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LANDBOUWHOGESCHOOL WAGENINGEN THE NETHERLANDS

EFFECTS OF STERILIZATION ON COMPONENTS IN NUTRIENT MEDIA

Papers presented at a symposium organized by the Department of Horticulture, Agricultural University, Wageningen, The Netherlands

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Organization of the Symposium

Scientific aspects	J. van Bragt R. L. M. Pierik
Technical aspects	International Agricultural Centre, Wageningen
Chairmen	J. F. Bierhuizen H. Veldstra
Public relations	Information Department, Agricultural University, Wageningen

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PREFACE

This symposium on the effects of sterilization on components in nutrient media is the realization of a suggestion put forward at a meeting of the Dutch Plant Tissue Culture Club. One of the basic problems met in plant tissue culture research is the sometimes uncertain fate of nutrient components, especially when heat-sterilized.

It was evident that similar problems confronted not only plant tissue culturists but also workers in the fields of animal and human tissue culture, medicine, pharmacology, technology, food preservation, radiobiology, etc.

We appreciate the readiness of the speakers to discuss the specific problems in their respective fields of research. However, we have not made an attempt to cover all sterilization topics and we are bound to have overlooked something. Although the subject of this symposium was rather specific, no less than about 400 participants could be welcomed.

The language of the symposium was Dutch. To facilitate communication with workers abroad, it was agreed to publish the papers of the symposium in the English language. The kind and generous support of the Board of the Agricultural University at Wageningen and the Editorial Committee for the Miscellaneous Papers is gratefully acknowledged.

J. VAN BRAGT
R. L. M. PIERIK

OPENING ADDRESS

F. HELLINGA

Rector Magnificus of the Agricultural University, Wageningen, the Netherlands

A great deal of preliminary work has already been carried out in the organization of this symposium. I do not intend to present a survey of the symposium, but rather prefer to explain why Wageningen University is such an excellent medium for diverse fundamental as well as applied sciences.

The Agricultural University at Wageningen consists of four main divisions of study e.g. basic sciences, agronomy, social and technical sciences. Within these four divisions at present 22 specialized studies are possible. These specializations are geared to a large extent for the demands of society. It has been a wise decision of the previous generation to stress the importance of basic sciences in the educational and research activities of the University. Special attention is given also to general problems such as the increasing pollution of land, air and water and technical know-how from various scientific fields is pooled to create the best possible chances for finding solutions. At present, therefore, many horizontal and vertical links within the University offer facilities for either specialization or generalization of the study programs for the students.

The research on tissue cultures at the Department of Horticulture is another example in which a specialized point of view may lead to generalization. Originally this research was initiated because of various horticultural problems arising from practice, such as vegetative propagation, dormancy, flowering and the production of virus free plants. Tissue cultures are also of great importance in animal and human physiology. Among the specialized branches of science various aspects of the preparation and sterilization of media for growth have analogies and differences, which might explain the wide interest in this symposium.

I wish the organizers and all the participants of the symposium a fruitful meeting.

PLANT TISSUE CULTURE AS MOTIVATION FOR THE SYMPOSIUM

R. L. M. PIERIK

Department of Horticulture, Agricultural University, Wageningen, the Netherlands.
Publication 337

SUMMARY

During the organization of this symposium it became evident that scientists from very different branches were interested in the problems arising from the sterilization of nutrient media. This can be explained by the fact that the participants in this symposium have a common interest in the study of the cell as the smallest living unit in man, animal and plant. Since the cultivation of cells, tissues and organs plays such an important role in fundamental as well as in applied research, a short review will be given of the history of human, animal and plant tissue culture. Although originally the culture of human and animal tissues was far ahead of the tissue culture of higher plants, it can be stated at present that due to the discovery of several plant growth regulators, the plant tissue culture specialists are rapidly making up the arrears.

A survey is given of what can be achieved today with tissues of higher plants cultivated in vitro: meristem culture to obtain virusfree plants; vegetative propagation via callus and suspension culture; organ formation (root and shoot formation, flowering, fruit set, seed formation); the acquirement of haploid tissues and plants; embryo culture to prevent embryo-abortion; the culture of unimpregnated egg cells and pollen grains and the realization of fructification in vitro.

In the coming years plant tissue and organ culture will expand and develop even more rapidly than it has done until now. Plant tissue culture may contribute to and open up new vistas on the solution of problems such as cancer, incompatibility, virusmultiplication, the testing of toxic substances and the production of substances which cannot be obtained synthetically. However, the possibilities for research-workers are limited by many problems, some of which will be discussed to-day by other speakers.

The first symposium on the sterilization of nutrient media brought together scientists from many different fields: parasitologists, technologists, tissue culture specialists, pharmacologists, toxicologists, physicians, dentists and many other specialists. Their common interest lies in the study of the cell as the smallest independent unit in the living organism (man, animal and plant). The regulation of a cell or a group of cells concerns various aspects: cell division, cell growth, deteriorated growth and growth inhibition, differentiation, the formation of organs, the production of hormones and toxic substances, the role of micro-organisms and preservation.

Since the cultivation of cells, tissues and organs plays such an important role in fundamental as well as in applied research, it is worthwhile paying some more atten-

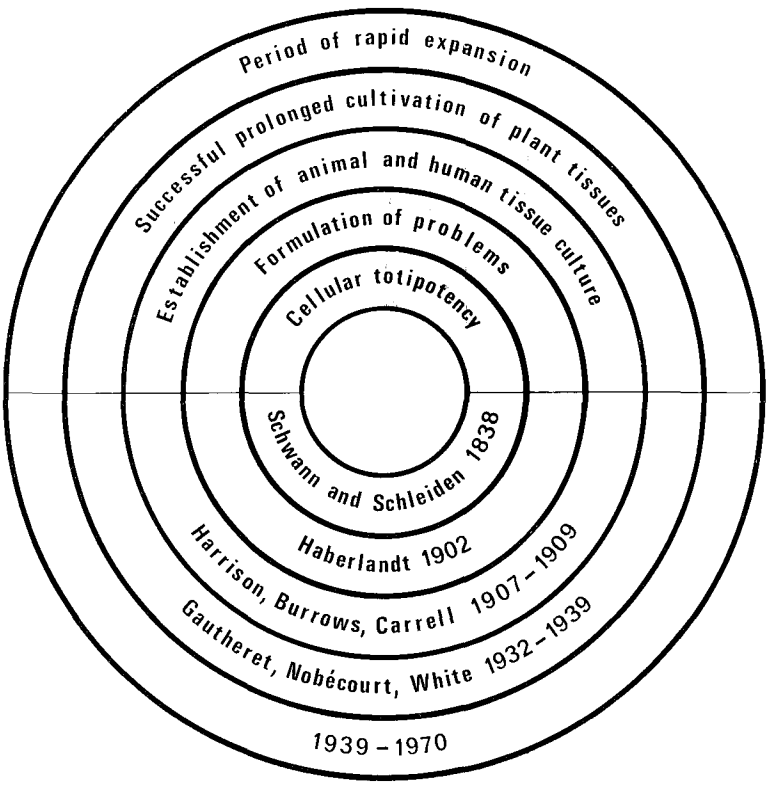

Fig. 1. The history of tissue culture.

tion to the technique. According to WHITE (1943), in his handbook of plant tissue culture, the history of science has been a history of methods, either experimental methods or methods of thinking. Plant tissue and organ culture is one of those methods or techniques, which cannot be detached from the history of tissue culture and from the way in which this symposium came about. Plant tissue and organ culture is the cultivation of plants, plant parts, tissues or cells on artificial culture media in sterile conditions. Figure 1 (PENSO and BALDUCCI, 1963; WHITE, 1943) shows the most important events in chronological order in the history of human, animal and plant tissue culture; specific important events and names in the history of biology, such as HOOKE, VAN LEEUWENHOEK, and BROWN, the discoverers of the cell and the nucleus, are omitted. The theory of totipotency as postulated by SCHWANN and SCHLEIDEN in 1938 focussed attention on possibilities which were to be realized much later, particularly with regard to plant tissues. The outstanding German botanist HABERLANDT (1902) clearly formulated the concept and stated the problems with which botanists were faced while cultivating plant tissues in vitro; but he did not succeed in bringing about the first plant tissue culture. The start of an animal and human tissue culture by HARRISON, BURROWS and CARREL (1907–1909) in the United

States can be characterized as the most important event in the history of tissue culture. However, plant physiologists were unable to bring about a real plant tissue culture until GAUTHERET, NOBÉCOURT and WHITE (1932–1939) succeeded in cultivating plant tissues effectively for some length of time at a stretch, thanks to the discovery of the first plant growth regulator auxin, and to their fortunate choice of plant material. Although the second world war strongly slowed down the further development of tissue culture, the period from 1939 to this day can be characterized as a period of rapid expansion.

From 1910 to 1930 much progress was made in the field of animal and human tissue culture (PENSO and BALDUCCI, 1963); VOLPINO (1910) cultivated in vitro cells from mouse adenocarcinoma; the problem of antibody production was investigated by CARREL and INGEBRIGTSEN (1912); it appeared that virus could survive for over a month in fragments of rabbit cornea (STEINHARDT and ISRAELI, 1913); FISCHER (1926) observed malignant transformation of cells in vitro, while CARREL and RIVERS (1927) were able to produce a vaccinia vaccine by growing virus in tissue culture. The most distinguished scientist in the period from 1910 to 1930, however, was ALEXIS CARREL,

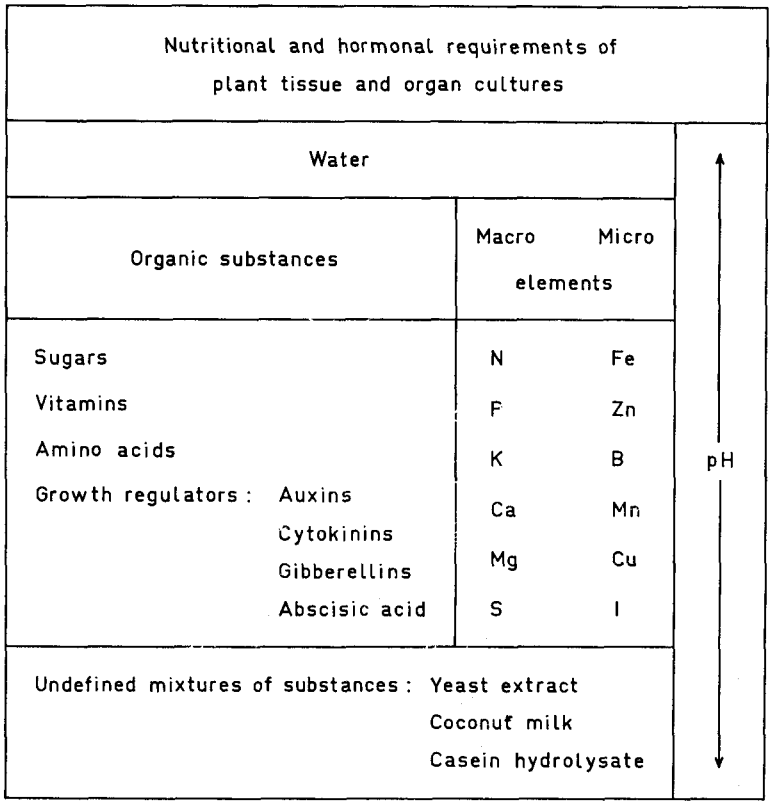

Fig. 2. Nutritional and hormonal requirements of plant tissue and organ cultures.

who more than anybody else contributed to the development and perfection of the techniques of cell culture.

Now, if we consider exclusively the history of plant tissue and organ culture, there can be no doubt that this branch of science strongly developed particularly after the second world war. The number of research workers markedly increased which can be illustrated by the fact that from 1964 to 1970 membership of the Dutch Plant Tissue Culture Club increased from 10 to 50.

In order to get a general idea of plant tissue culture and organ culture figures 2, 3 and 4 are given, showing the nutritional and hormonal requirements (fig. 2), a schematic representation of the culture of plants, plant parts and tissues in vitro (fig. 3) and of callus and suspension culture (fig. 4, WILMAR, 1969; WILMAR and HELLENDOORN, 1968).

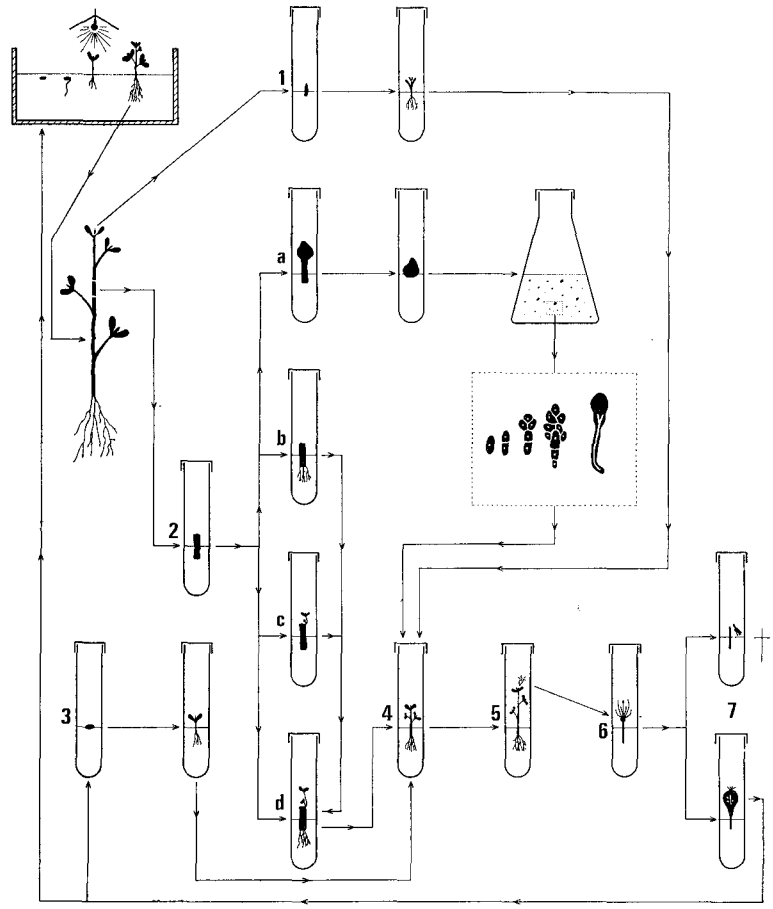

Fig. 3. Schematic representation of plant tissue and organ culture.

One should be careful in concluding from figure 2 that all components given would always be present in a culture medium. Figure 2 only shows the components which may be used in plant tissue and organ culture. A relatively simple medium on which as a rule plant tissues or organs will grow, consists of the following components: water, macro and microelements, a sugar, and a few growth regulators (like an auxin and a cytokinin). Addition of amino acids, yeast extract, coconut milk, plant extracts or casein hydrolysate to the culture medium rarely takes place. Plant tissue specialists will avoid using mixtures of substances which can be described as non-synthetic mixtures of vegetable origin as much as possible.

The most important results of plant tissue and organ culture are illustrated in figure 3, which first shows the development of a plant in vivo out of a planted seed. Tube 1 represents the isolation of an apical meristem in vitro with the object of obtaining a virusfree plant in tube 4. The basic principle of meristem culture is the fact that apical meristems are quite often free from virus. By the application of meristem culture QUAK (1966) was was able to obtain virusfree plants of potato, carnation, chrysanthemum, strawberry and iris. Tube 2 in figure 3 represents a test tube in which an explant out of a plant cultivated in vivo is isolated; the development and regeneration of the explant in tube 2 completely depends on the composition of the medium. In tube 2a callus formation takes place; callus tissues, partly undifferentiated, can be

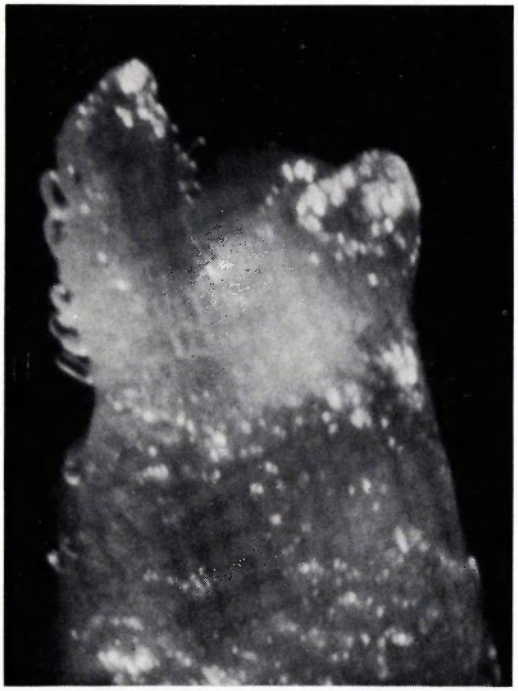

Photo 1. Terminal part of a potato shoot with the apical meristem (± 0.1 mm diameter) on top and 2 leaf primordia on the sides. Photo IPO (QUAK, 1966).

transplanted and cultivated in liquid media, where they form proembryos and embryo-like structures, which in turn can develop into complete vegetative plants (tube 4). The addition of an auxin may lead to root formation (2b), the addition of a cytokinin may cause shoot formation (2c), and in certain conditions a complete vegetative plant can be formed (2d and 4). The production of vegetative plants (from 2b, 2c or 2d to 4) was realized with chicory, lady's smock, biennial honesty, rhododendron and hyacinth (PIERIK, 1966, 1967a, 1967b, 1969, 1970b). The transition from the vegetative into the generative stage (PIERIK, 1966, 1967b) is also possible (tube 4–5), while the isolation of flower buds (PIERIK, 1970a) can lead to abscission (tube 7, upper part) and consequently to death of the plant material or to seed set and fruit set (tube 7, lower part) and seed formation. However, stage 4 can also very easily be realized by isolating a seed in a test tube (tube 3) and by seed germination in sterile conditions. Figure 3 shows in conclusion that all the processes from seed germination to seed formation can in principle be realized; it should, however, be mentioned that the scheme shown in figure 3 can only be realized with a restricted number of plant species.

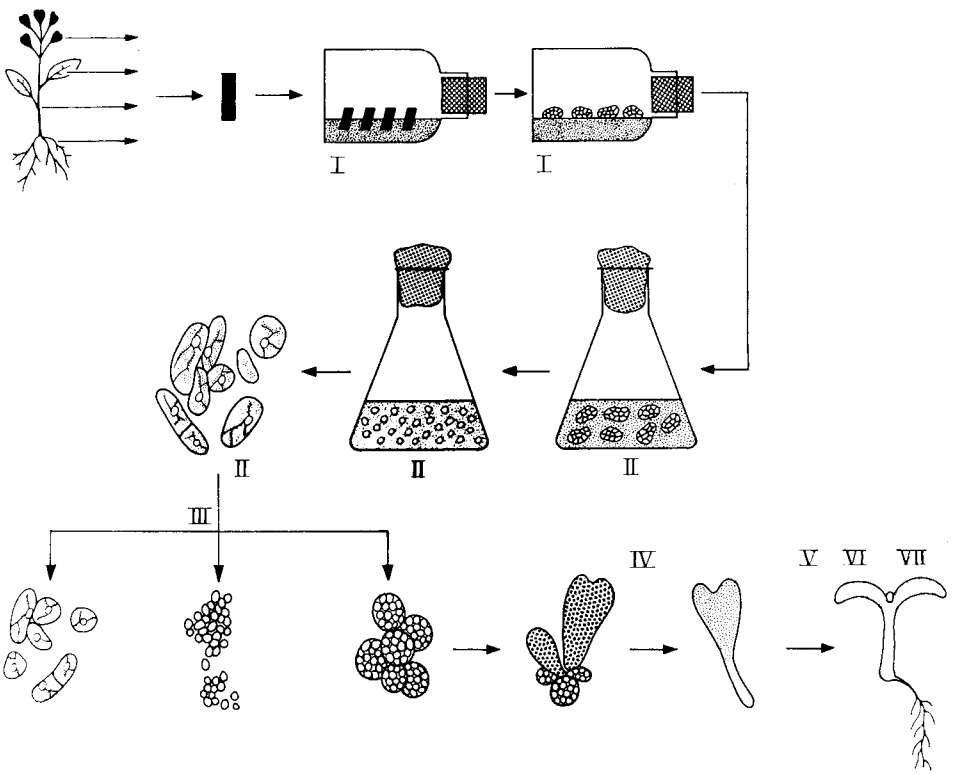

Fig. 4. Schematic representation of vegetative propagation via callus and suspension cultures (WILMAR, 1969; WILMAR and HELLENDOORN, 1968).

