each experimental group indicated there was no significant difference between fresh, frozen and freeze-dried fascia lata. There were, however, significant differences between individuals and between the groups studied, which were apparently due to age, cause of death and sex of the donors. These results indicated that the most suitable fascia lata was obtained from male donors in the younger age group and the tensile strength of the fascia appeared to be closely associated with the type of donor. Another method of preparation that may be considered involves sterilization and preservation by gamma radiation. Strips of fascia are placed into double-packed polyethylene envelopes with a little balanced salt solution. The grafts are sterilized by exposure to 2.5 Mrad of gamma radiation from a spent fuel source and stored in a normal refrigerator for up to eight weeks; a temperature of 4°C is maintained throughout. When required for surgery the grafts are ready for immediate use.

In 1967 Senning reported 90 consecutive operations in patients with aortic valve disease in which autologous fascia was used. Before this period homograft and heterograft aortic valves were being mounted into rigid support frames and used for aortic, mitral and tricuspid valve replacement. In more recent times heart valve replacement has been attempted with autologous fascia lata mounted in rigid support frames. Although early results do not appear to be too favourable, the follow-up period is far too short to draw any firm conclusions concerning the long-term fate of autologous fascia-lata valve grafts.

3. DURA MATER

In 1970 Abbott and Dupree reported the need for dura-mater grafts to close a meningeal defect in order to seal the cerebrospinal fluid and herniation of cerebral substance, to exclude contamination or infection in the wound, and to minimize cortical scarring and adhesion formation. Congenital defects were the most common in the young and trauma in young adults; the incidence of tumours increased with age. There were examples of almost every neurosurgical problem within the central nervous system, which led to the need for a dura-mater graft.

In 1958 Campbell et al. (1958) reported the successful surgical use of freeze-dried human dura mater in 5 patients. The results of this limited clinical study indicated that freeze-dried human dura mater serves as a suitable dural substitute. Its physical characteristics lend themselves to the rapid formation of a water-tight seal prior to infiltration by viable host cells. For dura taken under normal post-mortem conditions sterilization with 1% β-propiolactone at 37°C for 3 hours followed by washing in a sterile phosphate-buffered saline solution at pH 7.4 has been found suitable, as has sterilization by irradiation at 70°C by exposure to 2 million roentgens from
a Van de Graaff generator; in both instances sterilization was followed by freeze-drying (Abbott and Dupree, 1970).

4. BONE

Burwell (1969) describes the clinical use of the bone graft to provide a bridge of osteogenic tissue, either in a part of the skeleton that is deficient, or to establish bony fusion of a diseased joint.

The conditions that surgeons are called upon to treat by bone grafting include: (1) the delayed and non-union of fractures; (2) the arthrodesis and arthroereisis of joints; (3) the filling of cavities in bone; (4) the replacement of bone and joint loss; (5) the augmentation of skeletal deficiency in the forehead, nose, maxilla and mandible; and (6) the fusion of growth-plate cartilages (Burwell, 1969).

Rarely nowadays is a bone graft called upon to provide for its own internal fixation. Its functions are to establish an additional source of osteogenesis and to act as a scaffold, or trellis, for the ingrowth of new bone.

Two types of bone are generally used for grafting: autologous bone and non-viable foreign bone. The surgical techniques of bone autografting are now established. The current problems of the bone autograft relate less to technique and more to the cellular changes that occur in it after grafting (Burwell, 1969).

The use of foreign bone as grafting material obviously has greater convenience, both for the surgeon and for the patient. Unfortunately, however, the clinical results of using foreign bone have been, in general, less consistent than those obtained by using the patient's own bone as the grafting material. Fresh foreign bone is unsuitable for clinical use because of the immunological reactions that it evokes in the host (Burwell, 1969). Treated foreign bone (frozen or freeze-dried) is for practical purposes dead, and only future research can establish whether or not it will be possible to provide a foreign bone that is as consistent in clinical use as autograft bone (Burwell, 1969).

Despite the clinical advantages of using autologous bone, there still remains an urgent need for preserved bone stored in tissue banks. Spinal fusions for scoliosis are a typical example where bank preserved bone is necessary because insufficient autologous bone is available; this is particularly so in children.

In 1967 Dexter reported the preparation and distribution of preserved bone homografts to several orthopaedic units in the Leeds region. Donor bone procured at post-mortem was stripped of connective tissue and reduced to shapes and sizes (mainly cancellous) according to specific surgical requirements. The bone was washed free of marrow cells and sterilized with 1% β-propiolactone at 37°C for 2 hours. It was then snap-frozen to -80°C and freeze-dried; the final phase of this operation was the filling of each tube containing bone with oxygen-free nitrogen. Bone grafts prepared by this method have been used for the past 15 years. One of our collaborators (C.R. Berkin, F.R.C.S.) reported the grafting of 200 spines with a minimum average of six segments per fusion area; thus one can say that 1200 spinal segments have been fused. There are one or two areas of pseudarthrosis in approximately 20% of these patients, so the absolute results of fusion are
high. The general clinical results indicate 20% to 30% failures, which appears to be similar to others doing the same work; the successful grafting operations produced good union and rapid healing.

A second collaborator (H.D. Penney, F.D.S.) reported the use of this type of bone in oral surgery. Of the varied oral surgical procedures probably the most successful was by filling the large bone cavities that follow the enucleation of dental cysts. Provided that the soft tissues are completely closed over the grafted bone, X-rays show fairly rapid bone regeneration and presumably incorporation of the grafts in the new bone.

Frozen, frozen and sterilized by high-voltage electron beam irradiation, and freeze-dried homograft bone appears to have produced successful clinical results. However, high-voltage electron beam irradiation may not be available and a modification of this method is undergoing clinical trials. After bone has been freeze-dried under clean conditions it is packed into Polythene envelopes and sterilized by a dose of 3 Mrad of gamma radiation from a spent fuel source at 4°C. Although presentation of the bone grafts at surgery is much improved, more time is needed to evaluate the clinical results.

5. HEART VALVES

The successful grafting of a homograft aortic valve in the descending aorta was first reported by Murray in 1956. Ross (1962) and Barratt-Boyes (1964) pioneered the grafting of homograft aortic valves in the sub-coronary position. During the interim period a prosthetic ball valve was developed, which became widely used for heart valve replacement. The prosthetic (ball) valve has inherent disadvantages, the main one being a persistent incidence of arterial emboli. Degeneration with fracture of the ball or total dislocation from its prosthetic cage has also been reported (Barratt-Boyes, 1967). The risk of arterial embolism means that all patients undergoing replacement surgery with prosthetic valves require long-term anticoagulant therapy. The sub-coronary implantation of homograft aortic valves does not necessitate the use of post-operative anticoagulant therapy and arterial embolism does not occur (Barratt-Boyes, 1967; Donnelly, et al., 1971). Two alternatives to the use of mechanical prostheses for heart valve replacement are considered: free homograft or heterograft aortic valves; and homograft or heterograft aortic valves mounted into rigid support frames.

A general appreciation of current opinion indicates that there are three approaches to the problem of obtaining the best long-term results of implanted heart valve tissue: dead grafts chemically fixed to prevent ingrowth of host tissue, dead grafts treated so as to encourage ingrowth of host tissue, and viable grafts. Correct surgical technique is probably the most influencing factor overall. Without correct mechanical function of the grafted valve, it is unlikely that any other factors relevant to valve performance can be adequately assessed. The importance of an exact suturing technique on the fate of a grafted valve is well supported (Carpentier et al., 1969; Davies et al., 1968; Duran et al., 1969; Wright et al., 1968).

In 1969 Karp and Kirklin described the use of free homograft aortic valves for aortic valve replacement in 70 patients. In this series hospital mortality was 4.3%. There were no instances of infection, thrombo-embolism or late deaths and anticoagulants were not used. The homograft aortic
valves were procured from donors within 12 hours of death, trimmed in a non-sterile condition, measured, rinsed and packed in plastic bags for temporary storage at -70°C. The valves were sterilized by high-voltage electron beam irradiation and stored in deep freeze; the temperature of the valves was maintained at -70°C throughout. When required for grafting the valves were rapidly thawed by immersion at 37°C. Karp and Kirklin also stated that the assumption that groups with high hospital mortality rates are operating upon a higher percentage of seriously ill patients undergoing heart valve replacement surgery has been proven false. The need to master the technical operative details is the probable explanation; this also explains the relatively high incidence of homograft incompetence and infection. Either can lead to an undue incidence of calcification.

Recent developments in the preparation of frame-supported homograft valves have been reported by Donnelly et al. (1971). These studies support
FIG. 32. Homograft aortic valve support frame for use in the atrio-ventricular position.

FIG. 33. Preliminary stitching phase of a homograft aortic valve being mounted in a rigid support frame with the valve under pressure and the valve sinuses intact. Sutures for mounting heart valves to support frames were 3/0 Mersilene W6100, from Ethicon Ltd., Edinburgh 11, Scotland, United Kingdom.
<table>
<thead>
<tr>
<th>Valve No.</th>
<th>Valve mounted after dissection (d)</th>
<th>Cusp availability after mounting</th>
<th>Cusp availability 48 h. after mounting</th>
<th>Valve presented for surgery after mounting (d)</th>
<th>Cusp availability when presented for surgery</th>
<th>Loss of cusp surface area</th>
</tr>
</thead>
<tbody>
<tr>
<td>KB/59</td>
<td>4</td>
<td>+1</td>
<td>+1</td>
<td>48</td>
<td>+1</td>
<td>Nil</td>
</tr>
<tr>
<td>KB/54</td>
<td>3</td>
<td>+1</td>
<td>+1</td>
<td>8</td>
<td>+1</td>
<td>Nil</td>
</tr>
<tr>
<td>KB/71</td>
<td>3</td>
<td>+1</td>
<td>+1</td>
<td>8</td>
<td>+1</td>
<td>Nil</td>
</tr>
<tr>
<td>KB/72</td>
<td>3</td>
<td>+1</td>
<td>+1</td>
<td>18</td>
<td>+1</td>
<td>Nil</td>
</tr>
<tr>
<td>KB/73</td>
<td>3</td>
<td>+1</td>
<td>In tissue bank</td>
<td>In tissue bank</td>
<td>In tissue bank</td>
<td>Nil</td>
</tr>
<tr>
<td>KB/61</td>
<td>2</td>
<td>+2</td>
<td>+1</td>
<td>34</td>
<td>+1</td>
<td>-1 (slight)</td>
</tr>
<tr>
<td>KB/67</td>
<td>2</td>
<td>+2</td>
<td>+1</td>
<td>19</td>
<td>+1</td>
<td>-1 (slight)</td>
</tr>
<tr>
<td>KB/70</td>
<td>2</td>
<td>+2</td>
<td>In tissue bank</td>
<td>In tissue bank</td>
<td>In tissue bank</td>
<td>-1 (slight)</td>
</tr>
<tr>
<td>KB/65</td>
<td>1</td>
<td>+3</td>
<td>In tissue bank</td>
<td>+1</td>
<td>-2 (moderate)</td>
<td></td>
</tr>
<tr>
<td>KB/66</td>
<td>1</td>
<td>+1</td>
<td>-1</td>
<td>13</td>
<td>-1 (discarded)</td>
<td>-2 (moderate)</td>
</tr>
<tr>
<td>KB/69</td>
<td>1</td>
<td>+3</td>
<td>+1</td>
<td>19</td>
<td>+1</td>
<td>-2 (moderate)</td>
</tr>
<tr>
<td>KB/55</td>
<td>Immediate</td>
<td>+1</td>
<td>-2</td>
<td>2</td>
<td>-2 (discarded)</td>
<td>-3 (pronounced)</td>
</tr>
</tbody>
</table>

Valve cusp availability:
+3 Pronounced surplus cusp tissue, probably incompetent
+2 Moderate surplus cusp tissue, competent with good function
+1 Adequate cusp tissue, competent with perfect function
+ Minimum amount of cusp tissue, just competent
-1 Insufficient cusp tissue, valve incompetent
-2 Insufficient cusp tissue, cusps under tension and incompetent.