Figure 4 and photos 1–5 illustrate some work carried out by QUAK, WILMAR and PIERIK in the Netherlands. Photo 1 (QUAK, 1966) shows a potato shoot with the apical meristem on top and leaf primordia on the sides. The meristem (± 0.1 mm in diameter) is cultivated in vitro to obtain virusfree plants, whereas the two leaf primordia are broken off. Figure 4 (WILMAR and HELLENDOORN, 1968; WILMAR, 1969) shows the subsequent stages during the growth and morphogenesis of *Asparagus* cells cultivated in vitro: I. Isolation of explants and proliferation of isolated callus tissues which were formed on the explants; II. Cultivation of callus clumps in suspension culture, resulting is dissociation; III. When the level of growth regulators in the culture medium is reduced, small cells will be formed and nodule-like globular embryos will appear; IV–VII. The latter can develop into complete plants; but each stage of development will make its own specific nutritional requirements.

Photo 2 (PIERIK, 1966) shows the regeneration of a complete vegetative plant out of an isolated root explant of *Cichorium intybus* L. derived from a vegetative plant. Photo 3 (PIERIK, 1966) shows the regeneration of a complete generative plant out of an isolated root explant of a vegetative *Cichorium intybus* L. plant; flowering was induced by cold treatment (5 °C) of root tissue isolated in vitro during 8 weeks.

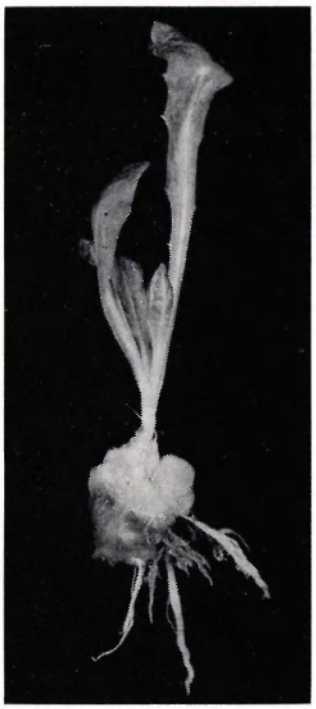

Photo 2. Regeneration of roots and a vegetative sprout at 25 °C from a root explant of *Cichorium intybus* L. (PIERIK, 1966).

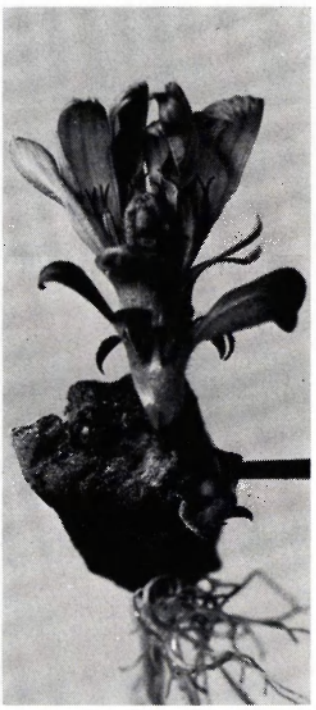

Photo 3. Regeneration of a flower on a root explant of *Cichorium intybus* L. which had been vernalized during 8 weeks at 5 °C (PIERIK, 1966).

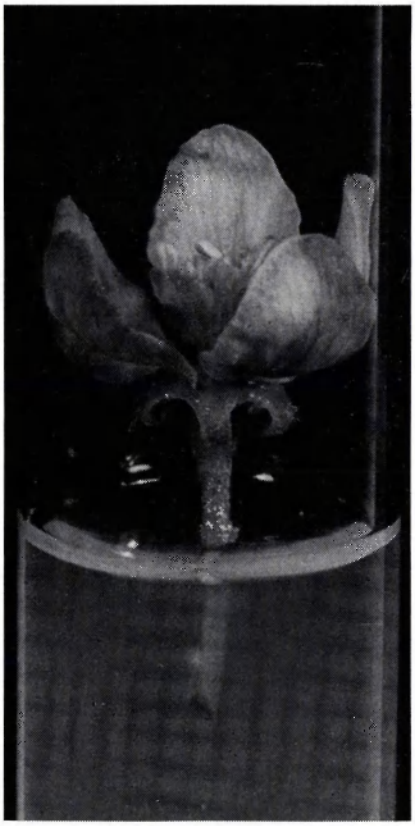

Photo 4. Isolated flower of the 'Cox's Orange Pippin' apple variety (PIERIK, 1970a).

Photo 5. Fruit set in an isolated flower of the 'Cox's Orange Pippin' apple variety, mentioned in photo 4 (PIERIK, 1970a).

Photo 4 (PIERIK, 1970a) shows an isolated flower of 'Cox's Orange Pippin' apple, which has been induced to parthenocarpic fruit set (Photo 5).

The results achieved with plant tissue and organ cultures are not restricted to the above mentioned applications. For the sake of completeness we should also mention:

1. Vegetative propagation of orchids by means of meristem and protocorm cultures.
2. The cultivation of haploid tissues and the production of haploid plants from anthers cultivated in vitro (SUNDERLAND and WICKS, 1969).
3. The transplantation of unimpregnated egg cells and pollen grains and the accomplishment of artificial fructification in vitro.
4. Embryo culture to prevent embryo abortion (LANGE, 1969), to produce viable hybrid plants and to accelerate plant breeding.

In future, plant tissue and organ culture will also be important to other specialists. We only have to think of the following problems: cancer, assaying in plant tissue cultures of substances toxic to man and animal, host-parasite-relationships, incompatibility problems and the production of substances which cannot be obtained synthetically. From this it is evident that the interests of the participants in this symposium are not so far apart as we might be inclined to think.

However, the possibilities for research-workers will be limited if we should not succeed in solving first a great number of problems coming up for discussion at this symposium. I should like to conclude my lecture by mentioning one of these problems: why are we still using a vegetable product like agar? To the gel-specialists present at this symposium I should like to put the question whether it might be possible to replace agar by a mixture of synthetic substances. Somebody can possibly answer the question whether a water-soluble synthetic polymer like polyvinyl alcohol together with another substance might not be used?

The problems we have to solve to-day are numerous. Each sterilization method has its own advantages and disadvantages. Gamma-sterilization for example has caused a great deal of discussion, particularly about the dangers attached to the consumption of gamma-irradiated food-stuffs, which may contain cancer-inducing components. But even if the sterilization-method does not implicate dangers, other problems arise. I should like to mention particularly the instability of various nutrients under the influence of light, heat and oxygen. The influence of light and heat (or temperature) on all sorts of processes in the plant is so evident, that during the second half of this symposium plant growth regulator-specialists will no doubt be happy to talk about the stability of plant growth regulators as affected by light and temperature.

At the end of my lecture I cannot refrain from mentioning that various colleages of mine, speakers and many other persons assisted at preparing and organizing this symposium. Because of the short time available for the organization of the symposium, not all of the important aspects of sterilization problems could be covered (SYKES, 1965).

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DISCUSSION

R. KOKKE: I have another argument to use a synthetic substance in stead of agar or gelatin as soon as possible. Agar, gelatin and other products such as yeast-extract and pepton are contaminated more and more by persistant toxic substances (antibiotics, chlorated hydrocarbons). For the time being, I suggest that manufacturers and dealers of agar are asked to provide a chemical analysis of their products which is provided in pricelists of pro-analysis chemicals. My questions to Dr. Pierik are: Do you know anything about the presence of toxic substances (1 ppm-1 ppb) detected by other specialists using agar, gelatin etc.? What is being done or what can be done to overcome the difficulties with toxic substances?

R. L. M. PIERIK: It is rather difficult to answer your question because in plant tissue and organ culture the influence of small quantities of toxic substances possibly present in agar has never been detected or cannot be detected. I can only say that in the past 20 years gelatin was hardly used in plant tissue culture media, because it was known to be toxic. In 1969 we asked Difco Laboratories in the U.S.A. to inform us about the chemical composition of Difco products and we received the following typical analysis of Difco-agars:

	Bacto-Agar	Noble-Agar	Purified-Agar
Ash	4.50%	2.60%	1.75%
Calcium	0.13%	0.23%	0.27%
Barium	0.01%	0.01%	0.01%
Silica	0.19%	0.26%	0.09%
Chloride	0.43%	0.18%	0.13%
Sulfate	2.54%	1.90%	1.32%
Nitrogen	0.17%	0.10%	0.14%
Iron	11 ppm	11.0 ppm	11 ppm
Magnesium	285 ppm	260.0 ppm	695 ppm
Copper	5 ppm	7.5 ppm	20 ppm

The table shows that no information is given or can be given about the presence of toxic substances or growth-regulating substances as plant growth regulators, vitamins, etc.

H. BELTMAN: I should like to comment on the substitution of synthetic substances for agar or gelatin. The advantage of agar or gelatin is that those compounds easily form gels. However, the disadvantage of agar is that a great number of undefined components is present (e.g. carbohydrates and microelements). This disadvantage can be prevented by using e.g. polyvinyl alcohol. Another possibility is to use a solid matrix such as a 'sponge' with capillaries like crosslinked dextran (Sephadex) or still better a synthetic polymer. It would be possible to infiltrate the sponges with water and nutrients by using microfilters.

R. L. M. PIERIK: We have already used sponges of foam plastic with great success. We used foam plastic as a matrix particularly in pH-experiments with rhododendron stem explants because liquefaction of agar takes place at low pH. The only difficulty with this matrix is that plant tissues, callus tissues and organs such as roots will grow in the capillaries, which is annoying.

MICROBIOLOGICAL CULTURE MEDIA AS ECOSYSTEMS

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SUMMARY

The functioning of a medium is determined by intrinsic properties as for instance: available nutrients, redox potential, pH, a_w and the presence of inhibitors; and also by implicit factors such as vitality of the inoculum and phenomena of antagonism. It means that extreme care has to be taken with the formulation, sterilization, storage and inoculation of media.

Methods are described for the ecometric evaluation of media in which regard is paid to the above mentioned parameters of media evaluation. For the measurement of the initial functioning of solid media a quantitative 'single plate' streak technique is described which reveals both inhibitory and productive effects. For the less frequently required evaluation of the course of functioning of solid media a replication technique is recommended. It is suggested to assess the functioning of liquid media by plating aliquots onto a set of suitable selective media at regular intervals during incubation.

1. INTRODUCTION

Large volumes of culture media are used daily in microbiological laboratories, though without much attention being paid to their quality and functioning – except in cases where suboptimal results are obtained. The performance of media, particularly of elective and selective ones, certainly depends on many variables. These, however, can be rather easily controlled and should indeed in practice be kept under control. In order to clarify the background of this quality control, the ecological occurrences in and on culture media will be reviewed in this paper.

For the functioning of the ecosystem, consisting of the medium and its inoculum, clearly the most important parameters are those connected with the medium itself, in other words its compositional characteristics. These are mostly indicated as intrinsic parameters. Obviously extrinsic factors such as temperature, time and gaseous atmosphere of incubation may play a decisive role too. In addition, the microflora of the inoculated material may influence the functioning of a given medium. Populations may grow more or less rapidly under otherwise identical conditions, while emerging populations may mutually influence each other's further development in a synergistic or antagonistic way. Such occurrences are called implicit phenomena (MOSSEL, 1969). In designing and using media, such phenomena should be taken into full account.

2. INTRINSIC FACTORS

The most important compositional properties of media that determine their functioning are the following.

2.1. *Available nutrients*

Usually these are grouped in four main classes: minerals, carbon sources, nitrogen sources and vitamins.

The need for availability of adequate amounts of minerals, some in mg-quantities others at μg levels, is generally observed. In addition, optimalization of the ion-balance is required. Particularly the anion composition is very important, since some cations can be precipitated or scavengered by particular anions and thus become unavailable for microbial nutrition.

The variety of carbon sources used in culture media is enormous. Alcohols, pentoses, hexoses, disaccharides, trisaccharides, various types of glycols, glycosides, acids and hydroxyacids are used, either alone or in combination – the latter deliberately or because impure preparations are used. Of great importance are also the so-called cryptic carbon sources. It has been demonstrated that sugars (VERA, 1950; KANDLER, 1960) and α-ketoacids (MACLEOD and MORGAN, 1959) occur in predominantly proteinaceous medium components of biological origin such as many peptones, meat extract, yeast autolysate and obviously in serum and whole blood. They may interfere with the functioning of the media to which they are added, particularly when the ingredients vary considerably. In such instances accurate chemical examination of every lot of component is inevitable.

The nitrogen sources used in culture media are also widely divergent. They belong to three classes: (i) well- defined inorganic salts, amino acids and peptides (TRITSCH and WOOLLEY, 1958; LEACH and SNELL, 1960); (ii) reasonably standardized (CHRISTIE, 1954; SCHWARZ et al., 1954; BARNES, 1955; HABEEB, 1959a, b, c; 1960a, b; BERNARD and LAMBIN, 1961) enzymatic digests of animal protein (BEDO et al., 1970; PARISI, 1970) or soya flour ('the peptones'), malt (BURBRIDGE and HOUGH, 1970) or yeast (GRANT and PRAMER, 1962; HOUGH and MADDOX, 1970); (iii) only crudely standardizable biologicals such as blood, serum, plasma, egg yolk, potato extract (BEEVER and BOLLARD, 1970), corn steep liquor (JOHNSON et al., 1971) or rumen fluid (BRYANT and ROBINSON, 1961; SIEBERT et al., 1968), which may have an additional value by supplying other growth factors.

As far as is known at present, the only type of vitamins required by micro-organisms are those of the B-group. They are added to culture media either as individual compounds or mixtures of those, or in the form of liver or yeast extract (STOKES et al., 1944). In some instances a more accurate control of the level of B-group vitamins in culture media is required than the usual routine attention.

It should be stressed that a richer medium is not always necessarily a more productive one. Practical experience in the field of the bacteriological examination of products such as soil, water and refrigerated raw milk has demonstrated that in such

habitats bacteria may occur that – without being autotrophs – proliferate well on poorer media, but not at all, or in much lower population fractions, on richer infusion media (WOLTERS and SCHWARTZ, 1956; SCHWEISFURTH, 1968; RUSCHKE and KÖHN, 1970). The term oligotrophic organisms has been suggested for these bacteria (NEHRKORN, 1968). Their behaviour may, in part, be caused by an unusually high sensitivity to e.g. certain nitrogen compounds with slight antimicrobial properties occurring in some components of nutrient media (BLOOM and BLAKE, 1948; BLOOM and PRIGMORE, 1952; HIRSCH, 1958; ASHTON and BUSTA, 1968). Also, lower recovery on rich media may be due to so-called inhibitory repression effects, i.e. blocking of assimilative pathways by amino acids and similar compounds (BEERSTECKER and SHIVE, 1947; ROWLEY, 1963; DATTA, 1969; KINOSHITA et al., 1969; JOSEPH, 1969) when these occur at rather high or unbalanced levels in some of the extracts and proteolysates customarily used in rich infusion media. Finally, such cells may be in a stress situation and it has been demonstrated that cells surviving e.g. exposure to sublethal ultraviolet irradiation, may make a much better recovery on poorer than on richer media (ROBERTS and ALDONS, 1949).

Media are often deliberately made elective by the incorporation of a limited supply of nutrients, so that the development of all organisms requiring the left-out growth factors is prevented. Nutrients with an elective effect used for this purpose are C-compounds such as ethanol, citrate, lactate, malonate and cellulose and N-containing nutrients such as arginine, urea, acetamide and glutamic acid.

2.2. *Redox potential*

This intrinsic parameter of nutrient media also requires permanent attention.

Very strictly anaerobic organisms may require Eh-values as low as – 200 mV (LOESCHE, 1969; MCMINN and CRAWFORD, 1970). In order to achieve the required low Eh-values sodium thioglycollate is quite often used as the redox potential reducing agent. Very many authors have demonstrated that this salt may be toxic to anaerobic bacteria (STERN, 1942; PORTWOOD, 1944; MALIN and FINN, 1951; HIRSCH and GRINSTED, 1954; GALESLOOT, 1961; MÖLLER, 1966; HILBERT and SPENCHER, 1970: LIN and LIN, 1970), particularly when sublethally impaired cells are involved (KOESTERER, 1964; DOYLE et al., 1968; SUTTON and HOBBS, 1969). The inhibitory effect of sodium thioglycollate is not always very consistent, since it seems to depend on factors such as (i) purity of the preparation used; (ii) mode and duration of storage of dried media, containing thioglycollate, prior to reconstitution and use; (iii) nutrient composition of the medium in which the salt is incorporated (CLARK, 1943; KLARMANN, 1956; MOSSEL and BEERENS, 1968). It is therefore advisable to avoid this sort of problems by refraining from the use of sodium thioclycollate in anaerobic media and to replace it rather by the physiologically entirely inert cystein (MOSSEL and BEERENS, 1968), suggested for this purpose as long ago as 1926 (QUASTEL and STEPHENSON).

Even when properly redox-adjusted media are used their handling requires attention Exposure of such media to air will result in an increase in Eh, the degree of oxidation

depending on the redox poising capacity of the medium. However high this capacity may be, oxygenation of such media for any period of time is to be discouraged, when strictly anaerobic organisms have to be cultivated. The pre-oxygenation effect (MOSSEL, van GOLSTEIN BROUWERS and de BRUIN, 1959) is a great handicap of the microbigenic technique for attaining anaerobiosis as introduced by FORTNER (1929). Even the much more rapid chemical methods for obtaining anaerobiosis may not always be entirely safe in this respect (de WAART and POUW, 1970).

2.3. *Acidity*

As a rule, sufficient attention is paid to proper adjustment of the pH of culture media. A notorious example of the opposite is, though, the generally held belief that any pH < 4.5 will be suitable for the cultivation or enumeration of all moulds and yeasts (SPLITSTOESSER et al., 1970). Yet, repeatedly evidence has been presented that quite some moulds and yeasts do in effect develop suboptimally at pH-values below 4.0 (PARFITT, 1934; von SCHELHORN, 1950; HOLWERDA, 1952; CHAMBERS and MCDOWELL, 1966; KOBURGER, 1970).

A second important aspect of the adjustment of the pH of culture media is the choice of the acids or bases used for this purpose. It has been demonstrated quite clearly that acids like lactic and acetic possess intrinsic antimicrobial properties, whereas e.g. citric and tartaric acid show no other inhibitory effects than those resulting from their reducing the pH of a medium (SHILLINGLAW and LEVINE, 1943; MORSE et al., 1948; MOSSEL and de BRUIN, 1960; CHUNG and GOEPFERT, 1970).