These results indicate that a 72-hour sensitization period against further shrinkage should be permitted to elapse before instituting any valve preparatory procedures.
the views of Karp and Kirklin and others on the importance of surgical
technique. A simple manual testing device (Fig. 31) was introduced per-
mitting visual appreciation of the function and competence of valves, both
during and on completion of mounting procedures. Additional appreciation of
the mounted valve was also made by the surgeon during the operation at the
time of grafting. Preliminary studies showed that of 25 successive valves
mounted to rigid support frames all were found acceptable for surgical use
when tested in a bowl of saline. However, when subsequently examined in
the new testing device, all were found to have functional faults resulting in
them being discarded. It was evident that the method of testing frame-
mounted valves in a bowl of saline had been proven inadequate since this
method failed to reveal the faults that were so clearly demonstrated in the
testing device. It was considered that dissection of the valve sinuses just
before mounting or the mounting technique had probably caused distortion of
the valve and loss of its true conformation. A new mounting procedure was
introduced permitting the valve to be sutured into its rigid support frame
(Fig. 32) with the sinuses intact and under fluid pressure (Fig. 33). It was
felt that the fluid pressure on the valve would produce apposition of the
cusps closely related to their original functional position. Only when the
valve had been sutured into its rigid support frame were the sinuses removed
and under these conditions it was accepted that correct apposition of the valve
cusps had been preserved. Final assessment of the new mounting procedure
not only demonstrated competence of the valves in the testing device but,
more important, that each valve produced good mechanical function.

A continuation of these studies (pre-publication) revealed that when the
mounted valves were presented for surgery pronounced shrinkage of valve
cusps resulted in some of them becoming incompetent. It was found that
when homograft aortic valves were dissected from donor hearts shrinkage
occurred during the subsequent 72-hour period. Homograft aortic valves
mounted to rigid support frames immediately after dissection resulted in
pronounced shrinkage of the cusps during the subsequent 48-hour period,
after which no further shrinkage occurred. If valves were mounted 24 hours
after dissection, shrinkage was moderate during the same period, having
already partially shrunk during the primary 24-hour period after dissection.
If 48 hours elapsed between dissection and mounting, a similar sequence
of events was experienced, but shrinkage was slight after mounting, con-
siderable shrinkage having occurred during the previous 48 hours. If a
72-hour shrinkage period elapsed after dissection of the valve from the heart,
no further shrinkage was detected with either free or frame-mounted valves
(Table XXXV).

In all instances valves were maintained in an isotonic solution with
antibiotics at 4°C. A similar sequence of events was experienced when
valves were maintained at 37°C. Under these conditions it appeared that
shrinkage of aortic valves dissected from hearts, or shrinkage of cusp tissue
of frame-mounted valves within the 72-hour period following dissection
seemed inevitable and irreversible in so far as our laboratory tests could
determine. It would seem advisable therefore that a 72-hour sensitization
period against further shrinkage should be permitted to elapse before
instituting any preparatory procedures.

It was also observed that approximately 10% of all homograft aortic
valves after being dissected from hearts were found to be incompetent.
A number of these were due to congenital defects that were clearly observed,
### TABLE XXXVI. EVALUATION OF HOMOGRAFT AORTIC VALVE FUNCTION

<table>
<thead>
<tr>
<th></th>
<th>Valve function</th>
<th>Reduction of surface area (%)</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Abnormal</td>
<td></td>
</tr>
<tr>
<td><strong>A. STERILIZATION</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Fresh</td>
<td>+</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>b. 1% BPL 3 h</td>
<td>-</td>
<td>Slight</td>
<td>0-15</td>
</tr>
<tr>
<td>c. Ethylene oxide</td>
<td>+</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>d. Gamma irradiation</td>
<td>+</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td><strong>B. STORAGE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Soaked in gauze</td>
<td>+</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>b. Deep frozen (-70°C)</td>
<td>+</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>c. Immersion in nutrient medium at 4°C for 2 weeks</td>
<td>-</td>
<td>Slight</td>
<td>15</td>
</tr>
<tr>
<td>d. Freeze-dried by quick technique, stored in vacuum</td>
<td>-</td>
<td>Slight to moderate</td>
<td>20</td>
</tr>
<tr>
<td>e. Slowly freeze-dried, stored in nitrogen</td>
<td>-</td>
<td>Moderate</td>
<td>25-30</td>
</tr>
<tr>
<td><strong>C. STERILIZATION AND STORAGE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Deep frozen and gamma irradiated</td>
<td>+</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>b. BPL sterilization, stored in nutrient medium</td>
<td>-</td>
<td>Slight</td>
<td>15</td>
</tr>
<tr>
<td>c. Gas sterilization, freeze-dried, stored in vacuum</td>
<td>-</td>
<td>Moderate</td>
<td>20</td>
</tr>
<tr>
<td>d. 1% BPL freeze-dried, stored in vacuum</td>
<td>-</td>
<td>Moderate</td>
<td>20-25</td>
</tr>
<tr>
<td>e. BPL sterilization, slowly freeze-dried, stored in nitrogen</td>
<td>-</td>
<td>Gross</td>
<td>50</td>
</tr>
</tbody>
</table>

although other anomalies could not be explained and might have been caused by variable shrinkage. It became necessary to pressure test all dissected aortic valves on completion of the 72-hour shrinkage period and subsequent results confirmed the original observations.

Although the new developments (Donnelly et al. 1971) provided a method of testing the competence of free aortic valves, the obligatory removal of the valve sinuses at the time of grafting disturbs the symmetry of the valve since approximately 50% of its aortic wall has been removed, and at the time of grafting its post-operative function must be regarded as uncertain. The successful mounting of homograft aortic valves into rigid support frames by this new technique provides an assurance that at the time of grafting the valves are not only competent but have good function and in this respect would at least be equal to the prosthetic valve.

The grafting of free homograft aortic valves in the sub-coronary position is well established. However, since this procedure has not been possible in the atrio-ventricular position, total prostheses or frame-mounted valves are the only alternatives. If it can be shown that frame-mounted aortic valves can be used successfully in the mitral position then, although there are haemodynamic differences relating to the two positions, it may well be that they would be equally successful in the sub-coronary position. It is clear that both surgical technique and the method by which valves are prepared are initially the most important factors in the preparation of heart valves for surgical use, but the priority of importance is significant as little can be assessed unless correct function of the valve is initially achieved.

Methods of preparation

The preparation of fresh homograft aortic valves is intended to retain tissue viability. In 1968 Mohri et al. reported thickening and limited mobility of fresh cusp leaflets after grafting. Similar studies by Angell et al. (1969) showed that fresh valves stimulated increased tissue re-activity and developed decreased cusp mobility and shortening of cusp length with resultant aortic insufficiency; in both instances the experimental subjects were dogs.

Homograft aortic valves that were chemically sterilized and freeze-dried developed thickening at the base of the cusps after implantation (Hudson, 1966). Chemical sterilization and freeze-drying also appears to have undesirable effects on the tensile strength of aortic valve tissue. Sterilization with 1% β-propiolactone for 3 hours produced a striking reduction in tensile strength by 35%, whereas freeze-drying reduced the tensile strength by 20%. Collagen is very sensitive to pH changes and is therefore vulnerable to chemical agents used in the preparation of aortic valves. Formalin-like compounds cause pH changes, which affect the structure of the collagen; in contrast, high-energy irradiation has little effect on the structural properties of collagen (Harris et al., 1968).

In 1969 Trimble et al. reported cinematographic evaluation of homograft aortic valve function using an internal pulse duplicator; the valves were sterilized and preserved by various methods (Table XXXVI).

High-voltage electron beam irradiation at -70°C combined with a meticulous surgical technique has produced probably the most encouraging clinical results of free homograft aortic valve replacement in the sub-coronary position (Donnelly et al. 1971). As this type of irradiation may not
be available and deep-freeze storage at -70°C may not be suitable for frame- mounted valves, a modification of this method is considered. Homograft aortic valves procured from donors less than 40 years of age are dissected from the heart by clean technique and immersed in a balanced salt solution with antibiotics at 4°C during the subsequent 72-hour shrinkage period. Whether prepared as free or frame-mounted valves, they are then re-immersed in a fresh isotonic solution without antibiotics and are individually double-packed into heat-sealed plastic bags. Sterilization of the valves is by exposure to 2.5 Mrad of gamma radiation from a spent fuel source during a dosage period of between one to three hours; a temperature of 4°C is maintained throughout. This method of preparation combined with the new mounting and testing technique is undergoing trials at the Regional Tissue Bank, Pinderfields General Hospital, Wakefield, and the Leeds Regional Cardio-Thoracic Unit at Killingbeck Hospital, Leeds, England.

Many other methods of sterilizing and preserving homograft and heterograft aortic valves may be considered but, without an assurance that the valve has good function at the time of grafting, its performance during the post-operative period must be regarded as uncertain. The introduction of a testing device for frame-mounted valves not only provides this assurance but ensures that only competent grafts with first-class mechanical function are used for surgery.
CHAPTER 22

THE USE OF ELECTRON STERILIZATION
WITH PARTICULAR REFERENCE
TO BONE AND NERVES

To bank human nerves for use in performing a neurorrhaphy or bone for grafting is relatively simple, provided one has suitable radiation facilities available.

1. SELECTION OF NERVE BY DIAMETER

It should be remembered that nerves procured from cadavers shrink in diameter due to escape of axoplasm and loss of blood supply. Therefore, when one is forced to bridge an irreducible gap in a peripheral nerve that cannot be compensated by joint angulation or resection of a section of humerus or femur with appropriate osseous fixation and repair, one must fall back on autografts or appropriately prepared and preserved homografts. If an autograft is available in the form of a pure sensory nerve, identical in diameter to the nerve in which the gap exists, and is easily obtainable, then this should be used. An example is the great postauricular nerve, which approximates the diameter of the accessory nerve so frequently sectioned by inexperienced surgeons obtaining lymph nodes for microscopic study from the posterior triangle of the neck. An electromyogram of the most caudal portion of the trapezius muscle has been published (Campbell, 1970) in which continuity of the accessory nerve was re-established with 6 cm of great posterior auricular nerve and function restored. Autografts can be used to bridge gaps in peripheral nerves under other circumstances if the diameter of the nerve in which the gap exists exceeds that of the autografts. In this case a compromise must be accepted and multiple cables are used. Each autograft is covered by epineurium and an outer perineurium. These anatomical layers occupy space and do not provide a scaffold for distal migration of axons, nor does the space between the autograft cables fixed in position to bridge a gap in a large-calibre peripheral nerve. Axons only migrate through pre-existing neurolemmal systems. For this reason, it would seem better to use homografts of identical calibre when one is forced to bridge a gap in a large-calibre nerve. For instance, after shrinkage the distal peroneal nerve is excellent for bridging a gap in the musculocutaneous nerve and a proximal portion of the posterior tibial nerve approximates the diameter of the ulnar nerve at the elbow or the median nerve at the wrist. Experience has shown that the length of a preserved homograft should not exceed 8 cm. Beyond this length capillaries do not penetrate. Bridging a gap in the main trunk of the sciatic nerve is not satisfactory with a homograft and we advocate femur shortening and joint angulation, plantar flexion of the foot, flexion of the knee to a right angle, and extension of the hip. In this way, after shortening the femur by 4.5 cm, we have been able to compensate for a 13-cm gap in the main
trunk of the sciatic nerve, just below the inferior fold of the gluteus maximus muscle, and re-establish function, both motor and sensory (Lusskin and Campbell, to be published). Below the knee tibia and fibula shortening is not satisfactory. At this level we think it is best to use a graft. For those interested in using autografts a paper by Sir Herbert Seddon (1963) is recommended.