Generally, thermal sterilization will change the pH of a medium, mostly lower it. Such a change may make a medium slightly inhibitory or modify its initial antimicrobial properties, which may lead to a different behaviour towards organisms inoculated upon it (PARISI and MARSIK, 1969).

Obviously the pH of a medium as a rule changes drastically with the development thereon or therein of micro-organisms. Where required this has to be controlled by adequate buffering and/or the use of acidulating agents that are not readily dissimilated by micro-organisms (PARFITT, 1934).

2.4. *Water activity*

The osmotic situation in a medium used to be expressed in its osmotic pressure. Mathematically as well as experimentally the use of osmotic pressure as a parameter presents some problems. It is much more convenient to use the parameter water activity instead, which is defined as the water vapour pressure over the medium at a given temperature divided by the vapour pressure of pure water at the same temperature (SCOTT, 1957).

With decreasing a_w the range of micro-organisms capable of developing on a medium decreases. Hence media are often made selective to more osmotolerant organisms by the addition of polar solutes like NaCl, glucose, fructose, saccharose and glycerol. It is essential to realize that the extent of growth inhibition resulting from a

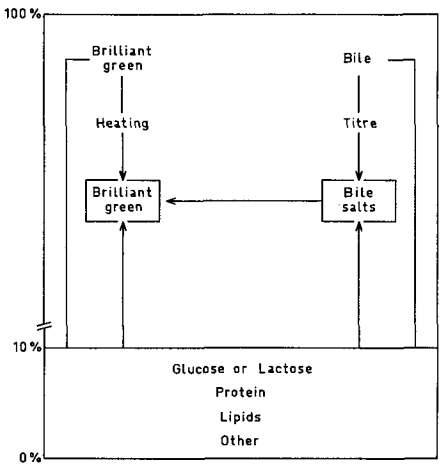

Fig. 1. Interaction between inhibitors and medium components. The activity, in the reaction kinetic sense, of the dye brilliant green and the surfactant bile salts in the commonly used medium: brilliant green bile lactose broth, depends on all the factors indicated including interaction between the two inhibitors.

given a_w is also, and often decisively determined by the character of the polar solute used to attain this a_w-value (SCOTT, 1957; KANG et al., 1969; MARSHALL et al., 1971; RODRIGUEZ-NAVARRO, 1971).

2.5. *Presence of inhibitors*

Although, as we have seen in section 2.1., selectivity of media is often based on the election of organisms with a specific anabolic pattern, selectivity of culture media is mostly achieved by the addition to a complete medium of one or more inhibitors of microbial growth with a certain degree of specificity.

The activity of such inhibitors under practical conditions is determined by the following parameters:

- Concentration added;
- Degree of inactivation during sterilization, e.g. hydrolysis or Maillard reaction type coupling during thermal sterilization and sorption or oxydation during filtration;
- Partial inactivation by binding to other medium components, by means of reactions which may occur at a significant rate even at temperatures between 20 and 40 °C and hence continue during storage of the medium and while it is incubated after inoculation (HANUS et al., 1967; KHAN and MARSHALL, 1969). Such interactions between inhibitors and other medium components can be of a very complex nature (Fig. 1).

Quite a number of selective agents used in modern media are antibiotics (Table 1). The activity of these inhibitors is obviously also influenced by the factors just discussed. But, in addition, the well-known resistance phenomena may limit the functioning of these factors as components of selective media.

Table 1. Selective media and strains used in the assay of their performance

Organisms looked for	Short description of selective medium	Test strains, of list (see page 22)	Temperature (°C) of incubation
Salmonella	Brilliant green lactose sucrose agar	2, 3, 4, 11, 15,	37
Arizona	Brilliant green sucrose agar	16, 18, 19, 25,	37
Shigella	Xylose lysine desoxycholate agar	26, 27, 28, 31,	37
Enterobacteriaceae	Violet red bile glucose agar	32,33, 43, 44,	37
Coli-aerogenes group	Brilliant green bile lactose broth		
	Lauryl sulfate broth		30
E. coli	Brilliant green bile lactose broth	11, 15, 16, 19,	44
	Trypticase NaCl-broth	26. 27, 43, 44	
Staph. aureus	Tellurite glycine egg yolk agar (Baird-Parker)	5, 7, 8, 9, 20, 23,	37
	Mannitol tellurite glycine broth (Giolitti and Cantoni)	26, 34, 37, 38	
Streptococci of the Lancefield D group	Crystal violet azide agar	2, 3, 6, 7, 9, 10,	37
	Azide dextrose Kanamycin agar*	10, 11, 20, 21, 22	
Streptococci of the *Mitis-Salivarius* group	Tellurite crystal violet trypan blue sucrose agar	23, 26, 27, 28, 30, 34, 35, 36, 37, 38, 39, 40	37
Clostridium group	Sulphite iron polymyxin agar*	4, 11, 12, 13, 26, 31, 32,	30
Cl. perfringens	Sulphite iron polymyxin neomycin agar*	37, 44	46
Bac. cereus	Mannitol egg yolk polymyxin agar	7, 8, 10, 11, 20, 21 23, 27,34, 37	30

Lactobacillaceae	Oleate acetate agar of pH = 5.5 (Rogosa)*	1, 3, 10, 11, 17, 20, 21, 22, 23, 27, 29, 35, 36, 37, 38, 39, 40, 42	30
Vibrio para-haemolyticus	Thiosulphate iron bile sucrose tylosin agar Trypticase starch salt agar*	2, 3, 4, 11, 25, 26, 27, 28, 32, 37, 43, 44	37
Pseudomonas aeruginosa	Glycerol mannitol acetamide cetrimide agar	1, 2, 3, 4, 5, 7, 11, 15, 16, 18, 19, 23, 25, 26, 27, 28, 32, 33, 37, 43, 44	42
Acetobacter sspp	Yeast extract ethanol agar	1, 2, 3, 5, 9, 10, 18, 22, 29, 42	30
Arthrobacter/ Brevibacterium	Blood nalidixic acid agar Glucose 5.5% salt agar	5, 7, 14, 15, 17, 18, 20, 23, 26, 30, 34, 37, 41, 42	30
Yeasts and moulds	Soya peptone dextrose agar, pH = 4.7 Yeast extract oxytetracycline agar	1, 5, 6, 10, 17, 20, 21, 22, 24, 26, 27, 29, 30, 37 38, 41, 42	24
Gram-negative rod shaped bacteria	Sugar-free crystal violet tylosin agar	1. 2, 3, 4, 5, 9, 11, 14, 15, 16, 18, 19, 23, 25, 26, 27, 28, 31, 32, 33, 34, 35, 37, 43, 44	19

* Incubation under anaerobic conditions

Legend to Table 1. List of test strains used.

1. *Acetobacter aceti*
2. *Aeromonas* sp.
3. *Alcaligenes* sp.
4. *Arizona* sp.
5. *Arthrobacter* sp.
6. *Aspergillus flavus*
7. *Bacillus cereus*
8. *Bacillus subtilis*
9. *Brevibacterium* sp.
10. *Candida pseudotropicalis*
11. *Citrobacter freundii*
12. *Clostridium perfringens*
13. *Clostridium sporogenes*
14. *Corynebacterium* sp.
15. *Enterobacter aerogenes*
16. *Escherichia coli*
17. *Fusarium* sp.
18. *Herellea anitratum* (Acinetobacter calcoaceticus)
19. *Klebsiella* sp.
20. *Lactobacillus acidophilus*
21. *Lactobacillus brevis*
22. *Leuconostoc* sp.
23. *Micrococcus* sp.
24. *Penicillium rubrum*
25. *Plesiomonas* sp.
26. *Proteus vulgaris*
27. *Pseudomonas aeruginosa*
28. *Pseudomonas fluorescens*
29. *Saccharomyces carlsbergensis*
30. *Saccharomyces cerevisiae*
31. *Salmonella gallinarum*
32. *Salmonella typhimurium*
33. *Shigella sonnei*
34. *Staphylococcus aureus*
35. *Streptococcus bovis*
36. *Streptococcus cremoris*
37. *Streptococcus faecalis*
38. *Streptococcus lactis*
39. *Streptococcus mitis*
40. *Streptococcus salivarius*
41. *Streptomyces* sp.
42. *Torulopsis* sp.
43. *Vibrio parahaemolyticus*
44. *Yersinia enterocolitica*

3. Implicit factors

In many instances media will be inoculated with pure cultures; their functioning will then, as far as implicit factors are concerned, only depend on the vitality of the inoculum. However, often the populations brought in or on media are not axenic, either per se or accidently. In such instances different organisms may influence each other's development by phenomena of synergism or antagonism. Culture media will always be composed in such a way that they allow the development of the sought organisms without relying on synergistic stimulation. However, antagonistic occurrences might escape our control.

3.1. *Vitality*

The vitality or growth intensity of an organism is expressed in the so-called Monod-Hinshelwood curve (Fig. 2). The parameters of these curves are: (i) the length of the lag-time, λ; (ii) the tangent of the logarithmic section, φ; (iii) the total cell yield, $\varkappa_f$. Obviously, these are implicit factors only as far as they are not unfavourably influenced by intrinsic (medium composition) and extrinsic (incubation temperature, gaseous atmosphere) culture conditions.

The values of λ, φ and $\varkappa_f$ are primarily determined by genetic influences. Some organisms are notoriously 'fastidious' or 'tardive', in that their λ-values are high, while φ may be rather small. Successful culturing of such organisms will have to be attained

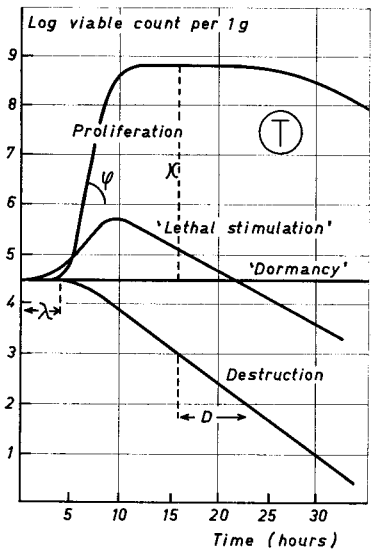

Fig. 2. Viable counts of micro-organisms as a function of time, as influenced by various parameters ('Monod-Hinshelwood curve').

by one of the following approaches. In the case of pure cultures prolonged incubation is inevitable, this in turn requires rigorously aseptic techniques (FAVERO and BERQUIST, 1968) to preclude interference by contaminants which, as a rule, are prolifically growing types tending to overcrowd the fastidious organisms looked for. When isolation from mixed populations is aimed at, a highly selective medium will have to be used that maintains its selectivity over a longer period of time than normally required; this demands that more attention should be paid to the parameters interfering with long-lasting selectivity, as discussed in section 2.5.

In addition to genotypically controlled influences on growth rate, instances of incidental modifications, particularly of λ-values, are observed repeatedly in practice; e.g. normally rapidly growing cells are found to be in a temporarily tardy condition, or populations of organisms, that normally tolerate customarily used inhibitors virtually without any loss in productivity, all of a sudden do not grow any more on such a selective medium. We have come to understand that such phenomena are caused by sublethal impairment of structural or functional properties of the cells, resulting from exposure to stresses such as moderate heating or irradiation, sojourn in an environment of subnormal a_w, pH or Eh, etc. After this had been established, restoration treatments, leading to normal functioning of such populations were found to be possible. Such a technique is indicated as a resuscitation treatment; e.g. most sublethally stressed Gram-negative rod shaped bacteria can be converted to normal cells by exposing them for 1–2 hours at room temperature and under reasonable conditions of aeration to a highly nutritious medium, as buffered glucose peptone broth (MOSSEL and RATTO, 1970).

It is of great importance that in daily practice full attention is paid to phenomena of

this kind. It involves that (i) where appropriate, pre-resuscitation of populations should always be carried out before inoculating them in highly selective media; and (ii) where suboptimal results are obtained with normally entirely satisfactory media the possibility that this is due to reduced vitality should be taken into account.

3.2. *Antagonism*

The functioning of many in itself excellent media is often impaired by phenomena of microbial antagonism. A fast growing organism can exhaust the supply of one or more nutrients and in this way suppress the development of slower growing organisms, which also require such factors (MOSSEL, 1969). Similarly one group of organisms may form quantities of acids or e.g. ammonia that soon become inhibitory to other organisms, occurring in the same habitat (MOSSEL, 1969). Or, a fast-growing organism may, due to its proliferation, reduce the Eh-value of a medium to a level that becomes suboptimal, if not fully inhibitory, to other organisms (DUBOS and DUCLUZEAU, 1969; TABATABAI and WALKER, 1970).

Such phenomena can be controlled to a certain extent in daily practice. Where exhaustion of nutrients might be a hazard, increased concentrations of growth factors may be incorporated in the medium; although, as we have seen in section 2.1. this may interfere with optimal growth of other types. Buffering of the medium will, as a rule, prevent the inhibitory suppression of organisms caused by an excessive increase or decrease of pH; although this may require attention in order to prevent that by too intensive buffering, the medium is made inhibitory by a not generally tolerated reduction of its a_w, or by direct impairment of growth resulting from intrinsic toxicity of the buffer itself. Where inhibitory anaerobiosis is to be feared, attempts should be made to maintain Eh-values at a rather high level by culturing in Roux flasks or by agitating or aerating the cultures.

Antagonism as a consequence of the formation of metabolites that act directly, i.e. not via pH or Eh, is not so easy to control. Two types of compounds may be involved in such phenomena: (i) those active in concentrations of the order $10^{-1}\%$ only, such as alcohols, certain ketocompounds, hydroxylamine and phenols; and (ii) the antibiotics, which show inhibitory action in concentrations as low as $10^{-4}\%$. A logical approach to the control of such phenomena would be: the use of media that inhibit the growth of strong antagonists. This is not always possible, though. To quote an example, it would be a very effective step in the isolation of clostridia or Lancefield group A streptococci from mixed populations if the growth of the powerful antagonistic Lancefield's group D streptococci (KAFEL and AYRES, 1969) could be precluded; however, there are no means to achieve this, because D streptococci show the greatest resistance against customarily used selective inhibitors (MOSSEL, 1959) as well as against most extrinsic physical parameters. Hence more involved measures may be required in such instances, such as the elimination of antimicrobial metabolites by dialysis (STERNE, 1958) or systematic dilution, as is achieved in continuous cultures (MEYNELL and MEYNELL, 1965).

4. PRACTICAL ASPECTS

As was demonstrated in the previous sections, the ecology of culture media is rather intricate. Practical measures aimed at strict quality control of the media, based on these considerations are particularly the following.

4.1. *Formulation*

It is necessary to verify the formulation, particularly of more complex media, at regular intervals by chemical-analytical methods (VAN DEN BEMD, 1970). For the determination of minerals, sugars and fatty acids the methods customarily used in the examination of foods are readily applicable. Peptones being very critical for optimal functioning of media as we have seen before, it goes without saying that they require regular examination; chromatographic (HABEEB and SHOTTON, 1956; SYKES, 1956; HABEEB, 1957) as well as microbiological methods (KHESHGI and SAUNDERS, 1959) are available for this purpose.

The extent of carrying out such analyses may be greatly limited by procuring almost complete media in dried or ready-for-use form. Such media are, as a rule, carefully checked by the manufacturers. Yet, the bacteriologist using such media may do wise by checking them too (REUSZE, 1971), albeit by a less cumbersome procedure, viz. the bioassay; cf. section 5.

In addition to a control on their composition, careful attention to the mode of medium preparation is necessary. Accurate instructions should be laid down with regard to the quality of the glassware and the water to be used in reconstitution, to the mode of dissolving dry ingredients (amount of heat required versus tolerated, degree of aeration and hence oxygenation that will occur, etc.) and to the way in which labile components have to be dissolved and added. Preventive supervision in the medium formulation room will do part of the job and bioassays of the prepared media may complete an almost 100% quality control.

4.2. *Sterilization*

Obviously any mode of sterilization used will in principle change the composition and hence potentially the functioning of media. The extent to which this occurs and the way in which this impairs the medium's productivity or selectivity vary of course greatly.

4.2.1. Thermal sterilization

The consequences of thermal sterilization of components of culture media are rather well known (FOSTER, 1952; EL MILADI et al., 1969; BRIDSON and BRECKER, 1970).

The main type of reaction occurring during heating is the so-called Maillard reaction in which NH_2 compounds are coupled with carbonyl groups (PEER, 1971). Such primary Maillard complexes and their decomposition products are not without

effect on micro-organisms (MC KEEN, 1956; JEMMALI, 1969; PEER, 1971).

Rather related to this type of heat-induced coupling reaction is the so-called caramellization of sugars, leading to higher molecular, often brown compounds (PEER, 1971). In this reaction hydroxymethylfurfural is formed, which has pronounced antimicrobial properties (DAVIS and ROGERS, 1939; TARKOV et al., 1942; CORPER and CLARK, 1946; INGRAM et al., 1955; FINKELSTEIN and LANKFORD, 1957). Hence the mode of heat sterilization may greatly influence the functioning of sugar-containing media through this mechanism.

Sterilization by heat will also inactivate B-group vitamins. The extent to which this occurs depends on the type of vitamin and the various parameters of the medium (FELICIOTTI and ESSELEN, 1957; FORD et al., 1969).

All the previously mentioned effects of heat may exert a detrimental influence on microbial growth. Heating of a medium may, however, also favourably affect the development of micro-organisms, viz. by partial inactivation of inhibitors such as brilliant green (MOSSEL and VINCENTIE, 1969), azide (MOSSEL et al., 1970), or antibiotics. The latter effect is, ultimately, also an unwanted one, since it reduces a medium's selectivity.

In this connection it is of great importance that the Q_{10}-values of the thermal reactions leading to the killing of microbes and that of the unwanted chemical conversions in media vary greatly, i.e. are of the order 10 for the first type and only approximately 3 for the latter. In practical terms this means that if a heat dissipation of a given, particularly sporicidal, effect is applied in the form of a high-temperature/short-time treatment, it will do much less harm to the chemical composition of a medium than when it should have been applied in lower-temperature/longer-time form (DAVIS and ROGERS, 1939; MOSSEL, 1951; HERRMANN, 1969). Therefore, in practice, heat-sterilization should always be carried out (i) at the highest feasible temperature, i.e. approximately 121 °C; and (ii) with heating-up and particularly cooling-down (BENTON and LEIGHTON, 1925) taking place as quickly as possible.

4.2.2. Sterilization at room temperature

Clearly, filtration of liquid media may, in principle, also lead to changes in composition affecting the biological value of the sterile filtrate. All filters will allow some adsorption of polar medium components, the degree of chemosorption obviously depending on the structure and thickness of the filter used, the composition of the medium, and the rate of filtration; in practice the effects will only be very slight, at least with the currently used type of filters (VAN BULCK, 1971). Less irrelevant are the effects of oxidation during filter sterilization. Particularly when reducing substances like sulphite are used in selective media, the degree of oxidation attained under practical conditions should be assessed and, if required, controlled (MOSSEL, 1959).

The possibility of rather significant changes in medium composition, resulting from sterilization of media by epoxidic compounds with strong alkylating properties

(KAYSER, 1971) is obvious. The same holds true for sterilization by irradiation: the oxidative and reductive changes occurring in aqueous environments as a result of the use of the required doses of the order 2.5 Mrad (BECKING, 1971) simply must impair the composition of media and hence their functioning. Consequently strict quality control is required in all instances where ionizing radiation is used for the sterilization of culture media.

4.3. *Storage of media*

The logistics of bacteriology require that as a rule rather large and varied stocks of media are stored for a longer or shorter period of time. Storage will inevitably lead to changes and these have to be kept under check.

Most well-known to bacteriologists is the loss of moisture during storage. Most freshly prepared media have an a_w-value of approximately 0.99 (KANG et al., 1969). In refrigerated rooms the relative humidity will not frequently exceed 0.85, whereas in stores at room temperature this value is mostly 0.60 or lower. Hence particularly agar plates will dry out at their surface and, because water diffusion in agar gels may be a slow process, the surfaces may soon have a_w-values not unlike the relative humidity of the environment. Organisms of low a_w-tolerance may suffer from this effect, unless they are inoculated with aqueous suspensions – as they fortunately often are.

The chemical changes in media, discussed in section 4.2.1. will continue during storage. Although the rate of the reactions will be very much slower due to the temperature difference of approximately 100 °C, long storage may partly compensate for this. Even more important are the reactions which are avoided during heat sterilization, by adding medium compounds subsequent to heating; particularly the oxidation of sulphite, the reduction of tellurite, the destruction of azide and the inactivation of antibiotics like penicillin and chlorotetracycline are reactions which have to be controlled carefully for optimal functioning of the selective media in which such inhibitors are used.

Sometimes the opposite effect is observed in that media which are slightly toxic when freshly prepared, will improve upon storage in the refrigerator (COOK, 1952). Such a detoxification process may be acceptable in a single instance; generally, it is much better to prepare media in such a way that they are exempt of any untoward effects immediately after preparation.

A most vexing potential effect of the storage of culture media is their contamination. Correctly plugged liquid media are almost immune from this effect, because their plugs are virtually impermeable to microbes, while any contamination occurring accidentally will rather soon become apparent from visible signs of microbial growth. Solid media stored in Petri-dishes will become contaminated much more easily, though. Sometimes a contaminant reveals its presence by forming visible colonies and such plates can then be discarded. However, we have observed that in many instances certain contaminants may grow out to micro-colonies of only circa 10^4 cells (MOSSEL

and VAN DE MOOSDIJK, 1964), probably due to storage of the plates at somewhat lower temperatures and as a result of local reduction of a_w. When such plates which therefore show no macroscopic signs of deterioration, are used for making counts by the spread-drop procedure or for isolating single colonies, the contaminating micro-colonies will be spread over the entire agar surface and, during subsequent incubation, lead to serious interference with the functioning of the plates.

4.4. *Inoculation*

In the ecological sense inoculation is one of the most essential facets of the use of culture media, because this part of its use introduces, in principle, both the organisms whose isolation or enumeration is the aim of the procedure and the contaminants. Because the numbers of wanted and unwanted organisms are almost as important as their attributes, let N_o^i be the number of intentionally inoculated organisms and ${}_1N_o^c$, ${}_2N_c^o$, ${}_3N_o^c$, etc. be the numbers of various contaminants. Only seldom, then, $\Sigma_i^n N_o^c$ will be nil.

When *pure cultures* are inoculated, even the best conventional procedures for what is called 'rigorous asepsis' will lead to $\Sigma N_o^c \neq 0$ as experience in many laboratories has shown (MUDGE and FOORD, 1940; WILLIAMS and CLARK, 1942; GAMBRELL and OSTROLENK, 1945; NOVEL, 1949; NICKERSON et al., 1956; MOSSEL and VISSER, 1960). Only when laminar air flow cabinets are used $\Sigma_i^n N_o^c = 0$ can be attained (FAVERO and BERQUIST, 1968).

In all *other* instances $\Sigma_i^n N_o^c$ is > 0 even without any environmental contamination occurring. What will happen when these strongly non-axenic populations are brought on to agar media will depend on many factors, intrinsic, extrinsic and implicit. Of primary importance is the population ratio, i.e. $N_o^i/_xN_o^c$ where x is the contaminant that either numerically or biochemically interferes most with the proliferation of the organism or organisms to be enumerated or isolated. But almost as important are $\frac{dN^i}{dt}$ and $\frac{dN_x^c}{dt}$. These parameters, in turn, depend on (i) the vitality of the seeded organisms; (*ii*) the initial growth potential and the inhibitory properties of the inoculated medium; (iii) the course of these characteristics as a function of incubation time at given temperature, oxygen partial pressure, etc.; (iv) changes in the composition of the medium due to growth – both of the organisms looked for and of the various contaminants; (v) antagonistic phenomena.

It is obvious that very little can be said *a priori* about the functioning of any particular medium in this sense. Rather are these most vital aspects of practical microbiology to be assessed empirically. Methods found useful for this purpose will be summarized in the following section.

5. Ecometric evaluation of media

5.1. *Principles*

The *initial* functioning of a medium is assessed by inoculating separate plates or tubes with a well conceived choice of wanted and unwanted organisms and studying their mode of proliferation and metabolism during incubation. This sort of assay requires careful standardization of the inocula, both with regard to (i) numbers of cells and amount and type of suspension fluid, transferred with the inoculum; and (ii) degree of vitality of the cells (Meynell and Meynell, 1965). It is obvious that the former parameters determine to what extent inhibitors used in selective media are neutralized by inoculation (O'Meara and Macsween, 1937; Schuhardt et al., 1950; Traxler and Langkford, 1957; Price and Gare, 1959; Mossel and Vincentie, 1969) and hence what the inhibitor activity per sensitive prosthetic group of micro-organisms may approximately amount to. As far as vitality is concerned, ideally the organisms looked for should be applied in physiologically impaired condition and the contaminants well into the stationary growth phase, where sensitivity to untoward external conditions is minimal (Stark and Stark, 1929; Sherman and Cameron, 1934; Hershey, 1939; Fry and Greaves, 1951; Stapleton, 1955; Goodlow and Leonard, 1961; Liston and Matches, 1968; Garibaldi, 1968; Mathieson, 1968; Palumbo and Alford, 1970).

The *course* of functioning of media is determined by inoculating them with mixed populations, again carefully chosen in the qualitative and quantitative sense, and examining the inoculated systems after e.g. 0, 3, 6, 10, 18, 24 and 48 hours' incubation. Liquid systems can best be plated out, in suitable dilutions, onto a set of selective media (Mossel and Vincentie, 1969). Solid media are to be examined by the replication principle of Lederberg and Lederberg (1952), using anew properly divised sets of selective enumeration media (Wiseman and Sarles, 1956; Corlett et al., 1965; Mossel et al., 1970).

5.2. *The quantitative single-plate streak technique*

The assessment of the functioning of a medium as it depends on incubation as described in the preceding section is not a routine method. However, checking on the initial quality should certainly be so (Banwart and Ayres, 1953; North and Bartram, 1953; Buchbinder et al., 1954; Reynolds and Wood, 1956; Stokes and Bayne, 1958; Black, 1960; Slocum et al., 1960; Read and Reyes, 1968; Stokes, 1968; Wilson et al., 1970; Taylor and Schelhart, 1971).

It can be carried out by inoculating the required number of plates with serial dilutions of a set of test strains – a few organisms that have to grow well and some that are to be entirely suppressed – incubating under the conditions used in practice, and comparing the numbers of colonies obtained on the medium under study and on suitable control plates. This method is the only one feasible in developing new media. However, it being rather material-consuming and time-consuming, various streak

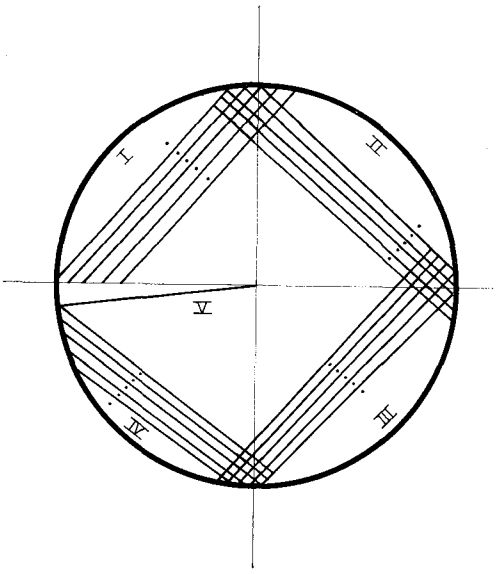

Fig. 3. The quantitative streak technique.

methods have been suggested for daily quality control instead (GILLISSEN and BIRKENHOVEN, 1958). These, however, lack to a certain extent the quantification, required in carrying out such tests (SCHWARTZ et al., 1961). Therefore, we prefer the following 'single plate' technique, suggested by BILLING (Personal communication, 1961).

As usually suspensions of the test strains, in their logarithmic phase, are made in a protective diluent (STRAKA and STOKES, 1957). The attempted colony count of the suspensions is 10^6–10^7/ml. Each suspension is now streaked, with a straight wire, onto the distal part of one quadrant of a Petri-dish, containing the medium to be assayed, applying five parallel streaks. The wire is then flamed and cooled, and subsequently five similar streaks are made in the next quadrant, using as the inoculum the ultimate (about one centimeter) area of the first set of streaks. This is repeated two more times. The fifth streak of the fourth set is, finally, prolonged so as to pass through the center of the plate (Fig. 3).

After suitable incubation of the plates, growth might occur or it might not; and in the former instance it could extend to the zones I, II, III, IV or V. The productivity of a plate may now be expressed in a figure between 0 and 5 dependent on which of the zones I–V still showed significant growth. In the case of the zones I–IV, this means that at least half of the streaks show closed lines of colonies.

It is our custom to use for the assay of each medium at least two sets of two test strains, viz.: (i) a robust and a weaker sought type; (ii) a readily suppressed and a harder strain amongst those which are to be inhibited. In the case of a plate, selective for *Salmonellae*, the first set would e.g. comprise *Salmonella typhimurium* and *Salmonella pullorum*, the second *Escherichia coli* and *Citrobacter freundii*. All strains are also streaked onto a good general purpose medium, e.g. blood-agar (Table 1).

The evaluation of a medium is expressed in the following way. To account for the

colony count of the suspensions used, for each strain the relative growth index (R.G.I.) on the test medium is determined and calculated with the formula:

$$\text{R.G.I.} = \frac{\text{productivity on test plate}}{\text{productivity on blood agar}} \times 5$$

The functioning of the plate under check is then expressed in a four number decimal figure in which the R.G.I.'s for the four test strains are recorded, rounded off to the nearest whole number, in the order listed above. For an ideal plate this index should be 5.5.0.0., but in practice it will obviously occasionally also be e.g. 5.3.0.1., etc.

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STERILIZATION KINETICS

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SUMMARY

Sterilization is commonly thought to obey the exponential 'law': $N_t = N_o e^{-kt}$ (N = viable count; k = rate constant; t = time). A simple theoretical model postulates that loss of viability is caused by a simple lethal event, occurring with equal probability in any cell at any moment. A second model, proposed by DEAN and HINSHELWOOD (1966) supposes the concentrations of various essential cell constituents to vary in a cyclic manner, these oscillations being superimposed on a general and gradual decline. Recovery is impossible when too many of the essential cell components reach a minimum simultaneously. The probability that this occurs is the same for any cell but is dependent on time. Deviations from the exponential 'law' can be explained in this way. On most practical occasions, however, the 'law' gives a satisfactory approximation.

Limiting the sterilization is necessary in most cases in order to reduce damage to the medium and to improve the heat-economy. The time necessary for a particular medium depends on: the initial count of heat resistant spores, the volume to be sterilized, the percentage of failures that may be accepted and the intensity of the treatment. An example of a calculation is given.

1. THE EXPONENTIAL 'LAW'

The kinetics of sterilization are commonly thought to obey the exponential 'law': $N_t = N_o e^{-kt}$. (N_o and N_t = viable count at time o resp. t, k = rate constant, t = time). This formula is obtained by integrating: $dN = -kNdt$, the latter expression being a formal way of stating that the probability of losing its viability is the same for any cell at any moment. Instead of the rate constant k, the decimal reduction time D is also often used. D is equal to the time during which the viable count has been reduced by a factor of 10. The relation between k and D is expressed by $D = 2{,}30/k$.

The exponential 'law' is considered to apply to sterilization by heat (wet and dry), radiation (ionizing and nonionizing) and chemical agents. A fully analogous 'law' can be formulated for sterilization by filtration through fibrous materials (Seitz-filtration, air filtration through glass wool). In that case the reduction of the viable count is not in the first place attributable to death but to adsorption on the fibres. Time (t) in the formula is replaced by the depth (d) of the filter. Here it is customary to express the rate constant as d_{90}, the decimal reduction depth.

The exponential 'law' is obviously not involved in sterilization by filtration through membranes having a pore diameter smaller than the germs to be removed, e.g. Millipore filters.

The exponential 'law' is very popular. Reasons are:

1. Its simple mathematical expression facilitates calculations.
2. It is symmetrical with the exponential part of the growth curve.
3. A satisfactory theoretical explanation of the 'law' can be given.
4. It is sometimes obeyed in practice.

In most cases deviations from the law are observed at the beginning and the end of the curve. The general form of the death curve with its most frequent deviations is given in fig. 1.

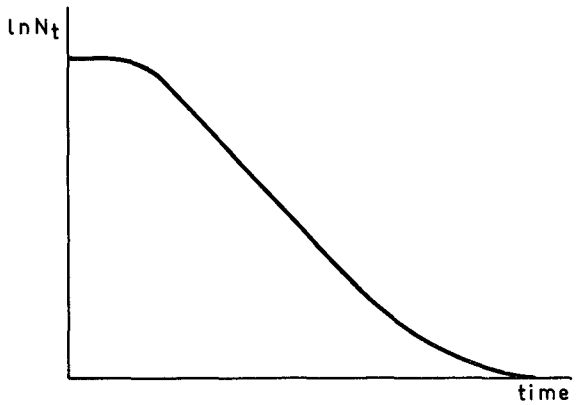

Fig. 1. General shape of the death curve. N_t represents the number of viable cells at time t on an arbitrary scale.

2. Explanations

A number of more or less plausible models have been proposed for the explanation of the exponential, 'law'. Two of these will be considered here.

2.1. *'Bullet'-theory*

The first rather crude model can be found in most textbooks and is referred to as the 'bullet'-theory. Loss of viability is thought to occur if one of a number of essential cell functions has been destroyed by the sterilizing agent. This lethal event must be thought of as sharply delimited in time and space. It is best compared with the action of a bullet hitting the head or heart. If these events occur at random, the probability of being hit is the same for any individual at any moment. The death rate will at any moment be proportional to the number of living cells present.

Sterilization by means of ionizing radiation may be adequately described by way of this model. The target area in the cell, the DNA, is spatially delimited. Irreparable damage, a double strand break, is the result of one event, which may be either the passage of a γ-quantum, or an α or a β particle. The statistical approach is also a good approximation for Seitz-or air filtration. The determining factor here is a collision between an organism and a fibre of the filter material which is also a spatially and temporily delimited event.

Sterilization by heat is obviously not adequately described by way of this model. The most important effect of heat is denaturation of proteins. Since a large number of molecules of any type of protein is always present in the cell, it is difficult to visualize the nature of the single, lethal event postulated by the theory. The same difficulty arises with sterilization by chemical agents.

2.2. '*Stress*'-*theory*

DEAN and HINSHELWOOD (1966) have constructed a model that gives a more satisfactory explanation in those cases where the bullet-theory fails. It could be called the 'stress'-theory. The model first considers the state of affairs in a culture which has entered the stationary phase due to the exhaustion of essential nutrients and then gradually loses its viability. Nearly all components in the living organism are constantly degraded and resynthesized. This process is continued in resting cells. A limited concentration of the exhausted nutrient will therefore always be present in the cells. A strong competition for the same scarce nutrients will ensue between the systems synthesizing a number of different cell components. Degradation of one cell system can supply building blocks for the resynthesis of another. The relationship between these cell components is thought to be analogous to that of predator and prey. VOLTERRA (1926) has shown that in such a relationship both populations can oscillate. DEAN and HINSHELWOOD postulate that the concentrations of different cell systems can also vary in a cyclic manner. Considering the large number of cell components and the large number of metabolites involved in their synthesis, these oscillations, when averaged for all cells in the culture, may be regarded statistically as occurring at random. Apart from these oscillations the cell as a whole will progressively degenerate with time as nutrients are used up, in order to provide energy for all the processes.

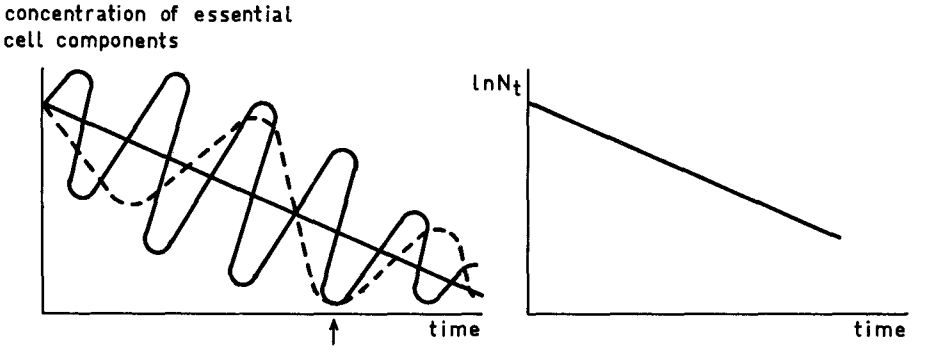

Fig. 2. Illustration of the stress-theory of Dean and Hinselwood for those cases when the exponential 'law' is obeyed (see right hand figure). In the left hand figure the curves represent the concentration in one cell of two essential cell components as a function of time on an arbitrary normalized scale. The straight line represents the average level of all concentrations of essential cell components. The arrow indicates the moment at which loss of viability occurs. Actually the oscillations stop at that moment.

Two situations may now be distinguished:

A. Cases in which the exponential 'law' is obeyed.

The amplitudes of the oscillations are large compared with the average level of the concentrations of the essential cell components. When too many essential cell components reach a concentration minimum simultaneously, recovery is impossible. The cell is no longer viable. The probability of this event is the same at any moment, hence the exponential 'law' applies. A schematic representation of this situation and of the corresponding death curve is given in fig. 2.

B. Cases in which the exponential 'law' is not obeyed.

Initially the amplitudes of the oscillations are small in comparison with the average level of the concentrations of the essential cell components, and with the rate of decline of this level. Even when a great number of essential cell components reach a concentration minimum simultaneously, the level of that minimum will still be high enough to enable the cell to recover. Loss of viability is therefore negligeable. When the average level has fallen to the same order as the amplitudes of the oscillations, the situation described in the preceding section (2.2.A.) prevails. Initially, the death curve will take a horizontal course. Eventually, after a transitional phase, the exponential 'law' will be obeyed. This is illustrated in fig. 3.