Once it is decided to use a banked homograft, it is recommended that a nerve of appropriate diameter be procured from a suitable cadaver. The donor should be young and healthy until the time of death, free of metabolic or malignant disease, and not have been a drug addict or the recipient of multiple transfusions. The last two circumstances increase the likelihood of inoculating the recipient of the graft with hepatitis virus. The nerve should be obtained under clean, but not necessarily aseptic, circumstances. Immediately upon removal, the nerve is packaged as illustrated in Fig. 34, using a heat-sealing device for all three coverings. It is then stored in dry ice, even when exposed to a source of irradiation for purposes of sterilization, and later retained under these conditions until ready for use in the operating room. Two small segments of the graft are similarly packaged. One is sent as soon as possible to the bacteriology laboratory to identify the contaminating organisms. The other piece accompanies the main graft to the source of irradiation and, once it has received this treatment, is examined by the bacteriologist. If the cultures aerobic and anaerobic are negative, the nerve is then made available for implantation in man. Even though precautions are taken in selecting donors, the patient who is to be the recipient of the graft must be warned of the risk of developing
hepatitis. The lethal dose of radiation for the hepatitis virus is not established. It can be said at this time that 2 Mrad delivered by a 3-MeV Van de Graaff accelerator is a consistent sterilizing dose. Further experience in evaluating reduction of antigenicity as a result of this dose of ionizing irradiation shows that 20 out of 20 guinea-pigs injected in the foot pads with $0.2 \text{ cm}^3$ of an emulsion of fresh cat sciatic nerve mixed with complete Freund's adjuvant did not develop experimental allergic neuritis. On the other hand, 20 out of 20 that received untreated nerve procured under aseptic conditions along with Freund's adjuvant developed or succumbed to experimental allergic neuritis.

2. CONSIDERATIONS IN THE SELECTION OF HIGH ENERGY ELECTRONS FOR IRRADIATION STERILIZATION

In interacting with matter megavolt electrons like X-rays and gamma rays possess physical properties that are capable of producing biological changes peculiarly applicable to the sterilization of tissue materials such as bone, heart valves and nerves. These forms of ionizing energy are capable of destroying bacteria and inactivating viruses (Lea, 1956) throughout the irradiated volume. Since electrons penetrate into the volume of the material, neither thermal conductivity nor gas permeability are of importance. With electrons sterilizing doses may be delivered in a fraction of a second. Since the absorbed energy required for sterilization produces a small temperature rise, material irradiated at dry ice temperature will remain frozen. Materials may be sterilized in the final packaged form in the liquid, dry or frozen state. Machine sources of radiation have been developed to provide high output capability with reliability.

Ionizing radiation, whether X-rays or gamma rays, high energy electrons, protons, alpha particles, neutrons or other heavy particles, is capable of producing chemical changes and with adequate doses destroying bacteria and viruses. The amount of ionizing energy required for sterilization depends on a number of factors: the nature of the energy itself, susceptibility and concentration of the organism in the specific medium and the conditions of irradiation. Such irradiation may produce changes in the material being sterilized. Some undesirable effects may be minimized by proper selection of the type of ionizing energy and the conditions under which it is applied. In considering the use of ionizing energy for sterilization the following criteria should be included:

1. The ionizing agent should be one that accelerates electrons within the absorber with relatively low density ionization tracks. Protons, alpha particles, neutrons and other heavy nuclear particles that produce high density ionization are ruled out by this criterion. High density ionization tracks are more likely to produce undesirable side-effects and are less efficient in their microbiological activity. To inactivate a virus a single or a few ionization events may be required. For these reasons the ionization from low density ionization tracks of secondary electrons produced by X- or gamma-ray absorption or by high energy electrons is preferable. Ultra-violet light consists of photons of about 5 eV, which, although selectively absorbed, produce low densities of excitation and ionization, and is acceptable by this criterion.
(2) The ionizing energy should be capable of penetrating to an adequate depth within the absorber and should deliver an approximately uniform absorbed dose. It should be practical to deliver the dose to liquid, frozen or dry material. Lack of adequate penetration rules out protons and other charged nuclear particles from practical sources. Ultra-violet light has been used for the irradiation of thin films less than 100 microns thick and would not be feasible to use for the thick sections encountered in tissue materials. X-rays, gamma rays and high energy electrons are capable of irradiating material throughout its volume in the frozen, liquid or dry state.

(3) In processing material accurate dosage control is essential. In principle, all forms of ionizing energy are capable of accurate application, measurement and control but in practice there may be a wide disparity in the practical attainment. For processing purposes, X-rays, gamma rays and high energy electrons may be used with accuracy in absorbed dose measurements and control.

(4) The radiation source must be capable of processing safely and economically the bulk quantities involved. For tissue materials the quantities may be quite limited so that a large processing capacity may not be necessary. If the source of ionizing energy is used for other projects, then the irradiation processing time may become important. For example, if efficiencies be assumed to be the same, then 1 kW of output electron energy is equivalent to the total gamma-ray energy released by 70 000 curies of cobalt-60. Since it is possible to concentrate the absorbed electron energy in a small volume, sterilization treatment times may be of the order of seconds, whereas for $^{60}$Co treatment times would be of the order of hours. Moreover, utilization efficiency of a directed scanned electron beam would generally be higher than for the more penetrating and divergent $^{60}$Co gamma-ray source.

3. PROPERTIES OF HIGH ENERGY ELECTRONS

High energy electrons are available for radiation processing from several different types of accelerators. Machines are available with electron beam power output up to tens of kilowatts. If a 50% utilization efficiency is assumed for an output power of 100 watts, then approximately 20 kilograms of material per hour may be irradiated to an average dose of 2 megarads. (The rad is defined as an energy absorption of 100 ergs per gram.)

Electrons have a range in material dependent on their energy and on the density of the absorber. In water the maximum range is about 0.5 cm for each million electron volts (MeV) of energy. An approximate formula for the maximum penetration of electrons in material in the 1 to 10-MeV energy range is $R_{\text{max}} \approx \frac{0.5 E}{\rho}$ cm, where $E$ is in MeV and $\rho$ the density in g/cm$^3$.

A typical distribution of relative ionization in depth versus range is shown in Fig.35 (Trump et al., 1950) for 3-MeV monoenergetic electrons at normal incidence to an absorber. The radiation source for these studies was a Van de Graaff electron accelerator. At high energies the relative ionization at the surface is more than that shown in Fig.35. At 10 MeV the relative surface ionization is 90% (Laughlin, 1954). Electron
energies should be less than 10 MeV in order to stay below most neutron production thresholds. The low energy neutron thresholds of beryllium and deuterium should not produce measurable induced radioactivity because of their low concentration and their low cross-section for electron-neutron interaction.

In the low MeV electron energy range the dose distribution in materials is affected by electron-electron and electron-nuclear scattering within the material. Backscattering may be used to enhance the dose on the exit side of the sample being irradiated. Studies have been carried out (Wright and Trump, 1962) to determine the number of backdirected electrons and the fraction of the incident energy that is carried back from materials of different atomic numbers. A portion of the backdirected electrons are true secondary electrons with energies of less than 50 eV and represent an insignificant portion of the backdirected energy. The major portion of the backdirected energy comes from primary electrons that have undergone nuclear elastic scattering and/or other scattering encounters and retain all or some of their original energy.

Elastic nuclear scattering is strongly dependent on the nuclear field and as a consequence becomes more important with high atomic number materials (Buechner et al., 1948; Miller, 1954a). For lead (atomic number 82) with incident 1-MeV electrons the backscattered primary electrons amount to 44% of the incident electron current with 34% of the incident energy backdirected. For an aluminium target (atomic number 13) the figures are 7.5% and 3.5% respectively. At higher incident electron energies the per cent of backscattered electrons diminishes — at 3 MeV with a lead target the percentages would be 33% and 18% respectively. With 3-MeV electrons incident on soft tissue or bone the backscattered energy would be less than 5% of the incident energy.

Some energy loss in material irradiated with electrons is encountered due to the production of X-rays. The efficiency of X-ray or bremsstrahlung production increases with electron energy and with the target atomic number. For example when 1-MeV electrons are incident on a gold target about 3% of their energy is converted into X-rays, while at 3 MeV this percentage would be about 10%. If the target were soft tissue, less than
1% of the incident energy of a 3-MeV electron beam would be converted into X-rays.

In personnel protection with electron beam installations great care must be taken to prohibit access to the irradiation area while machines are operating or have appreciable stored energy that may be released in the form of energetic electrons or X-rays. The shielding must be designed to stop electrons within the irradiation area and provide adequate thicknesses of material to protect personnel from X-ray exposure in accordance with recommended standards (ICRP, 1966). Scattering of electrons and X-ray production efficiencies should be considered in the choice of structural materials that may be bombarded by electrons. Particularly in high power installations control of air decomposition products must be undertaken to provide for personnel safety.

4. IRRADIATION TECHNIQUES WITH ELECTRON BEAMS

The absorbed dose distribution in a sample may be considered to be the same as the ionization distribution. The absorbed dose within the range of electrons depends primarily on the total number of electrons per square centimetre. The effect of increasing electron energy is to increase proportionately the depth to which the dose is delivered and to modify the relative distribution of dose in depth (Laughlin, 1954). At 3 MeV a total charge of 8 microcoulombs per square centimetre will deliver an average dose of 2 Mrad/g \((2 \times 10^8 \text{ erg/g})\) to a depth equal to 2/3 of the maximum electron range. The peak dose under these conditions would be 2.4 Mrad and the minimum 1.4 Mrad. Since damage to tissue is dependent on the maximum dose and persistence of microorganisms to the minimum dose, it is desirable to minimize the spread in these values. For thin samples absorbers may be placed to make use of the region near the peak of the ionization in depth curve. High atomic number materials may be placed to enhance by backscattering of electrons the dose on the exit side of the sample. Cross-firing techniques may be used to increase the useful thickness of materials to be irradiated.

In a typical accelerator the high energy electron beam emerges from the evacuated accelerator tube into air as a narrow beam. The beam cross-section is spread by the nuclear scattering of electrons as they pass through the thin window isolating the tube vacuum from air. The energy absorbed in the window is typically less than \(10^6\) eV per particle but results in local heating and X-ray production. The local heating may limit the beam current used with a fixed electron beam.