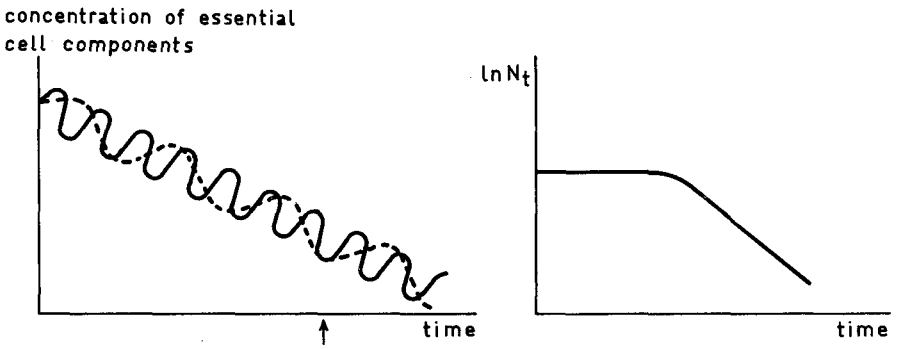

Fig. 3. Illustration of the stress-theory of Dean and Hinshelwood for those cases where the exponential law is initially violated (see right hand figure). Representation is the same as in fig. 2. Note the coinciding minima early in the left hand figure where no loss of viability occurs.

It is now supposed that any extreme condition will derange the normal cell processes and their regulation to such an extent that the same oscillations will occur with all the consequences discussed earlier. It can be expected that the less extreme a treatment is, the more pronounced the initial horizontal part of the curve will be. This is observed in practice. Compare for example the shape of the death curves obtained with *Bacillus* spores at different temperatures (fig. 4).

The appeal of the stress-theory lies in its explanation not only of the exponential

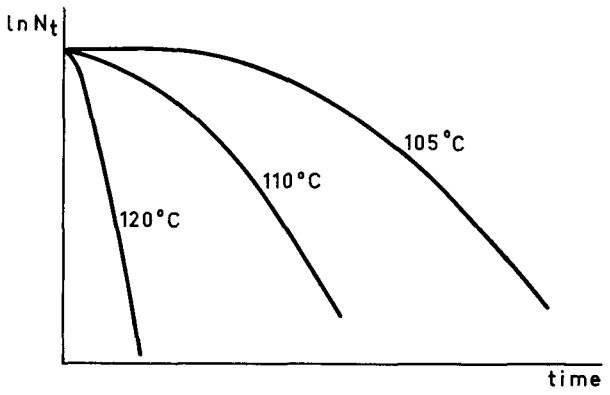

Fig. 4. Death curves obtained with *Bacillus* spores at different temperatures.

'law' but also of the most general deviation from this 'law'. However, it will definitely not be the final word on this issue. Firstly too many assumptions have been made which require experimental proof. Secondly it is difficult at first sight to visualize how the theory would apply to the metabolically inert heat-resistant endospores. On the other hand the conditions of a wet-heat sterilization cause a hydration of the spore and the systems that are normally involved in germination may then become operative.

2.3. *Deviations from the exponential 'law'*

Besides those already mentioned, other deviations from the exponential 'law' may be observed near the end of the treatment. Almost invariably the death curve then levels off and may eventually take a horizontal course. Some commonly occurring causes for this behaviour are:

A. Clumps of micro-organisms.

The presence of clumps of micro-organisms or spores is a frequent source of error in experiments designed to determine the shape of the death curve. Even when the test culture has been grown on a clear medium it is advisable to remove such clumps by means of a course filtration.

B. Clumping of the medium.

One should be very careful to preclude the formation of clumps, particularly when preparing media containing some form of starch. The penetration of heat in a dry clump of starch is poor and the internal relative humidity low, so that spores in the interior of such a clump will almost certainly survive sterilization. After inoculation, the clumps are dissolved by enzymic action and the surviving spores are free to germinate.

C. Differences in the physiological states of the cells.

Evidently, when the culture is inhomogeneous with regard to the physiological state of the cells, part of the population will die at a different rate from the rest. It may quite easily occur that the majority of the population has already lost its viability, while another part is still in the initial phase of the process discussed under 2.2.B.

D. Exhaustion of the chemical sterilizing agent.

Quaternary ammonium compounds e.g. are bound by phospholipid material released by the dead cells. The actual concentration of the agent is thus lowered. Other agents such as β-propiolactone (BPL) are decomposed by water and consumed in the reaction.

E. Non-inheritable adaptation of part of the population.

Especially when conditions are not too extreme, and during the initial phase of gradual decline, a part of the population can manage to bring the oscillations under control and thereby greatly reduce the rate of loss of viability.

3. Application

3.1. *Arguments in favour of restricting the sterilization*

A. Reduced damage to medium components.

During sterilization there is a risk of: a) decomposition of vitamins and other complex nutrients; b) reactions between sugars and amino acids; c) important pH changes; d) formation of toxic compounds etc.

B. Improved heat-economy.

On a small scale heat-economy is insignificant. It becomes of major importance, however, to an industrial process where the cost of energy is a substantial part of the total costs.

C. Saving of time.

This argument also, is very important where large scale processes are considered. The time during which a fermenter is not in production has still to be paid for with depreciations, wages, overheads etc. Where the autoclave space is restricted this factor may also be of importance on a small scale.

3.2. *Factors determining the length of the sterilization period*

The following example will be based on the exponential 'law' which, if its limitations are clearly borne in mind, will generally provide a good approximation in practice. The necessary duration of a treatment in a specific case is determined by:

A. The intensity of the treatment.

The dependence of the intensity of the treatment on the conditions of the sterilization will not be discussed here. As far as thermal sterilization is concerned this aspect is covered by Beverloo (1971). For chemical agents the rate constant in the exponential 'law' is as a rule linearly related to the concentration. This applies of course within the limitations discussed earlier and in the range of concentrations that is of practical value. In the examples it will be assumed that the intensity has been defined and that the corresponding value for k is known. The effect of the heating and cooling periods will not be evaluated as this aspect is also covered by Beverloo (1971). It should be borne in mind, however, that on a large scale their contribution is a major one.

B. The level of contamination of the medium.

The number of vegetative cells present is as a rule not interesting as the k values for them are at least an order of magnitude higher than those for heat resistant spores. (AIBA, HUMPHREY and MILLIS, 1965: vegetative cells $k \approx 10\ \text{min}^{-1}$; spores $k \approx 1\ \text{min}^{-1}$). Except for those cases where the vegetative cells enormously outnumber the heat resistent spores, the latter are the only ones to be reckoned with. The volume of the medium to be sterilized must also be considered as sterilization can only be successfull if less than one viable cell remains in a closed vessel (tube, flask, tank).

C. The acceptable rate of failures.

Sterility is an absolute concept, but, guided by the exponential 'law', we can only approach it statistically. We can only calculate how long a treatment should be pursued in order that the probability of one living cell remaining in the vat should attain a specified value. This value is determined by the number of failures we are willing to accept in a specific case.

3.3. *Examples*

Given values for the three variables discussed in the preceding sections, we are now able to calculate the minimum duration of the treatment that is necessary. This is illustrated by the two following examples. The values found, as discussed earlier, only apply if the medium is heated and cooled instantaneously. The effect of the heating and cooling periods which in most practical cases cannot be ignored, has to be calculated separately.

A. Tubes ad 5ml.

Consider a medium to be sterilized in tubes containing 5 ml. Further data are:

1. Spore count before sterilization: 10^3/ml.
2. Acceptable rate of failures: 0.1 %.
3. Sterilization at 121 °C, $k = 1\ \text{min}^{-1}$.

Calculation:

$$\left.\begin{array}{l} N_o = 5.10^3 \\ N_t = 10^{-3} \end{array}\right\} \ln(N_o/N_t) = kt \rightarrow t = \frac{1}{1} \times 2{,}30 \log 5.10^3/10^{-3} = 15{,}5 \text{ min.}$$

B. Fermenter ad 60,000 l.

The same medium has to be sterilized in a fermenter containing 60,000 liters nett. with the same probability of success.

Calculation:

$$\left.\begin{array}{l} N_o = 6.10^7 \times 10^3 = 6.10^{10} \\ N_t = 10^{-3} \end{array}\right\} t = \frac{1}{1} \times 2{,}30 \log \frac{6.10^{10}}{10^{-3}} = 31{,}7 \text{ min}$$

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DISCUSSION

F. M. ROMBOUTS: Could you elaborate on the effect on the logarithmic mode of death of physiological differences and inevitable differences in heat-resistance of individual cells?

P. P. SCHRAUWEN: The rather vague term physiological differences means that the cells have different initial concentrations of the essential cell components. It is evident that the subsequent oscillatory behaviour of these concentrations will also take a different course in different cells. The question remains whether these differences are really inevitable. In cultures obtained as an exponentially growing batch culture, a continuous culture at a reasonably high dilution rate or a synchronized culture, the differences in physiological state would be expected to become smaller in that order. This should of course be reflected in the shape of the death curves obtained. I know of no experimental work on these lines.

J. STADHOUDERS: Why is it impossible to explain a death curve that is, for instance, S shaped, by the natural distribution of thermal resistance of the cells (spores) in the population?

P. P. SCHRAUWEN: While referring to the answer to the previous question the answer is no. By assuming a definite predetermined lifespan for every individual organism under the conditions of sterilization, and further by assuming some statistical distribution of the lengths of that life-span for the organisms in the population, a death curve could be derived that, by variation of the standard deviation could be made to fit the experimental curves. This, however, would only give a mathematical description, not an explanation. The model would not explain, in terms of physico-chemical processes in the cells, why this mode of death should occur.

THERMAL STERILIZATION

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SUMMARY

A calculation method based on a simplified model of the thermal destruction of microorganisms is presented. The method, devised for the canned food industry, might also be applied to the sterilizing procedures inherent to the preparation of nutrient media.

The necessity of bleeding sterilizing autoclaves thoroughly, in order to drive out all noncondensable gas, is emphasized.

Thermal sterilization of sensitive organic material is applied on a large scale in the canned foods industry. This industry has to supply canned foods, wherein the hazard of the presence of pathogenic organisms is fairly nihil and the hazard of spoilage within a reasonable 'shelf-life' very small. On the other hand the consumer appreciates the canned food to have suffered as little as possible from the applied heat treatment. The vast interests involved in finding the optimal compromise have generated a reasonably accurate calculation method (BALL and OLSEN, 1957; CHARM, 1963; HERSOM and HULLAND, 1963; STUMBO, 1965). The method, however, has been developed and come into routine application while there has been no sufficient contact with people outside the canned foods industry: this caused a much too large collection of professional words and symbols, which are difficult to interpret even for qualified outsiders and easier to sense than to understand for insiders. In the textbook used for our Food Engineering course we tried to create some order, understandable for outsiders and recognizable for insiders (LENIGER and BEVERLOO, 1967).

The reduction of the number of living organisms at a constant temperature is considered to have a logarithmic course (Fig. 1). The rate is characterized by the decimal reduction time D, the time necessary to reduce the number tenfold. If log D is plotted against temperature it appears that in the range of temperatures applicable for thermal sterilization processes (say 90 °C– 150 °C) the results can fairly well be represented by a straight line (Fig. 2). Now the thermal resistance of a specific microorganism is characterized in the calculation method by $D_{ref.}$, that is the value of D at an arbitrary reference temperature $\vartheta_{ref.}$ (= 121,1 °C) and by z, that is the increase in temperature needed for a tenfold reduction of D.

Starting from a measured, expected or suspected initial contamination with some

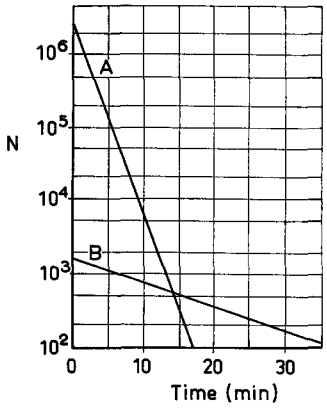

Fig. 1. Destruction of micro-organisms with different D-values.

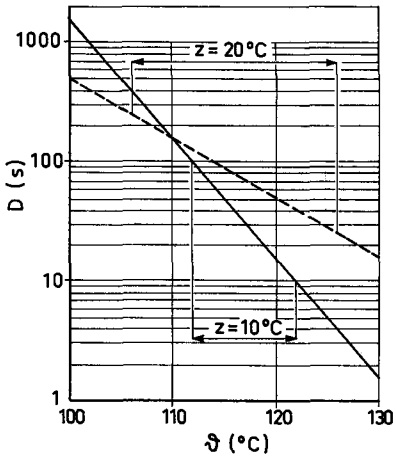

Fig. 2. Relation between D and ϑ; characteristic temperature difference z.

species of micro-organism on one hand, and from a specified, maximally tolerable final contamination or a maximally tolerable risk of surviving of one individual of the species in a can on the other hand, it is possible to calculate the time F needed at the reference temperature to perform the desired sterilization effect. Where F is a measure of the intensity of the required sterilization process it is called the 'process value' F. Times at other temperatures than $\vartheta_{ref.}$ can be converted into times at $\vartheta_{ref.}$ by multiplication with a factor L, the 'lethality'. The lethality $L = 10^{(\vartheta - \vartheta ref./z)}$ is nothing else than the ratio between D and $D_{ref.}$ Is the course of the temperature ϑ during the sterilization process known, either by measuring or by calculation, then F can be calculated from

$$F = \int_0 L \, d\, t = \int_0 10^{(\vartheta - \vartheta ref./z)} \, d\, t$$

wherein t is the time elapsed since the process started. Different temperature courses are mutually comparable in respect to their sterilization effects by their calculated process values, for the sake of completeness the value of z used in the calculation of F should always be specified; if no value of z is stated, it is mostly taken as $z = 10°C$. Special graph paper – 'lethality paper' – is available, where on the vertical axis L is plotted linearly with the temperatures belonging to the various values of L indicated on it; on the horizontal axis the time t is plotted linearly (Fig. 3). The process value F can be determined by measuring the area beneath the curve representing he temperature course on lethality paper.

When in the twenties of this century the calculation method was developed in the USA (BALL and OLSON, 1957), the micro-organism *Clostridium botulinum* was the microbial enemy en vogue as it produced a dangerous toxin, 'botulin', causing a deadly disease 'botulism', of which several cases induced by consumption of canned

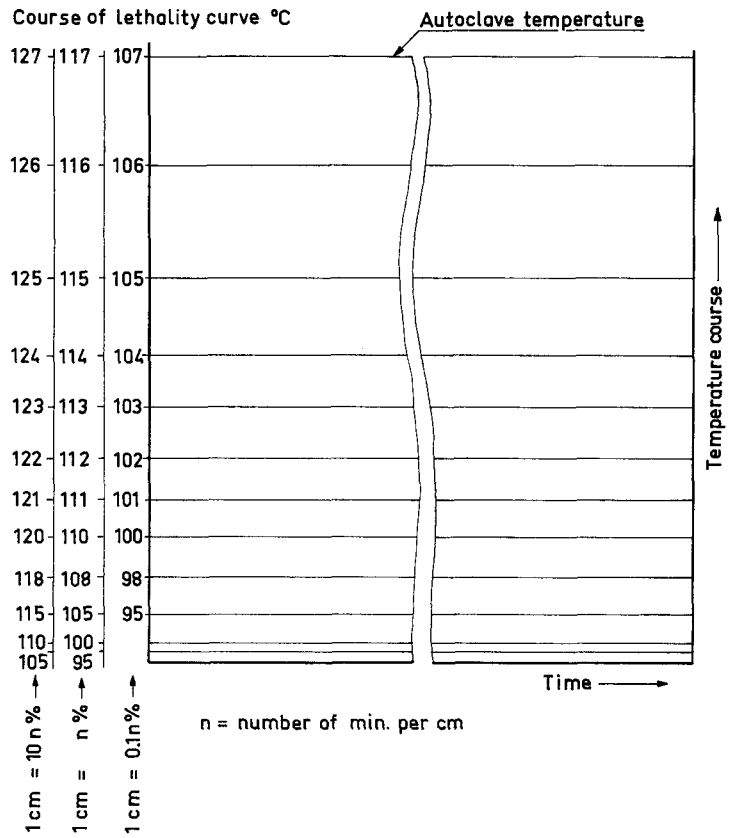

Fig. 3. Lethality graph.

foods had occurred. Since than the thermal resistance of strains of this organism have been the basis for process value specifications; in practice a reduction of the initial contamination by a factor of at least 10^{11} is specified, requiring a process value of at least 147 s. For the calculation of L, z is considered to be 10 °C. In practice it appears that the process values specified in this way also lead to a smaller but yet acceptable reduction of more resistant spoilage inducers which appear to be less dangerous to human health.

Precisely because L depends so closely on temperature and because rather drastic reductions are required, it is most important that all parts of a batch to be sterilized attain sufficiently high temperatures. If $z = 10$ °C a temperature difference of 3 °C causes the need of twice as much time for the same process value; of an expected reduction of *Clostridium botulinum* with a factor 10^{11} a reduction with a factor 3.10^5 actually comes off, so the risk that one organism survives- and multiplies itself during shelf life, producing botulin- becomes 3.10^5 times as high!

In many autoclaves the cans or other containers keeping material to be sterilized

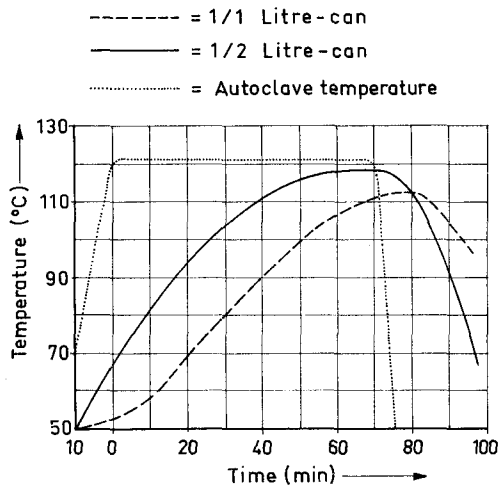

Fig. 4. Relation between temperature and time in the coolest point of large packages.

are heated by condensing steam, which is indeed the most adequate heat transferring medium in general use. However, in using steam or some other condensing vapour as heat transferring medium one has to be aware of the disadvantageous effect of non-condensable gas e.g. air present in the autoclave. Both the condensation temperature and the condensation rate are lowered by a layer of non-condensables in front of the heated surface; and noncondensables in the presence of condensables accumulate at the coolest places. So the presence of air causes the temperature at some places to rise slower than other temperatures do, this results in the accumulation of more air at the cooler places etc. In view of the risks of 'understerilization' it is good practice to bleed the autoclave well, especially in the early stages of the process and to charge the autoclave in such a way that air can easily be driven out.

The canned foods industry also has to handle rather large cans, wherein heat has to penetrate by conduction. This causes a considerable time lag in the temperature course of the material in the center of the can (Fig. 4). It is conservative practice to calculate the process value for the 'coolest point'. My impression is, that nutrient media are mostly 'packed' in rather small 'containers' and are liquid at sterilization temperatures, making 'natural convection' possible as a temperature equalizing mechanism. It will not be very important in sterilization of nutrient media to bother about the temperature distribution within the material.

In cases where it is important to find a compromise between sufficient reduction of the number of micro-organisms and limitation of some unwanted effect of the applied high temperatures, the application of a 'high temperature short time' ('HTST') process should be considered. The rate of chemical reactions in general, including the reactions that cause heat damage of sensitive materials, is much less temperature de-

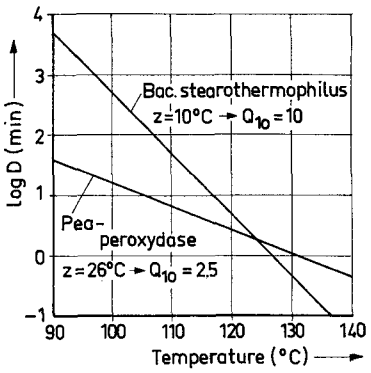

Fig. 5. Z-values for destruction of micro-organisms and for chemical reactions.

pendent than the rate of reduction of micro-organisms (Fig. 5). So a HTST-process with the same process value as a 'low temperature long time' process will give less heat damage. Technically a HTST-process is more difficult to realize. Both heating and cooling must be accelerated (Fig. 6); it may finally appear that the process cannot be realized for separate charges in an autoclave but should be performed continuously during flow through heated and cooled tubes. The product sterilized in this way must then be packed under sterile conditions, which raises the difficult problem of 'aseptic canning'. It might be interesting to design a small continuous flow sterilizer, adapted to the small quantities probably required, together with an aseptic filling system for nutrient media. The users of nutrient media themselves should consider the necessity of a compromise between sterilization and heat damage. A decision ought to be based on the knowledge about the kinetics of the destruction of micro-organisms and about the kinetics of unwanted heat damage reactions.

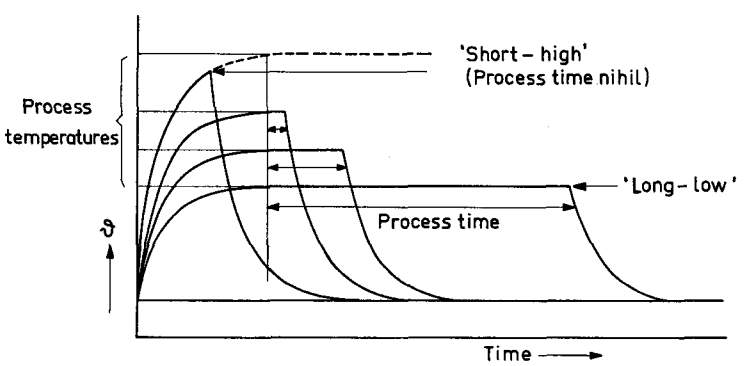

Fig. 6. Temperature-time programmes for long-low and high-short processes.

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RADIOSTERILIZATION OF NUTRIENT MEDIA

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SUMMARY

The physical properties of ionizing radiation and its chemical and biological effects are discussed. Some useful sources of irradiation and the essentials of dosimetry are reviewed.

A survey is given of the order of magnitude of the radiation sensitivity of various classes of micro-organisms and of the various intrinsic factors influencing sensitivity values. The effects of radiation on the components of the nutrient media are summarized. The radiosterilization of liquid nutrient media is discussed, but also that of foodstuffs and soils, because the latter substrates are often used as nutrient media by food and soil microbiologists.

1. PHYSICAL PROPERTIES OF RADIATION

Ionizing radiation can be distinguished in short-wavelength electromagnetic radiations like X-rays and γ-rays and in radiations of a corpuscular nature such as electrons (β^--rays), positrons (β^+-rays), helium nuclei (α-rays), protons, and neutrons.

The distinction between non-corpuscular waves and corpuscular particles is made for practical reasons only. It is certainly not fundamental, since a γ-ray of more than 1.02 MeV can produce a positron (e^+) and an electron (e^-) each having a mass equivalent to 0.51 MeV, indicating the creation of mass out of energy. On the other hand, the interaction of a positron and an electron can result in the production of annihilation radiation, which is an electromagnetic wave.

In principle there is also no difference between electromagnetic waves (such as X-rays and γ-rays) and radiation of longer wavelength like visible light. Moreover, the distinction X-rays and γ-rays is only based on their difference in origin. X-rays are initiated by mutations in the electron shells orbiting around the nuclei of the atoms and by interactions of electrons with the electric field of the nucleus of an atom (so-called Bremsstrahlung), whereas γ-rays are produced by mutations in the nuclei of the atoms itselves. In most cases, X-rays have less energy and consequently a longer wavelength than γ-rays. But there are also X-rays with higher energy than the γ-rays of some natural isotopes. Until fairly recently the most energetic X-rays used in biological experiments were obtained from 400 kV therapy tubes giving a spectrum reaching in wavelengths down to 3 pm (0.03 Å) and having an average wavelength of 6 pm,

whereas γ-rays obtained from radium with a wavelength of 1 pm correspond to X-rays of 1.2×10^6V.

The recent introduction of very powerful accelerators (linear ('Linac') or circular) with an energy of 6 MeV makes it possible to produce Bremsstrahlung (X-rays) of comparable energy, i.e. with an energy much higher than of ^{60}Co γ-rays. The only remaining fundamental difference between X-rays and γ-rays is that γ-rays produced by an isotope are mono-energetic, which means that they give rays of one (or more) particular energy(ies), while X-rays produced by a machine give a continuous energy spectrum of considerable range.

Beta-radiation may consist of electrons (β^--rays) or positrons (β^+-rays). They are both fundamental particles originating from the nuclei of radioactive isotopes during radioactive decay. The electrons are negatively charged particles like those orbiting around the nucleus of an atom and having a weight of about 0.05 per cent of the weight of a hydrogen atom. Whereas positrons are particles with a mass the same as that of an electron and a positive electric charge numerically equal to the negative charge of an electron.

Alpha-rays are relatively heavy particles consisting of two protons and two neutrons, in fact they are helium nuclei. They have two positive charges, so that their Atomic Number is 2 and Mass Number is 4. Alpha particles are in some instances ejected from the nuclei of radioactive heavy nuclides during radioactive decay.

Neutrons are uncharged fundamental particles. Therefore they can move close to an atomic nucleus without suffering electrical repulsion either by the negative charges

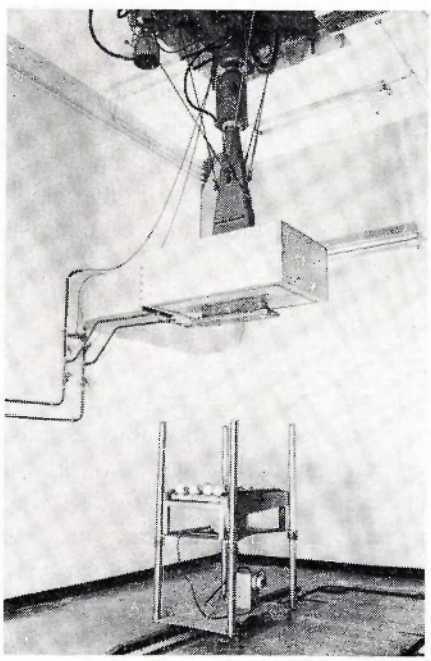


Photo 1. Van de Graaff electron generator of 1.5 MeV of the Institute for Atomic Sciences in Agriculture (Association ITAL-EURATOM) at Wageningen, the Netherlands. With this apparatus electrons of high energy (1.5 MeV) can be produced and by introduction of a target, preferentially a heavy metal such as gold, X-rays (Bremsstrahlung, Braking radiation) of somewhat less than maximum energy can be obtained.

of the orbital electrons or by the positive charge on the nucleus. The mass of a neutron is approximately equal to that of a hydrogen nucleus (proton). Neutrons are ejected during radioactive decay of some radioactive nuclides.

Important are the differences in degree of penetration of these various radiation types into matter. The penetration depth is dependent on the type of radiation, its energy, and the physical and chemical composition of the absorbing material. Gamma-rays of medium energy are not completely absorbed even by a relatively thick lead shielding, while β^--irradiation is unable to go through a thin aluminium foil and α-rays cannot even pass a sheet of paper. For this reason only electrons and γ-rays are of interest for sterilization purposes. Electrons (β^--rays) can only be applied for surface sterilization. By means of a Van de GRAAFF generator (Photo 1) or other accelerators electrons of a very high kinetic energy can be obtained. In spite of this, 15 MeV electrons can only penetrate biological material to a depth of 15 cm (CONSTANT, 1964). However, the use of very high energy electrons cannot be permitted, because an electron beam energy of more than 10–15 MeV can lead to a detectable induced radioactivity in the irradiated material. Most of this radioactivity is the result of photonuclear reactions produced by Bremsstrahlung, which is the secondary photon radiation produced by deceleration of the electron passing through matter, accompanying electron absorption. Some additional activity arises from secondary neutron-induced reactions and only a negligible contribution comes from direct electron interactions (MEYER and BURKHARDT, 1966).

For 'deep' sterilization, i.e. sterilization inside the material, in practice only γ-rays can be used. Therefore, this paper will mostly deal with γ-rays and their effects on biological material.

2. THE EFFECTS OF RADIATION

The energy of γ-rays is of the order of 10^4 higher than that of light. Therefore, there is little similarity between the interaction processes of γ-rays with matter and those of visible light.

But there are also some more fundamental differences between light waves and γ-rays. If light, e.g. ultra-violet light of 260 nm, is passed through a solution containing an equal mixture of nucleic acid and serum protein, more than 90 per cent of the light energy is absorbed by the nucleic acid and less than 10 per cent by the protein. Apparently, the absorption of light depends on the molecular structure of the absorbent and only to a small extent on its atomic composition. The spectrophotometrical determination of various chemical compounds is based on this principle as each substance has its own characteristic absorption peaks.

If, however, γ-rays (or X-rays) are passed through this solution equal amounts of energy of the γ-rays are absorbed by the protein and by the nucleic acid. In other words, the γ-rays are not selective for a particular compound. The absorption is primary re-

lated to the atomic structure of the irradiated compound and not to the way in which these atoms are configurated to molecules.

Moreover, when a quantum of visible or ultra-violet light is absorbed by a chemical compound, the energy absorbed is stored in the molecule. The molecule can then undergo one or more reactions, some of which will lead to chemical changes (such as molecular dissociation) and others to physical effects (such as fluorescence, heating, etc.). If, however, an atom absorbs a quantum of γ-rays, it loses an electron. Excluding extremely soft X-rays, which will not be considered, the energy taken up by the atom mostly amply exceeds the quantity necessary to produce this ionization (i.e. to eject an electron from the atom) and the surplus energy appears as kinetic energy of the ejected electron. In this case there are two possibilities. First, the ejected electron will obtain all the energy of the γ-ray, which effect is called the Photo-effect. Or the electron will obtain only part of the whole energy of the γ-ray, which is the so-called Compton effect. In the latter case a secondary γ-ray will appear having the remaining energy. In other words, it will continue its way as an electromagnetic wave of a longer wavelength.

In both cases the ejected electron, being itself the product of an ionization, is sufficiently energetic to produce ionizations in the atoms through which it passes. In fact almost all ionizations are produced by ejected electrons and the effect of the initial absorption of the quantum γ- or X-rays is usually negligible. Owing to this phenomenon, the ions produced are not distributed at random in the solutions as in the case of light absorption, but they are concentrated along the track of the ejected electrons. This represents another fundamental difference between ultra-violet or visible light absorption and ionizing radiation.

Radiation damage in living organisms can be divided into two mechanisms, which cannot be separated in the living cell. First, there is the direct action, i.e. the molecular damage in an essential molecule, which absorbs the radiation energy. Secondly, there is the indirect action, i.e. the formation of highly reactive free radicals in water, which radicals subsequently react with cell constituents. The formation of free radicals is far more destructive to the cell than changes in macromolecules (like DNA and enzymes) and other molecules (like ATP or co-enzymes) which lead to mutations without affecting in most cases the survival of the cell.

Since living organisms consist for about 80–90 per cent of water, it is understandable that radiation effects in aqueous solutions are of prime importance in radiobiology. For this reason, the indirect action of radiation, i.e. the radiolysis of water and the action of the free radicals on cell components, will be discussed in more detail.

The first mechanism for the formation of free radicals from water by irradiation was proposed by LEA (1947). A pair of positive and negative water ions was postulated as the direct product of ionization. These decompose on becoming hydrated within the relaxation time of water (i.e. 10^{-11} sec) to give radicals. The complete reaction is represented as follows:

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

$H_2O^+ + n(H_2O) \rightarrow H_{aq.}^+ + OH\cdot$ (i.e. decomposition on hydration)

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

$H_2O^- + n(H_2O) \rightarrow H\cdot + OH_{aq.}^-$

Overall: $H_2O \rightarrow H\cdot + OH\cdot$

According to this mechanism the two radicals are not formed close together as the slow electron has, on molecular scale, an appreciable free path before it is captured to give the negative ion.

More recently, SAMUEL and MAGEE (1953) using another model have calculated that the electron rapidly loses its energy to surrounding water molecules and that in fact it cannot escape from the strong electric field of the ion. After travelling a distance less than 2 nm the electron has lost sufficient energy to come into the attraction field, and approximately 10^{-13} sec is taken up by the charge neutralization process. This time interval is too short for the dissociation of the positive ion. Therefore, SAMUEL and MAGEE (1953) postulated that all radiation-induced chemical reactions in solids and liquids proceed via excited molecules and their free radical dissociation products. The following scheme was suggested:

$$H_2O \text{—}\sim\rightarrow H_2O^+ + e^- \xrightarrow[\text{(slow)}]{} H_2O^* \xrightarrow[\text{(highly excited)}]{} H\cdot + OH\cdot$$

The question which model describes best what actually happens is not yet solved. In the Magee-Samuel mechanism the H· and OH· radicals are formed at the same site, whereas in the Lea mechanism there is a significant spatial difference in distribution. The use of ultra-short radiation pulses (FIELDEN et al., 1970) and the measurement of the transitory free radicals in vitro and in vivo by electron spin resonance (ESR) during the irradiation process (KENNY and COMMONER, 1969) may provide a clue to decide between the two mechanisms.

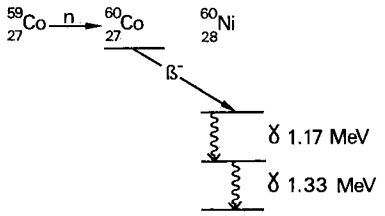
When the radiolysis of water occurs in the presence of oxygen the highly reactive perhydroxyl radical ($HO_2\cdot$) is formed as well. Its major sources are the reactions of hydrogen atoms and electrons with oxygen and the reactions of some organic radicals with oxygen (SCHWARZ, 1964). The perhydroxyl radical can act as an oxidizing agent to yield hydrogen peroxide, or as a reducing agent to form oxygen. Owing to these types of reaction it is often highly destructive to some cell constituents and to the survival of the cell as well. The so-called oxygen effect (see p. 67) is certainly related to this process.

3. RADIATION SOURCES AND UNITS

As previously explained radiation for sterilizing purposes should be done with well penetrating rays, i.e. γ-rays. Only for surface sterilization high-energy electrons may be used as well.

The most common sources of γ-rays are the isotopes ^{60}Co and ^{137}Cs. In certain shapes (rods or plates) and geometric conditions, they are commercially applied on a great scale. The half-lives of ^{60}Co and ^{137}Cs isotopes are 5.27 and 30 years, respectively. The long half-life of ^{137}Cs would be an advantage for the use of this isotope. But ^{60}Co has a four times higher energy output, since one desintegration of ^{137}Cs produces only one γ-quant of 0.66 MeV, whereas one desintegration of ^{60} Co gives rise in cascade to two γ-quanta, i.e. one of 1.17 MeV and the other of 1.33 MeV.

As shown in the diagram the desintegration of ^{60}Co proceeds in several steps. First a β^--ray (electron) is emitted producing ^{60}Ni, and then in two subsequent steps a γ-ray of 1.17 MeV and one of 1.33 MeV are emitted:



The higher energy output and the ready availability of ^{70}Co, which is a by-product of atomic piles, have certainly favoured the use of this isotope as radiation source. On the other hand, it appeared from radiation studies that only the amount of the absorbed energy determines the produced effects and this would favour the use of the ^{137}Cs isotope. Because of the lower energy of the γ-rays of ^{137}Cs these are absorbed by material to a greater extent than γ-rays of a cobalt source. For instance, the transmission through a 10-cm thick lead shielding is 1.0 per cent for ^{60}Co-rays, but only 0.001 per cent for ^{137}Cs γ-rays (Radiological Health Handbook, U.S. Dept. of Health, Education and Welfare, 1960 ed., p. 152).

The strength of a source is expressed in Curies; it is the number of desintegrations occurring in a certain time interval. The radiation unit one Curie (Ci) represents 3.7×10^{10} desintegrations per second or 2.22×10^6 desintegrations per minute (d.p.m).

In practice the efficiency of a source is much lower than the theoretical energy output, partly because of self absorption (about 10 per cent), but mainly due to the impossibility to utilize all radiation energy emitted, because of limited geometric efficiency and lack of absorption of all the γ-rays applied by the irradiated material. A source of 70,000 Ci ^{60}Co would give, theoretically, an output of about 1 kiloWatt γ-rays; but in practice its efficiency is only 200 Watt.

Units of radiation can be distinguished in exposure units and absorption units. For instance, the Röntgen-unit is a unit of exposure. One Röntgen (R) is the application of a dose, which produces 2.1×10^9 ion pairs per cm^3 of air at N.T.P. The most currently used absorption unit is the rad. One rad corresponds to an absorption of energy of 100 ergs per g of material. The application of approximately 1.07 R of X-

radiation or of γ-radiation to biological material results in the absorption of one rad, or 100 ergs of energy in each gramme.

The biological effects produced are related to the number of ionizations per unit of track length. In this context, the following fundamental relations have been established. The specific ionization of charged particles is proportional to the square of their charge and inversely proportional to their velocity. Protons and electrons, therefore, moving at the same velocity will produce the same number of ions per unit of length, whereas an α-particle with the double charge will produce four times as many ions. Moreover, neglecting relativity, the velocity of particles having a given amount of energy is an inverse function of their mass. For example, a 20 MeV proton has the same velocity and, therefore, the same specific ionization as a 10 keV electron, since the ratio of their masses is 1800:1. Thus, at comparable energies the number of ionization per μm of track is very much larger for α-particles and protons than for electrons.

For purposes of comparison of radiation effects produced by different types of radiation, relative units have been proposed. These relative units are based on the effects produced in matter by X-rays of an energy of 100 to 1000 keV, most commonly 200 keV. For instance, 1 rep (röntgen equivalent physical) is the same amount of energy released in water (or tissue) as 1 rad of X-rays of the above-mentioned keV's. The dose of 1 rem (röntgen equivalent man or medical) does the same amount of biological damage as 1 rad of X-rays. The dosage in rems is equal to that in rads, multiplied by the relative biological effectiveness (RBE) of the radiation, a factor that reflects the effects of different densities of ionization along the path of the radiation. Therefore, the relative biological effectiveness (RBE) is defined as follows: 1 RBE is the dose in rads to produce the effect with X-rays divided by the dose to produce the same effect with the radiation type under investigation. The RBE value for X-rays, γ-rays and β-rays is 1, but it is 10 for α-rays.

The energy output of a ^{60}Co-source of 1 Ci is about 0.016 Watt. Such a source will give in air at 1 m distance from the source a dose of 1.3 rad per hour. An industrial ^{60}Co-source, which must produce a radiation absorption of 0.5-3.0 Mrads per hour, must therefore have a capacity of several kiloWatts, or a radioactivity of several hundreds of thousands of Curies.

It is essential to know exactly the radiation dose absorbed by materials. This branch of radiobiology is called 'dosimetry'. In spite of many theoretical considerations and calculations, the most reliable method for determining the absorbed dose is the empirical approach. For dose-rate measuring ionization-chamber methods or calorimetric methods are most commonly applied. For biological experiments the Fricke dosimeter is used very frequently. This dosimeter consists of an aqueous ferrous sulphate solution (10^{-3} M), sometimes supplemented with cupric sulphate (HART, 1955; JARRETT, 1967). In this solution the nearly colourless (or light green) ferrous ion is oxidized to the yellow ferric ion which, by hydrolysis, produces the yellowish-brown $Fe(OH)_3$. Chemical reactions are usually expressed in terms of the number of molecules changed per 100 eV of energy deposited, this number being called the G-value.