To obtain a uniform dose over an area suitable to irradiate a stationary sample, further scattering may be obtained by using a high atomic number metal and an adequate distance to the sample. This method makes use of the central portion of the beam with quite low efficiency.

A shaped absorber diaphragm may be used to produce a uniform incident charge on a sample passed through the beam on a conveyor belt. This method is particularly suitable for solids and frozen liquids with low power or throughput requirements. If a liquid does not move relative to its container, then this method is satisfactory for liquids. Other methods may be used for flowing liquids but care must be taken in the design of the flow system.
If a number of overlapping beams were used with a conveyor belt, then uniformity of incident charge across a large width could be obtained with moderate efficiency and greater throughput capabilities. With a d.c. voltage source the beam may be scanned across the material being irradiated, transversely to the direction of motion of the conveyor belt. This method allows high efficiency use of the electron beam and the capability for high power operation.

A variety of methods using single or multiple beams to obtain the desired degree of uniformity and radiation economy have been presented in a paper by Tragessar (1960).

5. ELECTRON IRRADIATION OF TISSUE MATERIALS

Aortas

The results of studies by Gross, Meeker and Litwin (Meeker and Gross, 1951; Gross and Litwin, 1968), using frozen electron irradiated allograft segments of aortas to reconstruct thoracic aortas in 82 patients, have provided evidence of satisfactory function with follow-up to 20 years. There were no cases of aneurism formation; some had become partially calcified. After extensive trials both from the point of view of sterility and of host acceptance, the optimum conditions obtained were as follows:

(a) Radiation doses in the range of 2 Mrad
(b) Irradiation of the tissue in the frozen state over a bed of dry ice (-78°C)
(c) Irradiation carried out in 2 passes with half the dose from each side to improve dosage uniformity
(d) Irradiation time less than 1 min.

Bone

Bassett et al. (1962) and Cohen (1955) conducted studies on the use of electron irradiated bone. The bone used by these groups was irradiated in accordance with the procedures outlined above. A specified average dose of 2 Mrad was used in their clinical work. Where healing potential did not have to be augmented, irradiated bone has been used successfully in limited clinical procedures without adverse reactions. Detailed information is contained in the referenced articles.

Aortic valves

Malm, Harris and their associates (Malm et al., 1967; Harris et al., 1968) have carried out extensive studies on the strength of aortic valve wall material sterilized by a number of methods. Neither the normal architecture or the aortic wall tensile strength showed changes with irradiation according to the procedures above at an average dose of 2 Mrad. Other sterilizing methods showed significant reduction in tensile strength. Kirklin (private communication) has successfully used several hundred electron irradiated frozen aortic valves in patients with severe aortic valve disease.
Detailed information is contained in the reference articles and two papers included in the proceedings of the IAEA conference in Budapest in 1969 (Campbell et al., 1970; Wright and Trump, 1970).

Non-tissue materials

A variety of materials used in medical procedures are sterilized by radiation. Radiation has proved to be the method of choice for certain materials that are damaged by heat or chemical methods of sterilization. Surgical procedures have been developed for the control of some aneurysms (Selverstone, 1962; Selverstone et al., 1962; Callow et al., 1962) using electron sterilized latex and epoxy to prevent the adverse changes produced by heat sterilization. Plastic tubing and sheeting, surgical sutures, catheters, electrodes and a large variety of other medical materials (Campbell et al., 1970; Wright and Trump, 1970) and instruments, not amenable to other forms of sterilizing techniques, are sterilized by radiation methods.

The use of electron irradiation to produce polymerization has provided stimulus for the development of new materials in the medical field. For example, concentrated water solutions of polyvinyl alcohol may be cross-linked by electron irradiation to form hydrogels. These materials are being developed for use as synthetic articular (bone joint lining), as membranes for artificial kidneys and for blood conduits (Merrill, private communication). In addition to polymerizing materials by electron irradiation, sterilization of the finished product may be obtained in the final package.

Sterilization dose

Results of the radiation dose required to destroy microorganisms and those to produce sterility have been reported by Dunn et al. (1948), Proctor and Goldblith (1953a), Edward et al. (1954), Christensen (1970a) and many others. These studies have shown that each organism exhibits a characteristic resistance to radiation and that the number of organisms surviving decrease exponentially with increased absorbed dose. The conditions of irradiation may produce differing slopes of the survival curves for the particular microorganism studied. Since the survival curves are exponential, it is important to make certain that the initial contamination level is low so that the dose may be kept below the level of deleterious side effects. Basic sterility studies with tissue material should be carried out under conditions such that the doses are adequate to produce sterility at the initial allowable levels of contamination. For example if a dose of 100 000 rads reduces the bacterial count by a factor of 10, then 200 000 rads will reduce the count by a factor of 100 and 1 million rads will reduce the count by a factor of 100 million. With an initial count of 10 000 a million rads should safely produce sterility with but one chance in a million of survival.

6. CONTROL, PACKAGING, STORAGE AND SHIPMENT OF MATERIAL

Material is usually obtained under clean but non-asceptic conditions. Criteria for donors must be established to minimize the chances of
introducing high levels of hepatitis or other viral infections or unknown causative factors due to malignancies and to ensure mechanically sound material.

For bacteriological control it is recommended that two small segments of each graft to be banked be procured. One is to be used for identification and determination of the concentration of bacteria present at the time of banking; the second is packaged, frozen and stored with the graft material. If identification of the bacteria indicates a highly resistant organism or a high level of contamination, then the graft should be rejected. After irradiation the second sample is cultured for bacteria and if sterile, the graft material becomes available for clinical use. The necessary administrative and radiation procedures must be established to provide proper control and use of material.

Material is usually packed in heat-sealed polyethylene bags with an inner and one or two outer bags. In many instances the outer bag is a Mylar polyethylene laminate, which can be heat sealed and is stronger than polyethylene alone. The maximum size of the bags will be determined by the irradiation facilities used. If a conveyor belt system is used, then the length of the bags is not critical but the width will be limited by the characteristics of the particular installation. For efficiency the bag sizes should not greatly exceed the size of the samples. To show that a package has been irradiated a clear glass bead 1 to 2 mm in diameter or a small piece of glass, which will turn dark when irradiated, is packed in one end of the outer bag or taped to it.

Since electrons have a limited penetration range, criteria for total allowable thicknesses, packaging plus graft, will have to be established for the particular radiation installation. For material irradiated at 3 MeV with a distribution as shown in Fig. 35 the thickness should be limited to 1 cm of unit density material or proportionately less for higher density material such as bone.

Immediately after packaging the material is frozen to its storage temperature. A temperature of -50°C or lower is usually used, although some groups have found a temperature of -30°C to be satisfactory. The polyethylene bags and homografts may be brittle at these temperatures and should be handled carefully to avoid damage. The material is to be kept frozen until it is required for use. Dry ice (-78°C) is used to keep the material frozen during shipment and irradiation.

Suitable shipping containers, usually polyfoam boxes, are available that will keep the material frozen for several days when packed with dry ice. A typical container, about 30 cm on a side, will have wall thickness of 3 to 5 cm and use about 2 kilograms of dry ice per day. Buckle straps have proven to be more satisfactory than tape for packaging. To keep homografts uniformly frozen and prevent damage in shipment they should be packed in a small metal container surrounded by dry ice within the shipping container.

Shipment by both air and ground methods have been satisfactory. Care must be taken to ensure delivery on a working day within the safe holding period of the dry ice. Arrival on weekends or holidays may result in total loss due to thawing. Details of a planned shipment should be coordinated with the irradiation centre to ensure proper handling.
CHAPTER 23
SURGICAL ASPECTS

1. BLOOD VESSEL SURGERY

In the past twenty years considerable progress has been made in the use of arterial and venous grafts, both as a direct replacement of damaged or diseased vessels and that used as by-pass grafts. The methods and material used have changed considerably in the past few years because of the increasing use of artificial graft material. Fresh arterial and venous homografts have been used with fairly good clinical results but they are certainly difficult to obtain in most centres and as a result their use has been restricted. Various methods of sterilization and preservation have been used and a number of them have been discarded but there is no doubt that amongst the successful methods irradiated grafts followed by rapid freezing technique have produced satisfactory clinical results. In one long-term analysis Gross and Litwin (1968) found that irradiated homograft aortic grafts used to reconstruct the thoracic aorta have produced satisfactory function for over twenty years. Although some of these grafts have become partially calcified in this period of time, there has been no case of failure of the graft or of subsequent aneurysm formation. Similar results have been published for peripheral arterial grafts, especially when used to replace or bypass the femoro-popliteal arterial tree.

The use of man-made fibre grafts, such as woven Dacron, which are easily stored and sterilized, has made the surgery of the major arterial and venous vessels a more simplified procedure and although there is still a place for homograft material, there is now no doubt that in many centres the artificial grafts are used almost universally.

2. HEART VALVES

The development of heart-lung bypass techniques has enabled surgeons to operate with safety on diseased valves of the heart. The replacement or refashioning of disordered or diseased heart valves is part of the routine work of any cardiac surgeon. Where replacement of valves is concerned, there is no doubt that artificial valves are used by the majority of surgeons. Homograft valves and fashioned fascia lata valves mounted on a metal frame are used in cardiac surgical centres throughout the world and we have long-term results on many hundreds of patients. Comparison of these results with those obtained in similar cases with artificial valve insertions shows that the biological graft has a place in this type of surgery.

Homograft valves were first used by Murray (1956) in the descending aorta of patients suffering from aortic regurgitation. The patient's own incompetent aortic valve was left in situ and some of these patients have had a satisfactory clinical result for twelve years. The use of the homograft valve in the subcoronary position was proposed by Duran and Gunning (1962)
and further developed by Ross (1962) and Barratt-Boyes et al. (1962). The technique of insertion of the free homograft valve has been described in detail by these authors.

The problem of sterilization and preservation of homograft valves has always presented a difficulty. Initially valves were obtained in clean conditions and sterilized with betapropiolactone or ethylene oxide gas, often with a freeze-drying technique. A number of patients with freeze-dried valves have been shown to develop significant calcification in the homograft valve and other methods of sterilization have therefore been tried.

Malm et al. (1967) in the USA and Gibbons and Alladine (1967a) in the United Kingdom simultaneously produced reports on irradiated valves. Although the techniques differ in some respects, the long-term results have shown this to be a satisfactory method, which does possess advantages over other methods of sterilization, provided the technique of collection, sterilization and preservation is meticulously carried out. Valves should be obtained within twenty-four hours of death, dissected free of adventitia and most of the muscle present and then heat-sealed in double-packed bags. Irradiation giving a total dose of 2.5 Mrad should be done as soon as possible after dissection. The valves used by Malm et al. (1967) and Kirklin (private communication) are flash frozen to -70°C, irradiated and kept stored at -70°C. Gibbons and Alladine (1970) suggest that the valves are kept at 4°C but should be used within thirty days after processing. The clinical results of using either method of preserving homograft valve appear to be the same.

Some surgeons have sewn the homograft valve to a metal frame and then sterilized the complete valve unit by various means, which include soaking in antibiotics and irradiation techniques. Care should be taken that a suitable suture material is used if the valve is to be sterilized by radiation (Chapter 21). It has also been shown that Teflon is not a suitable material to be used on the valve seating but Dacron can be irradiated without any cause for anxiety (Gibbons, unpublished information). These frame-mounted homografts are suitable for the replacement of any heart valve. There are problems in using a free aortic homograft to replace an atrio-ventricular valve and, apart from the frame-mounted homografts, the methods used have not been entirely successful.