A large number of independent investigations in which the total amount of energy deposited was measured directly by calorimetry have established that the G-value for the oxidation of ferrous to ferric iron is 15.6 ± 0.2 for X- and γ-rays and for electrons, which have a higher energy than 25 keV.

The reaction is usually carried out in aerated solutions at a pH of less than 1.5. The yield is less at higher pH values and is reduced by 50% in the absence of oxygen (ALLEN et al., 1957).

The oxidation of ferrous sulphate in aerated acid solutions starts with radical formation in water in the presence of air:

$$H_2O + O_2 \rightarrow HO_2\cdot + OH\cdot$$

This reaction is followed by the subsequent steps:

$$Fe^{2+} + OH\cdot \rightarrow Fe^{3+} + OH^-$$
$$Fe^{2+} + HO_2\cdot \rightarrow Fe^{3+} + HO_2^-$$
$$HO_2^- + H_2O \rightarrow H_2O_2 + OH^-$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\cdot + OH^-$$
$$Fe^{2+} + OH\cdot \rightarrow Fe^{3+} + OH^-$$

Overall: $2H_2O + O_2 + 4\,Fe^{2+} \rightarrow 4\,Fe^{3+} + 4\,OH^-$

Hence for each ionization leading to radical formation 4 Fe^{2+} are oxidized. The produced Fe^{3+} concentration is measured spectrophotometrically with UV-light at 304 nm and the absorbed dose is read from a calibration curve. Regarding the G-value for Fe^{3+}, a simple conversion factor is useful: dose received by the solution in Krads = 29.2 × extinction coefficient due to Fe^{3+} at 304 nm as measured in a one cm cell. The Fricke dosimeter is mostly applied within the range of 4–40 Krads and it is a very reliable method.

Other dosimetric methods are the colour change in red 'perspex' glass (dose range 0.1–5.0 Mrads; WHITTAKER, 1964), the use of clear polymethylmethacrylate (PMMA) (dose range 20 Krads – 10 Mrads; CHADWICK, 1969), the use of blue cellophane (Du Pont 300 MSC light blue cellophane; dose range 1–10 Mrads; HENLEY, 1954 and HENLEY and RICHMAN, 1956) or even the darkening of ordinary window glass by irradiation. All these methods are in general less accurate, but they cover a higher dose range than the Fricke dosimeter (4–40 Krads) does. An exception, however, is the thermoluminescence dosimetry which uses calcium fluoride or lithium fluoride, and covers the dose range of 10 mrads to 50 or 100 Krads (PUITE, 1968; 1969).

4. THE CONCEPT RADIOSTERILIZATION

Sterility is an absolute concept: a product or a medium is either sterile or it is not. By sterility is meant that in a medium or in a product no viable organisms of any kind are detectable, when a given aliquot is examined by the best available method. In general practice, however, sterility turns out to be a statistical concept.

Sterilization involves the probability of a certain procedure to obtain a sterile product. When the number of trials and the size of the aliquots examined are made large enough, certainly non-sterile units will occur even after the conventional process for sterilization: heating. It is, therefore, required to lay down standards for the elimination of micro-organisms. Such a standard is the so-called 12-D concept for *Clostridium botulinum*. It means that the sterilization process will reduce viable *Clostridium botulinum* spores by a factor of 10^{12}; it is based on experience with heat-processed foods. In fact this gives a considerable safety margin. For example, a factory which heat-processes 10^8 cans per year, each containing ca. 10^3 grammes of food, and which works with a 12-D-process, will only once every ten years produce one tin out of its annual production which may contain a *Clostridium botulinum* spore.

In practice, the application of radiation for sterilization purposes depends therefore on the medium, the level of contamination of the medium and the presence of specific radioresistant spores and vegetative cells of micro-organisms. Knowing the bacterial contamination and the specific radiosensitivity of the components (See D_{10}-value in the next section), the applicable dosage and the duration of the treatment (dosage = duration of treatment $\times$ dose per unit of time) to obtain a certain effect can be calculated in a much simpler way than the calculations outlined by Schrauwen (1971) and Behagel (1971) for the heat process.

5. Radiosensitivity of micro-organisms

Micro-organisms show a very variable radiosensitivity. In the fungus *Phycomyces blakesleeanus* a response is already detectable with a radiation dose of 0.01 rad (Forssberg, 1941, 1964), while other micro-organisms such as some bacteria (*Micrococcus radiodurans*) can resist an extremely high radiation dose.

The radiation response in micro-organisms can be expressed by the decimal reduction dose, the so-called D_{10}-value, i.e. the radiation dose to produce 10 per cent survival or 90 per cent lethality.

By plotting the dose on a linear scale as the abscissa and the survival fraction on a logarithmic scale (or the logarithm of the survival fraction on a linear scale) as the ordinate, a sigmoid relationship is found (Fig. 1 and 4). At the lower doses there is a shoulder in the curve and at the higher doses there is a marked tailing effect. These effects become the more clear, the more precisely the experiments are performed. The middle part of the curve is linear. The linear part of the reduction curve has been explained by the random destruction of a key metabolite or a target molecule such as DNA, constituents of membranes or enzymes (Alper, 1965, 1967; Bacq and Alexander, 1966). The shoulder in the curve may be explained by a certain repair at the lowest doses and the tailing by a selection of mutants with high radioresistance. In some cases the tailing is very considerable and apparently correlated with the composition of the medium (Fig. 1, Anderson, 1969).

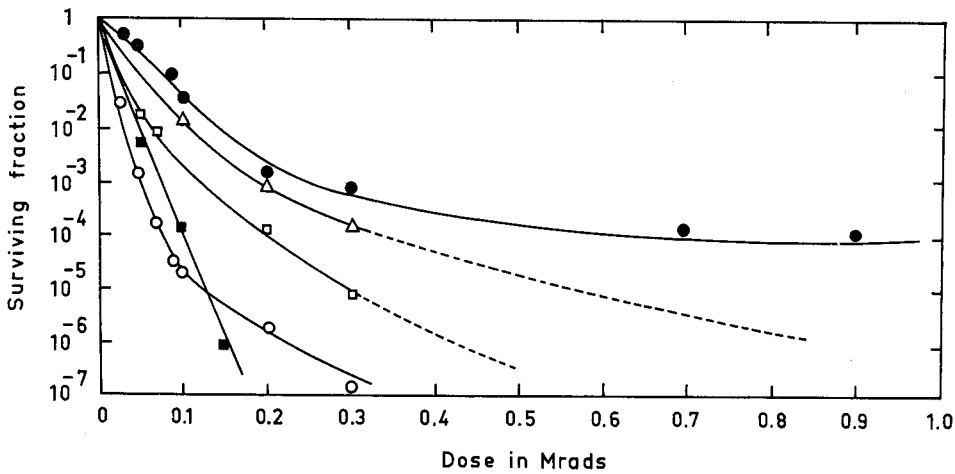


Fig. 1. Irradiation inactivation of *Salmonella typhosa* in different media.
Media: ○ Hartsells broth, △ crab, □ oysters, ● shrimp, ■ salmon (After ANDERSON, 1969).

The decimal reductions (D_{10}) values for a number of bacteria are tabulated in Table 1.

Table 1. D_{10}-values of some micro-organisms

Species	Krads
Pseudomonas spec	10 – 20
Escherichia coli (aerobic)	12 – 35
Escherichia coli (anaerobic)	20 – 45
Salmonella spec.	20 – 50
Streptococcus faecalis	50 – 100
Fungus spores (*Penicillium*, *Aspergillus*, etc.)	50 – 70
Bacillus pumilus (spores)	ca. 170
Clostridium sporogenes	160 – 220
Clostridium botulinum	150 – 250
Micrococcus sodonensis	ca. 195
Micrococcus radiodurans	600 –1200

The data presented are derived from the following sources: *Pseudomonas* (BRIDGES, 1962), *Escherichia coli* (ALPER, 1965), *Salmonella* species (LEY et al., 1963; COMER et al. 1963; ANDERSON, 1969), *Streptococcus faecalis* (ANDERSON, 1969), *Bacillus pumilus* (LEY and TALLENTIRE, 1964), *Micrococcus sodonensis* (MULLENGER et al., 1967), *Micrococcus radiodurans* (BECKING, unpublished), and *Clostridia* species (ROBERTS and INGRAM, 1965). The D_{10}-values of the fungi are from the work of POISSON and CAHAGNIER (1969). As evident from the table, the D_{10}-values for the various bacteria species range from 10 Krads in some *Pseudomonas* species to 1200 Krads in *Micrococcus radiodurans*. The strikingly low radioresistance of some pathogenic bacteria such as *Pseudomonas* species, *Escherichia coli* and *Salmonella* species is of great practical significance. For example, *Salmonella* contamination of a product is in general rather

low. With 0.65 Mrad a decimal reduction of 10^5 is obtained, which gives a commercial *Salmonella*-free product in the case of foodstuffs for human, animal or pet consumption (LEY, 1962, 1966, 1968).

Spores of spore-forming *Bacillus* and *Clostridia* species, are far more radioresistant than the vegetative cells. There is, however, no consistent relation between the radioresistance and the thermoresistance of spores. For instance, the spores of *Bacillus pumilus* are far more radioresistant than those of *Bacillus stearothermophilus*, the most thermoresistant *Bacillus* species known so far (DARMADY et al., 1961; BURT and LEY, 1963a, b; LEY and TALLENTIRE, 1964). The same holds for *Micrococcus radiodurans*, a very radioresistant bacterium, which is not al all thermoresistant (BECKING, personal observation).

The reason, why some micro-organisms are so very radioresistant and other are not, is still unknown. KAPLAN and ZAVARINE (1962) suggest a relationship between radiosensitivity and the base composition of DNA of certain bacteria (Figure 2). As shown in this figure, the most radiosensitive bacteria such as *Pseudomonas* species have a high guanine + cytosine (G+C) content of DNA, whereas radioresistant bacteria such as *Micrococcus pyogenes* var. *aureus* have a low G+C content of DNA. However, this cannot be valid in all cases, since the radiosensitive Gram-negative, aerobic, rod-shaped bacteria have a rather wide spectrum of G+C contents. For instance, *Pseudomonas* species have 58–70 per cent G+C, *Alcaligenes* species 56–70 per cent G+C,

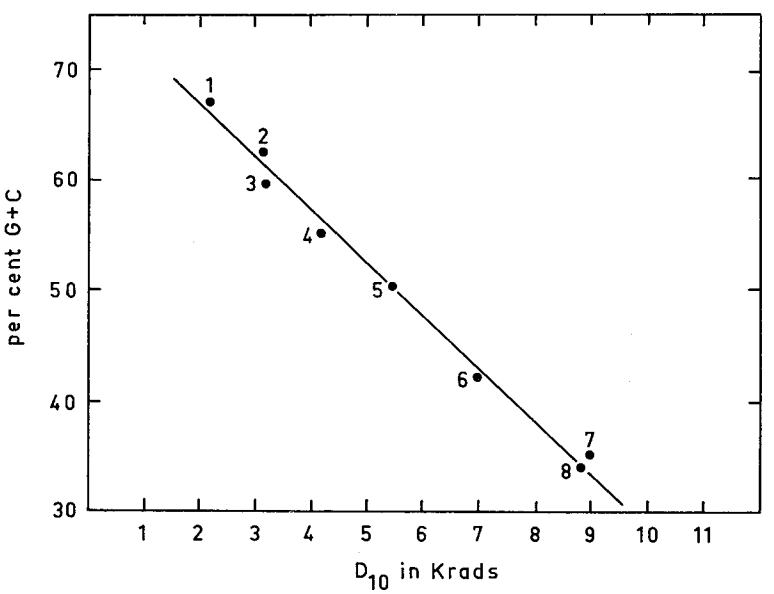


Fig. 2. Relation between G + C contents of DNA of different bacteria and the D_{10}-value in Krads of these bacteria.

Numbers: 1. *Pseudomonas aeruginosa;* 2. *Pseudomonas fluorescens;* 3. *Serratia marcescens;* 4. *Azomonas agilis;* 5. *Escherichia coli* B; 6. *Bacilllus subtilis;* 7. *Bacillus cereus;* 8. *Micrococcus pyogenes* var. *aureus* (After KAPLAN and ZAVARINE, 1962).

Xanthomonas species 63–69 per cent G+C, *Chromobacterium* species 63–72 per cent G+C, *Agrobacterium* species 60–63 per cent G+C, *Acetobacter* species 55–64 per cent G+C, and *Acinetobacter calcoaceticus* 39–46 per cent G+C. If *Micrococcus radiodurans* is identical to *Micrococcus roseus* mentioned in Bergey's Manual of Determinative Bacteriology (7th Ed., 1957), this species has a rather high G+C content of DNA, because its G+C value of DNA is 66.2–73.1 per cent. Thus this evidence is at variance with the view of KAPLAN and ZAVARINE (1962). On the other hand, BOHÁČEK et al., (1969) recently described two *Micrococcus roseus* strains with a rather low G+C content of DNA, i.e. 49.3 per cent.

All bacteria are eliminated at a 12-D-level, by a radiation dose of about 5 Mrads, but some viruses require radiation doses of about 15 Mrads.

In general, plant viruses are more radioresistant than animal viruses. LEA and SMITH (1940, 1942) measuring the inactivation of plant viruses, found a D_{10}-value for tobacco necrosis virus of 2.5 Mrads (X-rays, 0.15 nm) and one of 0.5 Mrads for tomato bushy stunt virus (γ-rays), both in dry condition and in aqueous suspension.

Animal viruses and bacteriophages are somewhat less radioresistant, since BRASCH et al. (1949) gave lethal doses ranging between 0.6 and 1.8 Mrads (Table 2).

It should be borne in mind that enzymes, especially when present in tissue, are extremely radioresistant, in this respect surpassing even viruses. For example, POWELL and POLLARD (1955) found that the effect of 1.4 Mrads on cytochrome oxidase and succinic dehydrogenase, and of 2.0 Mrads on invertase of intact *Bacillus subtilis* cells in aqueous solution was not measurable. Protection by cellular constituents must therefore be considerable. HUTCHINSON et al. (1957) observed an even higher radioresistance of enzymes in yeast cells in dry condition as compared to wet condition. In dry cells the 37 per cent inactivity dose of yeast invertase was 12 Mrads, that of alcohol dehydrogenase 28 Mrads, and that of co-enzyme A 160–190 Mrads. A general outline of the radiosensitivity of various organisms and enzymes is given in Figure 3 (Revised after MOSSEL, 1969).

Table 2. Sterilization doses (100 per cent lethality) of some animal viruses and bacteriophages (Revised after BRASCH, HUBER, FRIEDEMANN and TRAUB, 1949)

VIRUSES	Mrads
Encephalomyelitis (Murine SK strain)	1.7
Poliomyelitis	
(a) Aycock	1.5
(b) Lansing	1.6
Mumps	0.8
Influenza (human)	0.8
Rabies	0.9
Vaccinia	0.6
BACTERIOPHAGES	
E. coli Templeton	0.7
Staphylococcus V	0.6

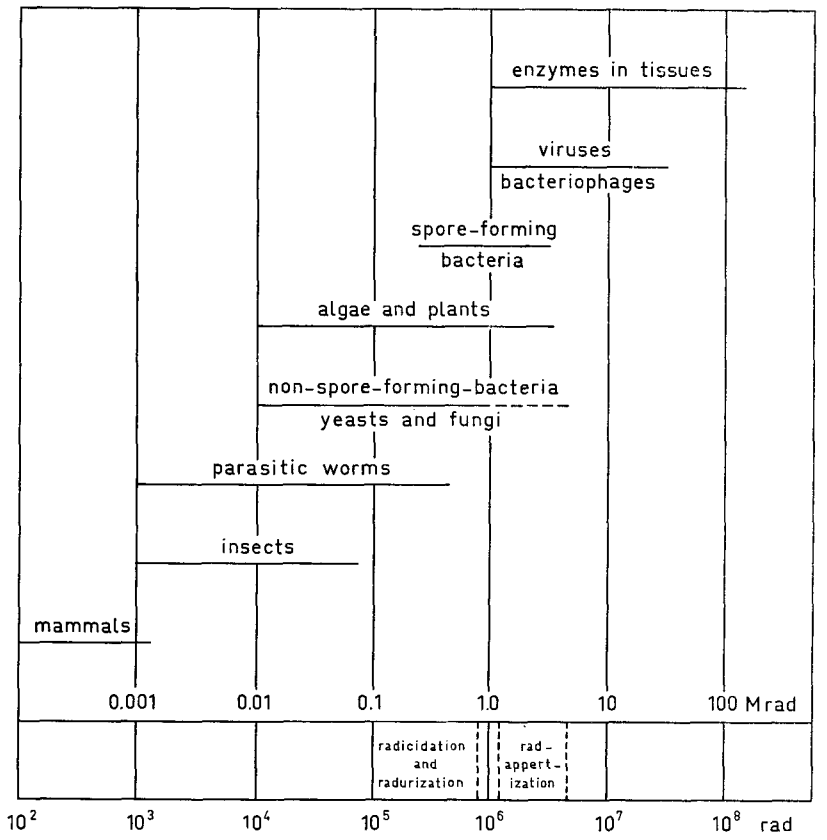


Fig. 3. Scheme of comparative radiosensitivity of living organisms and enzymes.

GORESLINE et al. (1964) have introduced new terms for the process of radiosterilization, i.e. 'radicidation', the process to kill the number of viable non-sporeforming micro-organisms other than viruses; 'radurization', the process to reduce the number of specific spoilage micro-organisms and thus increase the shelf-life of the product; and 'radappertization', the process to obtain a commercial sterile product.

6. FACTORS AFFECTING RADIOSENSITIVITY

6.1. *Oxygen effect*

The presence of oxygen (in the atmosphere or dissolved in solution) has a serious deleterious effect on the survival of irradiated organisms.

Under anaerobic (anoxic) conditions bacteria are far more resistant to irradiation than they are under aerobic (oxygenated) conditions.

For bacteria, the oxygen effect is not only observed with vegetative cells but also with spores in resting condition. A typical example of the oxygen effect on vegetative cells is the work of ALPER (1965) with *Escherichia coli* (Figure 4), which shows a marked increase in radioresistance under anoxic conditions.

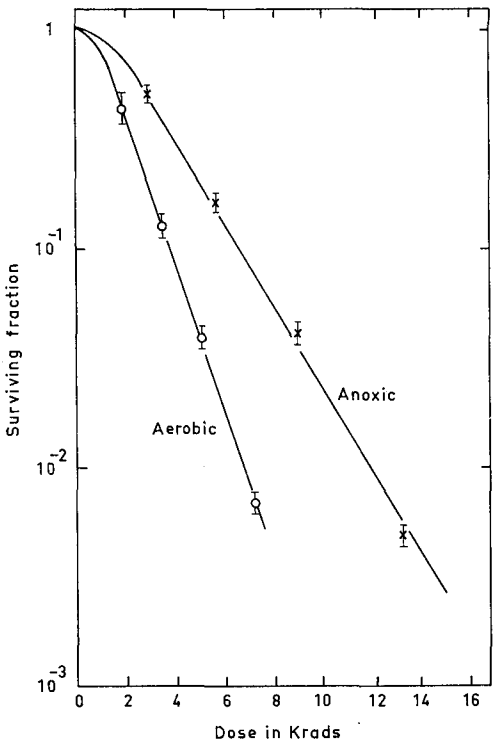


Fig. 4. Effect of anoxic and aerobic conditions during irradiation on the survival of *Escherichia coli* Bs exposed to X-rays (After ALPER, 1965).