Heterografts, being more easily obtained, have been widely used in the past. Many surgeons using these valves, often mounted on a frame, were very enthusiastic at first, but the clinical results, except in a few units, do not seem to be as good as those obtained with artificial and homograft valves.

Another technique used during the past two years has been the fashioning of fascia lata mounted on a metal frame to form a valve suitable for insertion. The results to date seem satisfactory but we do not yet know the long-term results so that no comparison can be made with other methods. This type of valve can be sterilized with radiation by gamma rays, but too few valves have been inserted so far for the method to be generally advised.

3. PERIPHERAL NERVE GRAFTS

The problem of repairing defects in peripheral nerves is one that has perplexed surgeons for many years. Attempts using homografts and autografts have, in the main, proved unsuccessful. In most cases the foreign
body response to the implanted nerve tissue and the subsequent rapid ingrowth of fibrous tissue at the anastomotic site has resulted in a fibrous graft without function. Removal of the graft has shown that the regenerating axons have been prevented from growing down the neural sheath by the fibrous tissue that has invaded the anastomotic site.

Some success has been achieved with the use of autogenous cable grafts and the use of pedicle nerve grafts (Seddon, 1963). Seddon showed, using this technique, that distal lesions do better than proximal and that delay in operation does prejudice the final result. The problem of using this type of graft is that it must be of sufficient calibre to accommodate that of the transected nerve. The disadvantage of using peripheral sensory nerve autografts, such as the lateral cutaneous nerve of thigh or the sural nerve, is that they are of relatively small diameter and leave the patient with a loss of skin sensation where the cutaneous nerve has been excised.

As stated, little success has been reported using unirradiated homografts or heterografts. In most experimental work using these types of graft there have been few examples of return of any function and the graft has been reduced to a fibrous band. The use of irradiated grafts by Marmor (1964) and Bassett et al. (1959) has been much more encouraging in both experimental animals and man. Marmor's technique has been to remove grafts under clean conditions and then heat-seal them in Polythene bags. The bags and grafts then were frozen to -12°F (-25°C) and irradiated with 2 Mrad, thus sterilizing the graft and reducing the inflammatory response when used at operation. These grafts were stored at -12°F (-25°C) for some four to eight weeks before use. In one of two patients described by Marmor (1963) a 5.5 inch (13.8 cm) irradiated homograft was inserted in a nineteen-year-old man who had lost 5 inches (12.7 cm) of his ulnar nerve from a gunshot wound. Some five months later he had a return of sensation to the palm and ulnar side of the fourth finger.

Campbell et al. (1970) have described irradiating nerves after freezing to -76°C using 2 Mrad by a 3-MeV Van de Graaff accelerator. In experimental work closing defects of 1.0 cm in the sciatic nerve of the cat good voluntary function and sensation returned. In man, grafts have been encased with Millipore, which barricades the junctions of the graft so that the outer sheaths of the nerve heal in a linear fashion. The Millipore is removed at two to two and a half months and the grafted area encircled with silastic. From experience of the first thirty grafts in man those over 7.0 cm in length have not proved successful but good results were obtained with nerve grafts bridging gaps smaller than 7.0 cm.

4. TENDON GRAFTS

Homograft tendons have been used for some years to repair both extensor and flexor tendons where the use of autografts was not possible. Seiffert (1971) has removed tendons from cadavers within twenty-four hours of death and then placed them in an organic mercurial antiseptic called 'Cialit' (1 in 1000) for twenty-four hours. They are then stored at 4°C in the same solution (1 in 5000) for about two months. At the end of this period of time no cellular material can be demonstrated in the tendons and they are then used for transplanting. Seiffert reports the use of these
homograft tendons in 43 cases with good clinical results and no evident deterioration after several years.

These tendons appear to be free of an antigenic response, presumably because the cellular material has disappeared. The host cells migrate into the graft and take up the position and shape of normal tendon cells. Using $^{14}$C-labelled proline Seiffert has shown that the collagen of the graft is replaced by the host in six to nine months and the end result is that the tendon homograft becomes living autogenous tissue.

Experimentally tendons have been irradiated with gamma irradiation at 4°C and this sterilizes the graft, kills any living cells yet does not appear to affect the strength of the tendon. To date there is no recorded case of their clinical use but there seems no reason why irradiated tendon material could not be stored and used in tendon repair surgery.

5. BONE GRAFTS

Bone graft material was first used in an operation over one hundred years ago. Since that time an increasing number of orthopaedic operating techniques have developed where the use of bone grafts is involved. Autogenous grafts have often only limited application because the amount of graft material that can be obtained from ribs or the ilium of the patient is quite limited in quantity and may not be sufficient for many operative procedures. The advantage of this fresh autogenous graft material is that there is no antigenic response that could delay or prevent incorporation of the graft. This graft material also contains living bone cells and this may be a factor why this type of graft is usually well incorporated in the graft site. A disadvantage is that an additional operative procedure is required with all the attendant risks of surgery, even though these are minimal. Possible complications include stress fractures across the upper tibia when this is used as a donor site and there is always the problem of haematoma formation and sepsis.

In operations where a large amount of bone is required homogeneous and heterogeneous grafts are particularly valuable. Bone can be obtained from suitable cadavers or animals, then sterilized and stored in a bone bank for future use. Because much larger cortical grafts can be obtained, they can be prefabricated at operation to fit into the graft bed. The cortical bone used is normally obtained from ribs or tibia, whilst the cancellous bone is most easily acquired from the ilium.

The collection of homogeneous and heterogeneous bone graft material does mean that there is a possible risk of sepsis. Cancellous or cortical bone should be obtained within twenty-four hours of death under clean conditions where refrigeration is also available. All soft tissue should be removed before the bone is treated and sterilized. Several methods of treating the bone to remove fat and other material are now accepted practice. The use of hydrogen peroxide and acetone has been used by some investigators. A more complicated procedure with detergent extraction, prolonged washing, lyophilization and sealing in sterile glass containers has been used by other workers in this field. In recent years bone irradiated with 2.0 to 3.0 Mrad and subsequently stored frozen has been used in several centres both in Europe and North America. Usually cancellous bone cut to 1-cm fragments has been irradiated. There are only minor changes in bone
structure and the grafts have been found to be well incorporated and consolidated after twelve weeks. Seeding, using a few chips of autogenous bone with the homogeneous cancellous graft, has proved effective in practice.

It is known that much of the antigenic properties of transplanted bone is due to the presence of blood vessels or marrow tissue. It would appear that irradiated and frozen bone loses its undesirable antigenic properties and is thus a very suitable preparation for clinical use. Irradiation undoubtedly kills the living bone cells but the survival of living osteogenic cells does not seem of prime importance. The grafted bone acts as a scaffold, provides mechanical fixation and allows bone cells of the host to lay down new trabeculae, especially in the case of cancellous bone, which allows easy infiltration.

In any surgical procedure it is the end result that is of prime importance and the use of irradiated bone grafts certainly appears effective. The experience of both Bassett and Cohen, as quoted by Wright and Trump (1970), shows an infection rate of only 1% in 3000 grafted cases. The clinical results in these patients are satisfactory and compare favourably with other reported series of cases where autogenous bone or homogeneous bone prepared by other means has been used.

The use of irradiated calvarium has recently been described by Campbell et al. (1970). From 1954 they used frozen irradiated split ribs and iliac bone to repair skull defects. Calvarial grafts, perforated at 2-cm intervals with 4-mm holes, have been used where defects occur at points of maximum curvature of the skull. The 4-mm holes were filled with living autogeneous bone chips, thus becoming the seeding areas for remodelling of the bone implant. The clinical results are extremely satisfying and compare well with the use of artificial materials used to bridge skull defects.

6. FASCIA LATA AND DURA MATER

Fascia lata obtained under clean conditions has been sterilized using 2 to 5 Mrad and stored for future surgical use. This fascia has also been mounted on a valve frame for use in heart valve replacement. To date there are insufficient reports to assess its suitability for surgical work.

Similarly, dura mater has also been sterilized with gamma radiation and used in clinical practice. We must await further authoritative reports before its use can be generally recommended, but initial reports are encouraging (see Chapter 21).

7. CONCLUSION

Surgical procedures are ever changing and new methods of treatment, new procedures and materials are brought into use year by year. To gain general acceptance with medical practitioners any new procedure must be of benefit to the patient and produce long-term results as good as, or better than that currently considered the best treatment. In recent years the introduction of new artificial materials that are non-toxic and accepted by the host has revolutionized certain branches of surgery. Likewise, tissue grafts are now widely used in surgical treatment. With some very specialized
tissue such as nerve and bone man has not produced any artificial material that can be compared with tissue graft material for clinical use. In other fields, such as arterial grafts, man-made fibres have gained almost universal acceptance, although homografts have functioned quite successfully in patients for over twenty years. Where heart valve surgery is concerned, homografts and fashioned fascia lata valves are being used side by side with artificial valves and the results, so far, are encouraging.

Much research on tissue grafting still needs to be done and it is possible that with improved methods of collection, sterilization and storage more surgical use will be made of this type of graft material. Irradiation of biological tissue grafts is now accepted as a standard procedure that has many advantages when compared with other methods of sterilization and subsequent storage of tissue. It is to be hoped that much wider use will be made of irradiated biological material in surgical practice for the clinical results have been shown to be quite satisfactory in many instances and compare well with most other procedures.
CHAPTER 24

OBSERVATIONS ON THE CLINICAL USE
OF FREEZE-DRIED, IRRADIATED ALLOGRAFTS
IN BONE TRANSPLANTATION

1. INTRODUCTION

Bone grafting is a well-known operative procedure with many applications in orthopaedic surgery. There is an increasing need for transplantable material, i.e., bone grafts from the patient who is going to be operated on (autografts), from other human donors (allografts) or from different animals (xenografts). Allografts and xenografts are usually provided from 'bone banks'.

The development of a sufficient stock of preserved bone grafts, mostly allografts, began long ago. Various methods of sterilizing and preserving these have been developed and many countries of the world have laboratories that deal with such problems.

Fresh bone autografts are the best material for transplantation and the best bone bank for each person is in his own iliac crest and tibia (Hyatt, 1960b). Preserved allografts are distinctly superior to similar xenografts. This superiority has been proved by many experimental and clinical observations and applies not only to bone, but generally to all kinds of organ or tissue transplants.

The main problem of bone transplantation lies not only in the choice of auto-, or allo-, or xeno-graft — since the quality of each has been well established — but rather in the choice of a graft that has been prepared by the best method or procedure.

The critical points of bone graft sterilization and preservation are: sterilization; immunological inactivation; and long-term preservation of the grafts.

Grafts should be treated so that they are sterile, well tolerated by the potential recipient, and capable of preservation by the simplest means for a long time. In addition, the graft should not lose its biological value nor become harmful to the recipient.

There is now quite a large body of experience with freeze-dried and irradiated bone allografts and we may therefore consider that lyophilization and sterilization by high doses of gamma irradiation do not appear to produce any serious damage to bone, on the contrary they make it a very useful transplantable biological material.

In a series of experiments Turner et al. (1956) found that the use of irradiated bone allografts in experimental animals was highly satisfactory. The authors pointed out that with this kind of graft there was an acceleration of bone healing in the recipient as a result of bone growth induction from the graft. They concluded that these results prove that irradiated bone grafts are nearly biologically equal to fresh autografts.

De Vries et al. (1958) found that freeze-dried irradiated bone implanted in experimental animals stimulated new bone formation. In clinical
applications the same authors reported very good results in 104 orthopaedic operations. Many other authors (Bassett et al., 1956; Swanson et al., 1963; Tillman et al., 1963; Ostrowski et al., 1967) have reported encouraging results from the use of frozen or freeze-dried irradiated bone allografts.

Tarsoly et al. (1969) found through experiments that the formation of the new bone induced by long massive cortical allografts in dogs was greatly improved by opening holes before transplantation through the mass of the graft. These authors believe that this improvement is the result of more rapid revascularization of the cortical graft. Campbell et al. (1970) also obtained good results when using freeze-dried irradiated bone allografts for bone defects of the skull. They opened holes at equal distances through the mass of the graft and filled them with bone marrow elements of the recipient just before the bone transplantation. This method was found to improve revascularization of the graft and new bone formation considerably.

The beneficial effect of opening holes in the graft and their subsequent filling with bone marrow elements from the recipient could be correlated with the observation of Burwell (1966) who found that fresh bone grafts, when impregnated with elements of bone marrow or with autologous bone chips, greatly improved the quality of the bone graft.

In an analysis of 3000 cases of bone transplantation reported by Wright and Trump (1970) 85% showed very good results. This percentage is easily comparable to those after transplantation of fresh autologous bone grafts. Similarly, very encouraging results have been reported by Cohen (1955), Artzimanoglou (1961), Triantafyllou et al. (1970) and others.

The method of lyophylization and irradiation has been used for three years now by the Human Tissue Bank in Greece for the preservation and sterilization of bone allografts. The main points of the method are as follows:

1. Collection of bones from fresh cadavers or amputated limbs under the best hygienic conditions. Collection must take place in the first 8 hours after death or amputation from the donor.

2. Special attention is given to careful selection of the donors. They must be young (20-40), with no history of any malignant disease, infection, hepatitis, blood diseases etc.

3. All the stages of the procedure, from the collection of the bone to sterilization of the grafts, are carried out under the aseptic conditions of an operating theatre.

4. Soon after collection the bones are kept at the temperature of dry ice.

5. The cancellous and cortical parts of the bone are separated and divided into standard sizes (sticks and cubes) and the fat, bone marrow elements etc. are carefully washed out.

6. The grafts are placed into vials and submitted to the lyophylization procedure. Subsequently, and since the vials are filled with a N atomosphere, the vials are hermetically sealed in a double polyethylene envelope.

7. Sterilization takes place in a gamma-ray source at a dose of $3.5 \pm 20\%$ Mrad for 10 hours.

8. After sterilization, the packages are stored and are available for distribution to the surgeons after two weeks but only if the results of the histological, microbiological and dosimetry controls are absolutely satisfactory.
The Human Tissue Bank of Greece has established a register of all the important details of the donor, the stages in the sterilization and preservation procedure and the condition of the recipient are carefully recorded. This register works in close co-operation with the surgeons who use the grafts.

2. AUTHOR'S MATERIAL

From the data available from the register of the Greek Human Tissue Bank we have selected for this report 104 cases of orthopaedic operations in which bone allografts were used. These 104 cases were treated by the same team of surgeons and in the same hospital. Table XXXVII shows the kind of operation performed and the number of cases.

**Sex and age**

Of the total number of cases 62 were female and 42 male. This slight difference is not of great significance since in our clinic we have more beds for females than for males. The youngest of our patients was 7 years old at the time of the transplantation and the oldest 62, while the majority of them (88) were between 19 and 45 years old.

**Clinical picture**

Most of the diseases suffered by the patients are shown in Table XXXVII. Apart from the cases of tuberculosis of the spine, the group also included 2 cases of TBC of the hip joint and 1 of the wrist. In addition to the cases of TBC of the spine, spinal fusion was performed in 22 cases of various other diseases of the spine such as protrusion of intervertebral disc,

**TABLE XXXVII. NUMBER AND TYPE OF OPERATIONS**

<table>
<thead>
<tr>
<th>Type of operation</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Spinal fusion, posterior, TBC spondylitis</td>
<td>14</td>
</tr>
<tr>
<td>2. Spinal fusion, posterior, for other reasons</td>
<td>10</td>
</tr>
<tr>
<td>3. Spinal fusion, after laminectomy, posterior</td>
<td>12</td>
</tr>
<tr>
<td>4. Spinal fusion posterior, for idiopathic scoliosis</td>
<td>4</td>
</tr>
<tr>
<td>5. Arthrodesis of the hip joint</td>
<td>5</td>
</tr>
<tr>
<td>6. Self-operation of the hip for CDH</td>
<td>1</td>
</tr>
<tr>
<td>7. Filling of bone cysts</td>
<td>8</td>
</tr>
<tr>
<td>8. Benign tumours of bone</td>
<td>12</td>
</tr>
<tr>
<td>9. Pseudarthrosis of long bones</td>
<td>18</td>
</tr>
<tr>
<td>10. Other operations</td>
<td>20</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>104</strong></td>
</tr>
</tbody>
</table>
spondylolisthesis, fractures, localized spondylosis etc. In all the cases of spinal fusion the posterior route was followed. Arthrodesis of the hip, except for TBC arthritis of the joint, was performed as also in 3 cases of severe secondary arthritis of the hip. All the cases of pseudoarthrosis of the long bones were of post-traumatic origin. Under the item "other operations" we have included cases of chronic osteomyelitis, arthrodesis of the small joints of the extremities for various reasons, shortening of the femur, various kinds of osteotomies, tibialization of the fibula etc. No malignant tumour was diagnosed in any of the patients and none has since suffered from a generalized bone disease as osteoporosis, fibrous dysplasia, Paget's disease, or eosinophilic granuloma. Apart from the orthopaedic disease for which our patients were admitted to the hospital, all were in a good general condition. No case of hepatitis was observed in any of these 104 patients either before or after the operation.

All the operations were performed by the same team of surgeons and under the same environmental conditions (theatre, hospital). The operations were performed under general anaesthesia and under the same aseptic rules that are usually applied to all the orthopaedic operations. In all the cases of spinal fusion blood transfusion was given during or after the operation. This was also the case for the operations of the hip and a few other cases. Special attention was paid during transplantation to ensuring the closest possible contact between graft and the bone bed of the recipient. As a rule a systematic antibiotic treatment was given to all patients for 8-10 days post-operation.

3. RESULTS

In evaluating the results obtained, attention was focused on the following three main points:

1. The incidence of infection
2. The radiographic appearance of a sound callus or new bone formation
3. The phenomenon of graft rejection.

The incidence of infection

In this series of 104 cases 4 patients showed post-operative signs of infection in the area of operation. These cases are shown in Table XXXVIII.

In the first case of TBC of the spine ($T_6$-$T_7$) a rather extensive necrosis of the tissues surrounding the surgical trauma appeared. Surgical intervention was necessary to remove the necrotic tissues. A subsequent drainage of pus persisted for two months. In this case we presume that the infection was a secondary development to the necrosis of the skin. This necrosis, we believe, was due to the pressure of the patient's back against his bed.

The same necrosis of the tissues surrounding the surgical trauma developed in the third case, the pseudoarthrosis of the tibia. While suturing the skin the tissue available was not enough and the assistant applied rather strong tension to the margins of the wound to complete the suture. Subsequent to necrosis a septic osteitis developed at the site of the pseudoarthrosis. We believe that in this case the infection was iatrogenic due to errors during the surgical procedure.
TABLE XXXVIII. CASES OF POST-OPERATIVE INFECTION

<table>
<thead>
<tr>
<th>Operation</th>
<th>Number of infections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinal fusion for tuberculosis</td>
<td>1</td>
</tr>
<tr>
<td>Benign tumour</td>
<td>1</td>
</tr>
<tr>
<td>Pseudarthrosis of bone (tibia)</td>
<td>1</td>
</tr>
<tr>
<td>Osteomyelitis chronic</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>4</strong></td>
</tr>
</tbody>
</table>

In the case of the chronic osteomyelitis the bone bed of the recipient was already septic and so the post-operative inflammatory processes could be attributed to this cause.

The only case in which infection developed in an aseptic background and without obvious external factors is the case of a growing fibroma of the greater trochanter, which was removed surgically and the remaining cavity filled up with bone allografts. In this case extensive inflammation appeared at the site of the previous operation, on the 20th day, and we had to repeat the curettage and remove all the grafts that had been placed in the previous operation, after which the infection gradually subsided.

The radiographic appearance of a sound callus, or of new bone formation.

The time of the radiographic appearance of a sound callus, or of a new bone formation is shown in Tables XXXIX and XL.

Table XXXIX only includes the cases of spinal fusion, scoliosis and pseudarthrosis of the long bones because to some extent they are rather typical operations and their results are quite easily comparable with the results of similar operations in which fresh autologous bone grafts have been transplanted (group A). Table XL includes 84 cases in which the same operations have been performed, but with the use of fresh autografts (group B). The sex and age distribution of both groups were approximately the same. All the operations on group B were performed by the same surgical team and under the same conditions as for group A.

Rejection of the graft

In only one case did we observe the phenomenon of graft rejection. This case was a boy of 14 with a severe idiopathic scoliosis of the spine. A long massive cortical allograft was used during the operation, as is usual in the case of scoliosis, but this time instead of lyophilizing the graft before irradiation a frozen-irradiated one was used. Four weeks after the transplantation the radiographs of the patient began to show a progressive absorption of the graft. The rapidity of this absorption was so great that radiographs taken 3 months after the operation showed that at least one third of the graft was absorbed. Six months after the first operation we re-operated and this time used fresh autograft taken from his tibia. In some parts we found small pieces of the old allograft, but the
TABLE XXXIX. TIME OF RADIOGRAPHIC APPEARANCE OF SOUND CALLUS: GROUP A

<table>
<thead>
<tr>
<th>Operation</th>
<th>Weeks after operation</th>
<th>Failures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-8</td>
<td>8-10</td>
</tr>
<tr>
<td>Spinal fusion, no laminectomy</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Spinal fusion, after laminectomy</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Scoliosis</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Pseudarthrosis of long bones</td>
<td>2</td>
<td>9</td>
</tr>
</tbody>
</table>

TABLE XL. TIME OF RADIOGRAPHIC APPEARANCE OF SOUND CALLUS: GROUP B

<table>
<thead>
<tr>
<th>Operation</th>
<th>Weeks after operation</th>
<th>Failures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-8</td>
<td>8-10</td>
</tr>
<tr>
<td>Spinal fusion, no laminectomy</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Spinal fusion, laminectomy</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>Scoliosis</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Pseudarthrosis of long bones</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

whole area of the previous transplantation was covered with a dense connective tissue. We think that it is important to point out that a week after the initial operation and until the fourth month after there was a permanent slight elevation of the temperature of the patient from 37.3 to 37.6°C. We attribute this rapid absorption of the graft to a rejection process.

4. DISCUSSION

The true evaluation, from the clinical point of view, of preserved bone autografts is a difficult task (Burwell, 1969). More difficult is the evaluation of the different methods of bone preservation and sterilization.

Schwier (1960) found that clinically frozen allografts are superior to chemically preserved ones. Burwell (1969), summarizing the results of the use of bone allografts preserved by freezing and other methods, points out that frozen bone is more effective than merthiolated bank bone and that freeze-dried b-propiolactone-treated bone and boiled bone are inferior to either frozen or freeze-dried bank bone.

The efficacy of high doses of gamma irradiation is examined in detail elsewhere in this Manual. In our experience irradiation fulfills all the requirements for sterilization of bone allografts. All sterility controls, whether direct or indirect, that we have applied to bone grafts have shown
that irradiated grafts are practically sterile and can be used as transplantable material. This is in agreement with the observations of other authors (Wright and Trump, 1970; Christensen et al., 1967b; Bonet-Maury et al., 1967) who emphasize that with a range of 2.5 - 4 Mrad practically all bacteria pathological to man, and probably all viruses, are fully eliminated. This material should therefore be considered as sterile.

We do not think that the cases of post-operative infection that appeared after the use of preserved bone allograft in four of our patients show the real incidence of infection after the use of this type of graft. We believe that at least two of the four cases must be excluded since the cause of the inflammatory processes in them could be attributed to necrosis of the tissues around the surgical wound. Thus in 104 orthopaedic operations in which preserved bone allografts were used only two cases of infection could be explained as probably due to the grafting process. We do not consider this percentage, approximately 2, as being considerably higher than that usually observed in any kind of orthopaedic operation. In 4000 orthopaedic operations performed over the last few years under the same conditions as the bone transplantations discussed here we have had a post-operative infection rate of 1.5 - 1.9%. To this encouraging result can be added the results of Swanson et al. (1963) who mention a rate of 1.9% for infections in the 368 bone transplantations of grafts preserved and sterilized with a similar technique.

Some authors (Christensen, 1970a; Little, 1970a) have recently suggested that it is necessary to increase the dose of gamma irradiation to 4 - 4.5 Mrad or more. We do not believe that there is evidence for such an increase for the sterilization of bone autografts taken from healthy donors under the best hygienic conditions.

Judging from the results of the comparison between the transplantations of fresh autologous bone grafts (group B, Table XL) and preserved and sterilized by the method we use (group A, Table XXXIX), we think these results are quite comparable. Of course our results in both groups have been evaluated only (as far as the callus or new bone formation is concerned) by radiographic criteria and possibly our findings are not absolutely accurate. We agree with Burwell (1969) that an absolute assessment of the value of preserved bone grafts needs more detailed criteria, for example, the surgical exploration of the site of previous bone transplantation. But for clinical assessments we believe that the radiographic demonstration of a sound callus or new bone formation is quite convincing evidence that the implanted graft has accomplished its aim.

Of the 104 cases of the Group A (Table XXXIX) only 8 cases of graft failure were observed, i.e. 7.6%. This incidence is probably small in comparison with that of Wright and Trump (1970) where the failures among 3000 cases were found to be 15%. We attribute this 'optimistic' incidence of our own to the small number of cases.

Apart from the four cases of infection and the one of graft rejection, we noticed another three cases of failure due to the fracture of the graft.

Actually we have noticed that grafts of bone so preserved and sterilized become brittle and are easily broken down. Other authors have also made the same observation. Possibly the cause for this change in the mechanical properties of the bone is due to the destruction of the collagen fibrils in doses of over 2 Mrad (Buring, 1970). We do not think that the discoloration of the graft after irradiation has any clinical importance. Moreover, as
far as we know, there is no published case of neoplasia or other disease that can be attributed to the presence of free radicals in the irradiated bone graft (Stachowicz, 1970a).

We are rather sceptical about the beneficient effect, noted by Tarsoly et al. (1969), of cutting holes through the mass of massive cortical bone grafts, as far as the revascularization of the graft is concerned. We have applied the method in scoliosis, where we cut such holes in order to introduce a wire suture to fix the graft (a massive cortical one) to the spinal processes. Of course our experience is very limited, but we have not noticed any important difference. On the contrary, we think that by opening such holes the graft is weakened further. We have no experience with the method of Campbell et al. (1970) in filling the holes with bone marrow elements from the recipient.

As far as the immunological properties of freeze-dried and irradiated bone allografts is concerned, we agree with the other authors that in the various stages of the procedures to which it is submitted the bone loses the greater part of its antigenic properties. We are not in a position to give any substantial explanation of the case of rejection noticed in the one patient of our series. Perhaps the fact that this case was the only one where a frozen-irradiated graft instead of the usual freeze-dried one was used has something to do with the rejection.

5. CONCLUSION

In the conclusion, we believe that freeze-dried and irradiated bone allografts fulfil all the necessary requirements for successful bone allotransplantation. Some very important points must always be kept in mind:

(1) Cancellous grafts are always better than cortical ones.
(2) The quality of cortical grafts can be improved by their impregnation with autologous bone marrow elements, or by cancellous chips taken from the recipient during the operation.
(3) New bone formation with the cortical grafts takes place chronologically later than with cancellous grafts.
(4) Orthotopic application of the grafts is absolutely necessary for the success of an allotransplantation.
(5) Freeze-dried and irradiated bone graft, being more brittle than fresh autologous bone, is not very suitable for use alone as support material in the skeleton.
(6) The allograft must have the closest possible contact with the bone bed of the recipient. Better results are obtained if the graft is in close contact with the cancellous bone of the recipient. This graft material is therefore recommended for operations where such close contact is feasible, e.g. filling bone cysts, filling various bone cavities, pseudarthrosis of bone, osteotomies, spinal fusions etc.
(7) When covering or bridging large bone defects or gaps or replacing extensive parts of long bones, we think that cortical and cancellous allografts should be supported by other means such as internal fixation with endoprosthesis, or external supports such as plaster of Paris, various frames etc. The need for support is not only because the allografts are brittle
but also because during the period of revascularization and remodelling they become progressively weaker and are easily broken down.

Finally we believe that till now the best methods of bone preservation and sterilization are freeze-drying and high doses of gamma-irradiation. More experience and systematic experimental study are needed to confirm the clinical findings.
CHAPTER 25

ORGANIZATION OF TISSUE BANKS
BASED ON THE RADIOSTERILIZATION TECHNIQUE

1. INTRODUCTION

The following Chapter is based mainly on the authors' own experience in organizing and running the Central Tissue Bank in Warsaw for more than 7 years. The operating principle of this bank is radiosterilization of tissues processed in such a manner that they can afterwards be kept at room temperature. The other types of banks, based on radiosterilizing frozen tissues and distributing them in the frozen state, will also be taken into account. Banks that do not apply radiosterilization as a final step in tissue preservation will not be described.

The preserved tissues are of course biostatic grafts, i.e. devitalized tissues that serve as biological prostheses and in some cases are substituted by the host's own tissues.

2. LEGAL AND FINANCIAL ASPECTS

As the first step in the organization of any tissue bank the legal aspects of tissue collection from cadavers must be carefully studied since they may considerably influence the organizational scheme. In certain countries access to human tissues may be difficult for traditional or religious reasons. In Roman Catholic countries autopsies and removal of parts of the human body are authorized by a papal bull.

In most countries tissue banks are organized as non-profit institutions supported by the government or a foundation. The bank itself, as an independent organization, is responsible for the free distribution of preserved tissues. In our opinion no financial remuneration for the family of the donor should be provided, which considerably simplifies the moral and human aspects of the problem.

It seems that from the economic point of view tissue banks based on radiosterilization of preserved tissues should be organized as large units supplying the whole country or region. The stability of preserved tissues at room temperature allows their distribution by the normal postal service. The need for grafts depends on many factors but it seems that once the surgeons become convinced of the merits of such material, the bank may expect a demand of at least 2 - 3 thousand grafts per annum and population of $10^7$.

3. METHODS OF PRESERVATION PRIOR TO RADIOSTERILIZATION

From among the tissue preservation methods available, those presented here seem to be most suitable if the tissues are to be sterilized by irradiation. Human bone is usually preserved by lyophilization (Flosdorf and
Hyatt, 1952; Manning, 1960). Since bone represents the largest item among the products of most tissue banks, this procedure will be elaborated in more detail further. Xenogenic bone, usually partially deproteinized for removal of its antigenic proteins (Hancox et al., 1961; Maatz and Bauermeister, 1961) may also be sterilized by irradiation. Human costal cartilage used in plastic surgery is best preserved by irradiation in saline (Dingman and Grabb, 1961) since its resorption is then slower than after preservation by other methods. Human or calf cartilage used to fill bone cavities (Piskorski, 1968) are preserved by lyophilization to speed up their resorption, which must precede the healing of the defect. Trials are undertaken to apply radiosterilization to lyophilized blood vessels (Flemming et al., 1966). Fascia, meninges and tendons are fixed in 70% ethanol, rinsed and irradiated in saline. The same procedure is used for skin preservation. It is based on the observations of Koontz and Kimberly (1961) that fascia fixed in ethanol remains unresorbed after transplantation longer than unprocessed irradiated fascia.

Grafts preserved by the above-mentioned methods and sterilized by irradiation offer the following advantages:

1. Long durability of preserved tissues
2. Lowering or complete suppression of tissue antigenicity
3. Sterility
4. Easy transport, distribution and storage in hospitals
5. Relatively low cost of graft production and handling;

and the disadvantage of changes in the mechanical and biological properties that occur, e.g., in the case of compact bone.

4. OUTLINE OF TISSUE BANK ORGANIZATION

In banks that sterilize tissues by irradiation the following steps should be organized:

1. Collection of appropriate tissues from the cadavers selected
2. If tissues are collected outside the bank's territory, then their transport under adequate conditions must be ensured
3. Cleaning grafts of adhering tissue and cutting them into properly modelled fragments
4. Lyophilization, fixation in ethanol or storage in saline
5. Radiosterilization
6. Control procedures to check the sterility, antigenicity, mechanical and biological properties and the presence of induced free radicals
7. Distribution
8. Documentation - involving data concerning the tissue donor, the number and type of grafts produced, results of control, data on distribution of grafts and retrospective analysis of graft performance based on clinical reports.

Some of these steps are discussed below.

4.1. Collection of tissues and selection of cadavers

Since the effectiveness of radiosterilization depends on the degree of initial contamination (see Chapter 3), it would be preferable to collect
tissues under sterile conditions and use radiosterilization as an additional security only. On the other hand, an attempt to collect tissues under conditions of complete sterility would increase very considerably both the organizational effort and the cost of graft production. The actual practice of many banks, including our own, is to collect tissues under clean but non-sterile conditions. After radiosterilization the material is acceptable for clinical use.

Tissues may be collected within the bank, which involves the troublesome organization of cadaver transport to the bank. The alternative solution is to collect the tissues in a department of forensic medicine or in hospital prosectories. The main source of human tissues in many banks is the victims of traffic accidents. Tissues should be collected not longer than 6-8 hours after death. If the body is preserved at low temperature, this limit may be extended to 48-72 hours. The age of the donor should not exceed 60 years in the case of bone and 30-40 years in the case of rib cartilage to be used in plastic surgery, since its degeneration starts very early (Quintarelli and Dellovo, 1964).

It is absolutely necessary to provide prostheses for the removed bones and repair all incisions as carefully as possible.

Anamnesis and anatomo-pathological examination should exclude neoplasms, viral hepatitis, septic diseases, tuberculosis, syphilis, certain acute poisonings, etc. Syphilis is excluded for social reasons and protection of the technicians modelling grafts, although Spirocheta pallidum is one of the most susceptible organisms to any kind of sterilization. Opinions differ as to whether the presence of brain tumours should exclude the possibility of taking bone for preservation. In our opinion it is safer not to use such material, particularly in view of the known resistance of some viruses to radiosterilization and the possibility of viral etiology of some tumours.

4.2. Lyophilization

Lyophilization of tissues frozen to the temperature of dry ice is run according to the type of the apparatus used, but the details of the procedure must be determined empirically. The problems involved in determining the amount of water to be left in bone have been discussed extensively by Hyatt (1960a). It should be stressed that excessive removal of water influences the mechanical and biological properties of bone very unfavourably. Occasional criticisms of the properties of lyophilized material may have been evoked by an improper choice of lyophilization parameters. The control of the mechanical properties of bone will be described further.

4.3. Packing the material

Modelled fragments of tissues packed in glass containers may be sealed in vacuo or closed with non-toxic rubber stoppers and sealed with gelatine gel. Containers for lyophilized material should be filled with inert gas, for instance nitrogen, to prevent the oxidation of fat. The advantage of rubber stoppers is that the surgeon can rehydrate the material before use without opening the container.
4.4. Radiosterilization

Since the radiation sources that are suitable for tissue sterilization are rather expensive, it is more economic to send containers with grafts to an appropriate source outside the bank. Then the irradiation and physical dosimetry are done by the staff operating the radiation source. Nevertheless, bank personnel should be aware of the geometry and homogeneity of irradiation. Various radiation sources are suitable for graft sterilization. Cobalt-60 sources (Manning, 1960; De Vries et al., 1956), Van de Graaff generators (Cohen, 1955; Turner et al., 1956) and X-ray apparatus (Arct and Klimek, 1967) have been used. The use of fuel channels in a shut-down nuclear reactor for graft sterilization has also been described (Czerniewski et al., 1965).

The initial contamination of the tissues to be sterilized is unknown and so sterilization represents a compromise between the application of as much radiation as possible to ensure the highest probability of killing all microorganisms and the necessity of preserving the biological and mechanical properties of tissues. A dose of 3 - 4 Mrad is usually applied. In some banks special dosimeters are attached to containers as an additional control of the dose received.

4.5. Checking preserved and radiosterilized tissues

Bacteriological control

It is hardly possible to devise an absolutely dependable control of graft sterility. The dependence of the effectiveness of radiosterilization on the initial contamination is described in Chapter 3. Trials to culture aerobic and anaerobic organisms from randomly chosen sterilized grafts may be undertaken. It should nevertheless be borne in mind that some bacteria present, for instance, in vascular spaces may not come in contact with the bacteriological medium. Attempts to pulverize sterilized bone involve the serious danger of external contamination. Another solution is to attach to all or to representative containers 'sporotests' containing radioresistant spores and to study their possible survival after irradiation. Such control is particularly important if the radiation dose received by single grafts is not determined by other means. It is based on the assumption that the initial contamination does not exceed a level comparable to the number and concentration of spores in the sporotest. Spores of B. subtilis or B. pumilus may be used for this purpose.

There is at present no routine control that can be used to detect viral contamination of grafts.

Antigenicity of preserved tissues

All products of tissue banks are used in practice as allo- or xenografts and may sensitize the host. Therefore it is useful to understand to what extent various steps in the preparation of a graft may influence its antigenicity. Determination of the degree of antigenicity in patients would be too complicated so it is better to use animals. An example of such determination performed in our bank is shown in Fig. 36. Tissues preserved by various means and radiosterilized were grafted in an allo- and xenogenic
system into rabbits and the blastic reaction evoked by transplants in the peripheral lymph node was determined quantitatively (Kossowska-Paul, 1966). As can be seen from Fig. 36, both the whole procedure and the individual steps involved in tissue preservation considerably diminish the antigenic properties of tissues.

Mechanical properties

There is no doubt that lyophilization and radiosterilization influence the mechanical properties of tissues. Some quantitative measurements performed in our bank (Komender, 1970) are shown in Fig. 37. The diagram presents the values of breaking stresses applied in bending, pressing and twisting standardized samples of compact human bone. It appears that the breaking stress values do not change uniformly after different procedures. Lyophilization does not diminish the resistance of bone to bending but lyophilization and irradiation do. Resistance to pressing is increased after lyophilization and to twisting is considerably diminished after rehydration in saline of lyophilized and irradiated bone. High doses (6 Mrad) of irradiation diminish the resistance of bone to all stresses.

The changes in the mechanical properties of bone after heavy irradiation set an upper limit to the dose that can be reasonably used for radiosterilization. The mechanical properties of bone after lyophilization should be checked occasionally to estimate the degree of damage and eventually to correlate it with the degree of bone dehydration.
Determination of bone graft resorption rate

Preservation and irradiation of bone may considerably influence the rate of its resorption (Boyne, 1968). It is therefore useful to have some information about the resorption rate of bone grafts produced under standard conditions. In the case of irradiated bone data can be obtained relatively easily owing to the stability and chemical areactivity of paramagnetic centres in hydroxyapatite (Slager and Reilly, 1964, Termine et al., 1967, Stachowicz et al., 1970b). It is possible to calculate the total number of paramagnetic centres present in standardized fragments of irradiated bone. Similar fragments are subsequently transplanted and the decrease in number of paramagnetic centres is recorded at various time intervals after grafting (Ostrowski et al., 1970). Such a procedure is less tedious than the histological estimation of bone graft resorption.

5. RADIOSTERILIZATION OF FROZEN TISSUES

Some banks use radiosterilized frozen tissues as material of choice (Turner et al., 1956). The advantage of such procedure, if the radiation dose is not too high (Fig. 37), is that it causes little mechanical change in bone. The disadvantage is the more difficult transport and storage of tissues, which must remain frozen until used to prevent autolytic changes. For radiosterilization of frozen tissues large Van de Graaff generators are more suitable than cobalt-60 sources since the shorter time of irradiation simplifies the maintenance of the low temperature of the specimens. Unfortunately such generators are available in a limited number of countries only. In addition to the tissues listed in section 3, the radiosterilization of frozen nerves (Marmor, 1963) and heart valves (Malm et al., 1967) has also been reported.
6. CLINICAL EXPERIENCE

Clinical experience indicates that the results obtained with radio-
stereilized tissues justify their widespread use. On the basis of reports on
the clinical application of ca. 10,000 bone and 5,000 other tissue transplants
from our bank it appears that lyophilized and irradiated (3.3 Mrad) spongy
bone behaves after transplantation similarly to fresh autogenic bone as far
as its substitution is concerned. Substitution of compact grafts is distinctly
slower than that of fresh autogenic material. However, the majority of
compact grafts are used for vertebral column fixation and in this case delay
in substitution is not detrimental to the final results. The resorption of
cartilage is moderate and it is useful in plastic reconstruction. Favourable
reports on the application of meninges, fasciae and tendons have been
received from various surgical centres.
Most vaccines used for prevention and therapy of infectious diseases are administered parenterally. As with any other pharmaceutical preparations administered parenterally, the vaccines must also be sterile. Sterilization by heat cannot be used for the vaccines as the high sterilizing temperature destroys the essential antigens and thereby renders the vaccine non-immunogenic and non-functional. For the sterilization of already prepared vaccines the applications of ionizing radiation have proved successful and will lead to the broadening of the overall scope for the techniques of radiosterilization in the medical industries (Christensen et al., 1967b). A considerable amount of research is currently underway to investigate the effects of irradiation on vaccines as a means to further improve the efficiency and economy of the radiation sterilization techniques.

Vaccines are usually prepared under aseptic conditions, filtered through bacterial filters and are put into ampoules, in which form they are distributed for use in the relevant medical practices. This procedure involves an extensive and stringent aseptic condition for the manufacturing process, which becomes more expensive. Furthermore, a high risk of contamination of the product during ampoulation and packing sometimes lowers the quality of the vaccine. Irradiation provides an alternative technique for sterilizing the vaccine when it is already prepared and packaged. This process, in addition to being convenient, precludes contamination of the product during ampoulation and packing.

The preparation of vaccines, both chemical and corpuscular, are generally carried out in a production environment of good hygienic standard. In addition, the techniques involved in the process preclude the chances of high contamination of the vaccine from the environment. The radiation sterilization of prepared vaccines involves an exposure to high doses of radiation, which kills even highly radioresistant bacteria including the spore-forming types (Proctor and Goldblith, 1953b). Therefore there is no need to measure the initial pre-sterilization level of bacterial contamination of the prepared vaccines for the choice of an effective sterilizing radiation dose.

In both corpuscular and chemical vaccines a sterilizing dose of 2.5 Mrad has been applied successfully. Irradiation of a typhoid vaccine with a dose below 10 Mrad has not significantly affected the immunogenic properties (Tumanyan et al., 1958; 1969), whereas its toxicity has been reduced as compared to the non-irradiated vaccine preparations. Since the immunogenicity of typhoid vaccine depends on O, H and Vi antigens, it is essential to know the effects of sterilizing radiation doses on these antigens. It has been proved that at doses of up to 3 Mrad of gamma and X-rays O and Vi antigens are not destroyed and little effect is produced on H antigen. Thus to sterilize corpuscular and chemical vaccines for use against intestinal infections, already prepared and packed samples should be irradiated at a dose range of 2.5 to 5 Mrad. These doses not only
provide for sterility together with preserved immunogenic properties of the vaccines, but also a lower vaccine toxicity.

Radiation-sterilized vaccines must be tested for bacterial sterility, toxicity and the presence of the desired immunogenic properties as the quality control measures of the final products. Sterility is tested by inoculation on media with radiosterilized vaccine followed by 7 days incubation at constant temperature. It is considered as sterile if after the seventh day there is no growth of colonies either of contaminant bacteria or of those used for preparing the vaccine, wherever applicable.

The toxicity reducing effects of radiation have been noticed during the preparation of chemical vaccines from typhoid bacteria killed by irradiation (Tumanyan et al., 1969). Data were obtained concerning a significant decrease in the toxicity of various bacterial antigens removed from the bacterial cells irradiated with a dose of over 2 Mrad. If the bacteria from which the antigens had been removed were exposed to a larger radiation dose the antigen (Vi; lipopolysaccharide), toxicity decreased. When bacteria were irradiated with doses of 2.5 to 5 Mrad the toxicity of the antigens removed from them decreased by 2-5 times for Vi radioantigens; 2-4 times for lipopolysaccharides irrespective of whether the various test animals were injected with the antigens by intravenous, intraperitoneal or subcutaneous means.

So far it has been difficult to account for the observed radiation-induced decrease of toxicity of the bacterial antigens. It is assumed to result from a decrease in a lipid component in the antigens resulting from the radiation exposures. There is evidence (Kasai and Nowotny, 1967; Kim and Watson, 1967) to confirm that the toxicity factor is associated with the lipid component of the bacterial endotoxin.

The instances of the decrease in toxicity of the antigens derived from the irradiated bacteria may be of great significance for the successful preparation of low-toxicity radiosterilized chemical vaccines.

Tests for the toxicity of radiosterilized vaccines are carried out by injections in experimental animals. In such tests control standards are provided by heat-sterilized vaccine samples or those containing a complex of adequately purified highly immunogenic and non-irradiated bacterial antigens.

The antigenic and immunological properties of radiosterilized vaccines are tested by the immunization of rabbits followed by the determination of antibody titre in the blood serum of those animals (Tumanyan et al., 1958).
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