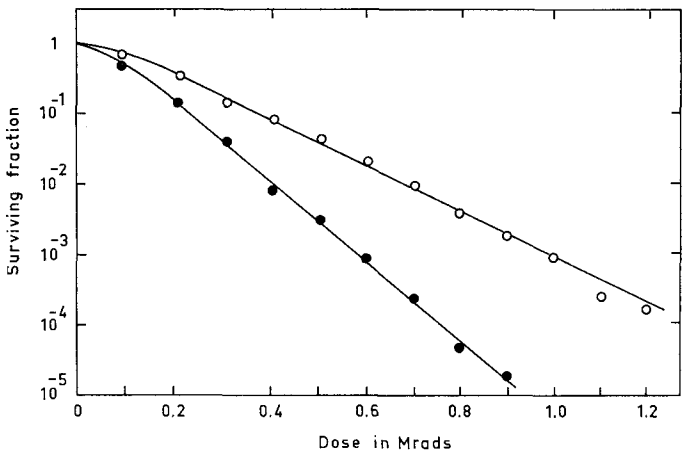


Fig. 5. Relationship between surviving fraction and radiation dose for spores of *Bacillus pumilus* E.601 suspended in phosphate buffer exposed to gamma-irradiation.

Symbols: ○, spores irradiated under anoxic conditions; ●, spores aerated during irradiation (After BURT and LEY, 1963a).

The same is true for spores of *Bacillus pumilus* irradiated in phosphate buffer under anoxic and aerated conditions as shown in Figure 5 (BURT and LEY, 1963a). Under anoxic conditions the D_{10}-value of these spores was 0.306 Mrads, but it was only 0.175 Mrads with aeration during irradiation.

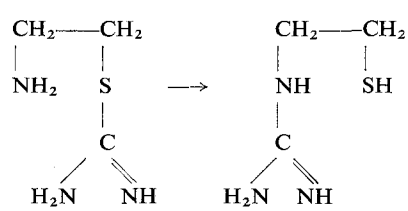
The oxygen effect can be partly explained by the fact that formation of oxygenated radicals in water and organic molecules takes place during irradiation, but probably the effect is more complex and other factors are involved as well.

For the sterilization of culture media by means of irradiation the oxygen effect is of considerable significance. Micro-organisms present in media which are sealed in plastic foils, etc., may be considerably more resistant to irradiation than in normal, i.e. aerobic, condition. Even when media are not sealed anaerobically, anoxic conditions may develop as a result of oxygen consumption by micro-organisms and components of the media. Oxygenated conditions during irradiation are therefore favourable for obtaining a sterile medium. On the other hand the presence of oxygen enhances the formation of oxygenated radicals, which are often deleterious to the chemical compounds of the medium. For this reason, a compromise condition between both extremes should be applied for the process of sterilization by means of irradiation.

6.2. *Effects of protective and sensitizing substances*

Evidence is accumulating that chemical substances containing sulphydryl groups (-SH) are often very good protecting agents, whereas compounds which have the tendency to bind sulphydryl groups are often active sensitizing agents of radiation effects.

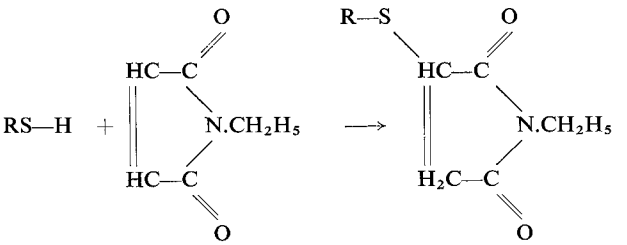
A typical example of an active radioprotecting substance is the simple molecule of cysteamine. The activity of this compound disappears or is strikingly reduced, if the hydrogen of the -SH function is substituted by a firmly held radical (-CH_2, for instance). Another radioprotective substance is β-aminoethylisothiouronium (AET). AET in aqueous solution is changed into mercaptoethylguanidine (MEG), by a process of intratransguanylation and MEG is in fact the protecting molecule (DOHERTY et al., 1957). The reaction is as follows:



Therefore substances like isothiouronium compounds, which do not rearrange into guanidothiols do not protect against radiation (PLZAK et al., 1958).

The first demonstration of the sensitization of a micro-organism by a sulphydryl-

binding agent was the action of N-ethylmaleimide (NEM) on the gamma-sensitivity of *Escherichia coli* (BRIDGES, 1960). The ability of NEM to bind sulphydryl groups can be illustrated as follows:



Although in a large number of chemical compounds the presence of sulphydryl and sulphydryl-binding groups can explain their action as a protecting or sensitizing agent, there exists a number of other compounds with this ability, which do not possess these groups. Their activity must therefore be due to another mechanism. A list of well-known radioprotecting and radiosensitizing agents is given in Table 3.

Remarkable is the specificity of certain compounds for certain bacteria. Both N-ethylmaleimide (NEM) and iodoacetamide (IAAM) are sulphydryl-binding compounds, yet NEM is active in *Escherichia coli* and not in *Micrococcus radiodurans*. *Micrococcus radiodurans* can, however, be sensitized with IAAM or mercury compounds such as p-hydroxymercuribenzoate (HMB). A typical result of the action of iodoacetamide (IAAM) on the survival of *Micrococcus radiodurans* is presented in Figure 6 (BECKING, unpublished).

Table 3. Chemicals affecting radiation sensitivity of micro-organisms

Protecting:	*Sulphydryl agents* (SH or thiol groups) CSH (cysteine), CSSH (cystine), GSH (glutathione), GSSG (glutathione-disulphide), MEA (β-mercaptoethylamine), AET (β-aminoethylisothiouronium), MEG (mercaptoethylguanidine), GED (oxidized form of the latter compound), diethyldithiocarbamate and other dithiocarbamates, etc.
	Other compounds Ascorbic acid (vitamin C), glycerol, cyclohexanolsuccinate, TACE (chlorotrianisene), amines (tryptamine, adrenaline, histamine), sex hormones (oestrogens), etc.
Sensitizing:	*Sulphydryl-binding agents* NEM (N-ethylmaleimide), IAA (iodoacetic acid), IAAM (iodoacetamide), CAM (chloroacetamide), PMA (phenylmercuric acetate), HBM (p-hydroxy-mercuribenzoate), vitamin K derivates, quinones and hydroquinones, sulphurous acid, nitric oxide, methyl glyoxal, neoarsphenamine, etc.
	Other compounds Cupric chloride, potassium iodide, sodium iodide, choralhydrate, methyl hydrazine, ethyl acetate, ethyl methanesulfonate, sodium methanesulfonate, divinyl sulfone, glycine, sodium hydrosulfite, phenol, 2-fluoroadenosine, di-t-butyl-nitroxide, t-butyl-2,6-dimethoxy-phenyl-nitroxide, acrylamide, 5-bromouracil, etc.

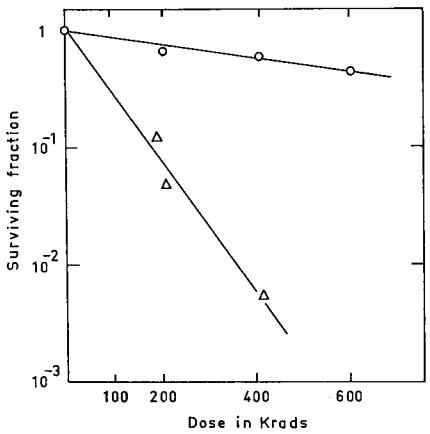


Fig. 6. Effect of 10^{-3} M iodoacetamide (IAAM) during irradiation on the aerobic radiosensitivity of *Micrococcus radiodurans* E exposed to electron radiation.
Symbols: ○, without IAAM; △, with IAAM.

As evident from this figure a concentration of IAAM of 10^{-3}M reduces the initial D_{10}-value of *Micrococcus radiodurans* of 1200 Krads to 200 Krads.

In experiments with sensitizing agents an oxygen effect was also observed. As shown in Figure 7 the effect of N-ethylmaleimide (NEM) is much smaller in anoxic conditions than in aerated conditions (BRIDGES, 1961). Moreover, there is an interaction of these compounds with the metabolism of the micro-organism, since BRIDGES (1969) found a synergistic lethal effect of NEM (10^{-3}M) in the presence of urea (1.5 M) on the survival of *Escherichia coli* in physiological saline solution (Figure 8).

Moreover, the medium in which the radiation is performed has a marked influence on the lethality of the micro-organisms. In addition, micro-organisms cultured under

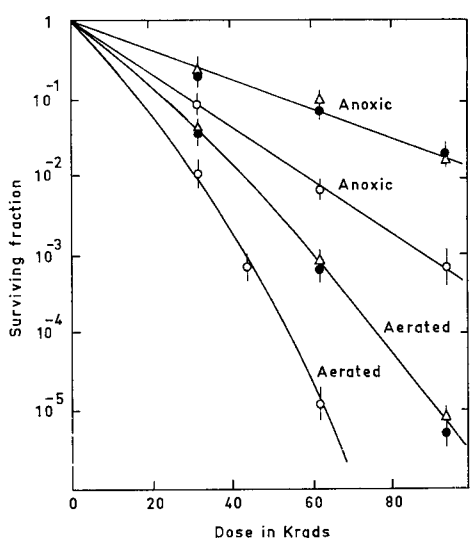


Fig. 7. Effect of 10^{-3}M N-ethylmaleimide (NEM) during irradiation on the gamma-irradiation sensitivity of stationary phase *Escherichia coli* B/r in the presence and absence of oxygen.
Symbols: ●, Control; ○, NEM; △, NEM added after irradiation (After BRIDGES, 1961).

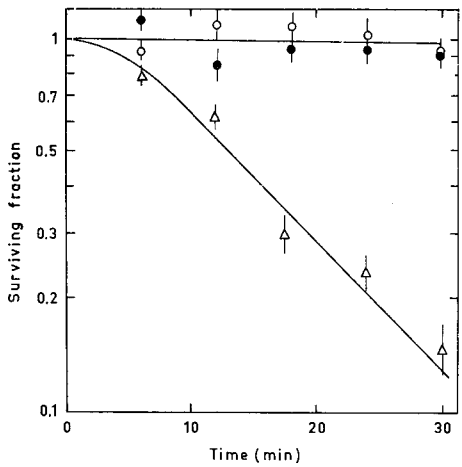


Fig. 8. Effect of urea (1.5 M) and NEM (10^{-3} M) on the survival of *Escherichia coli* B/r WP 2 suspended in physiological saline at 20 °C.
Symbols: ●, Urea; ○, NEM; △, NEM + urea (After BRIDGES, 1969).

various conditions give different results. Therefore, the nutrient status of the micro-organisms, which is related to the composition of the medium, influences their survival. Finally, plating irradiated micro-organisms on an optimal medium does not necessarily enhance their recovery. In fact, the reverse is often true, because lethality can be greatly suppressed by prolonging the lag-phase of growth of the micro-organism by plating it on a sub-optimal medium.

In all cases a sensitizing effect of the chemical compound is obtained only, if the sensitizing agent is present during irradiation. The presence of this substance before irradiation (and its subsequent removal), or the exposure of the micro-organisms to this substance after irradiation, has little or no effect. So far there has been no evidence whether the sensitizing agent actually enters the cell. Experiments performed with ^{14}C-labelled NEM showed that this substance enters the cell, although in a very low concentration (HARTOG and BECKING, in preparation).

Two recent reviews concerning the knowledge of the working mechanism of radio-protecting and radiosensitizing agents are 'Radation damage and sulphydryl compounds', IAEA-Panel Proceedings, Vienna, 21–25 October 1968 (1969) and an article of BRIDGES (1969).

7. EFFECTS OF RADIATION ON VARIOUS ORGANIC COMPOUNDS

For many compounds the effect of ionizing radiation on aqueous solutions has been tested.

Some examples of biological important compounds such as carbohydrates, proteins and amino acids, lipids, nucleic acids, and vitamins will be given.

Chemical reactions are, as explained in section 3, customarily expressed in terms of the number of molecules changed per 100 eV of energy absorbed, this number is the

Table 4. Radiolytic destruction of some organic compounds in aqueous systems

Carbohydrates:	Hydrolysis of the polysaccharides by cleavage of the glucosidic bond. Selective oxidation of the terminal OH-group of the mono-saccharides giving the corresponding acids (e.g. gluconic acid, maltobionic acid, arabonic acid). Reduction and decarboxylation to less C-atoms containing compounds: aldehydes, acids, carbon dioxide, etc. Viscosity decrease of polysaccharides. Colour changes and organoleptic changes.
Proteins:	Formation of amino acids by cleavage of the peptide bond. Degradation of the amino acids by deamination and decarboxylation producing ammonia, aldehydes, fatty acids, amines, carbon dioxide, hydrogen, methane, etc. Selective oxidation of SH-groups of S-containing amino acids. Viscosity decrease by hydrolysis, but also aggregate formation and polymerization.
Lipids:	Formation of fatty acids. Decarboxylation and breakage of the carbon chains, giving lower mono- and dicarbonic acids, hydroxy-acids, epoxy-acids, aldehyde-acids, keto-acids, aldehydes and carbonyl compounds. Formation of aliphatic hydrocarbons such as alkanes, alkenes (the off-odors: hexene, heptene, octene), internal alkenes, alkadienes. Sometimes re-polymerization of the fatty acids. Serious organoleptic changes, especially in the presence of oxygen.
Nucleic acids:	Depolymerization: decrease in viscosity and changes in the optical characteristics. Splitting off of the pyrimidine and purine bases, and inorganic phosphate. Destruction of the bases by deamination, ring cleavage and oxidation.

so-called G-value. In some of the results presented this G-value will be used. The main results are summarized in Table 4.

7.1. *Carbohydrates and related compounds*

Irradiations studies on polysaccharides, including the trisaccharide raffinose, and the disaccharides sucrose, maltose and cellobiose have shown that the glucosidic bond is particularly sensitive to irradiation. The extent of hydrolysis increases with increasing dosage. Qualitatively, hydrolysis by irradiation was found to be the same as hydrolysis by acid (WOLFROM et al., 1959).

EHRENBERG et al. (1957) found that when starch was hydrolized by ionizing irradiation, there was some protective effect of water, since the number of chain breaks produced in the polysaccharide decreases with increasing water content. This effect was explained by assuming that in material with low water contents excitations lead to chemical changes more efficiently than they do in materials containing more water.

Irradiation of cellulose gave a progressive decrease in viscosity at higher doses

(GLEGG, 1957). Soluble collagen irradiatied with gamma-rays also showed a decrease of viscosity by fission of the peptide bonds, but without significant destruction of the amino acids in both neutral-salt-soluble and acid-soluble collagen (DAVIDSON and COOPER, 1968).

Selected carbohydrates underwent colour changes and organoleptic changes when irradiated in aqueous solution and there was in general a selective oxidation of the terminal hydroxyl-group (WOLFROM et al., 1959).

Monosaccharides are partly oxidized to the corresponding uronic acids and decarboxylated to less carbon-containing reducing and acidic substances. For instance, the irradiation of the disaccharide maltose yielded glucose, gluconic acid, maltobionic acid, etc. That of glucose gave gluconic acid, 2-oxo-d-arabino-aldohexose, 2-oxo-d-arabino-hexonic acid, arabinose, etc. Irradiation of glycolic acid (= hydroxyacetic acid), a constituent of cane sugar juice, yielded glyoxylic, tartaric, oxalic and formic acids, formaldehyde and carbon dioxide (BARKER et al., 1959). Thus, apart from a destructive effect, radiation may also produce new substances by polymerization.

7.2. *Proteins and amino acids*

The major effect of radiation on proteins or polyamino acids is the cleavage of the peptide linkages with the formation of amide and carbonyl groups. The yields of amides tend to increase with increasing oxygen tension during irradiation. Also, many other reactions may take place; among others: reductive deamination, decarboxylation and recombinations. SOUTHERN and RHODES (1967) succeeded in detecting residues of several new amino acids in irradiated poly-α,L-glutamic acid, poly-D,L-alanine, poly-L-proline, while LIEBSTER and KOPOLDOVA (1964) mentioned recombinations involving the methylene and methyl groups of higher amino acids.

SHARPLESS et al. (1955) studied the radiolytic behaviour of individual amino acids like alanine. They observed the formation of many smaller molecules such as ammonia, acetaldehyde, pyruvic acid, propionic acid, ethylamine, volatile acids, carbon dioxide, hydrogen, and carbon monoxide (Table 5). Moreover, there was a good agreement in

Table 5. Effect of radiation on alanine in neutral solution (1.0 M alanine; anaerobic). After SHARPLESS, BLAIR and MAXWELL (1955).

Products	Molecules/100 eV
Ammonia	4.48
Acetaldehyde	0.59
Pyruvic acid	1.92
Propionic acid	1.04
Carbon dioxide	0.59
Ethylamine	0.17
Hydrogen	1.10
Methane	0.01
Carbon monoxide	0.01

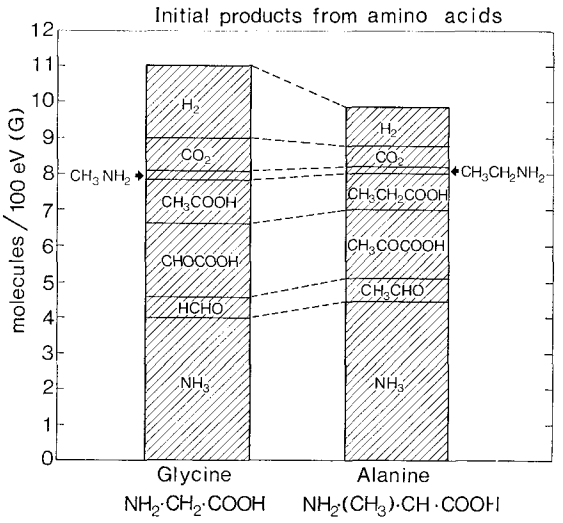


Fig. 9. Comparison of initial yield of products obtained by irradiation of glycine and alanine solutions (1.0M) in vacuo with X-rays (After SHARPLESS, BLAIR and MAXWELL, 1955).

the proportionate yields of the several components produced by irradiation, if a 3-carbon amino acid such as alanine was compared with the 2-carbon amino acid glycine (Figure 9).

Sulphur-containing amino acids such as methionine are often particularly sensitive to irradiation. This is especially important, since irradiation causes the formation of hydrogen sulphide, mercaptans and disulphides, which are mainly responsible for the off-flavours and off-odours of the irradiated medium or foodstuff. In model systems HARLAN et al. (1967) studied the production of methyl mercaptan (CH_3SH) from methionine by irradiation. The formation of mercaptan was not inhibited by anoxic conditions, but only by a very drastic reduction in temperature (from +20 °C to −80 °C or −196 °C).

7.3. *Lipids*

The most prominent radiation effects in fat are decarboxylation and the cleavage of the carbon chains. The latter will produce, together with recombinations, the formation of shorter, longer or branched carbon chains of the fatty acids. Irradiation in the presence of oxygen results in the formation of oxygenated radicals producing peroxides, which will induce autoxidations of other substances. These oxidations will produce mono- and dicarboxylic acids, hydroxyacids, epoxyacids, aldehyde acids, ketoacids, saturated and unsaturated aldehydes, and carbonyl compounds. In addition, a shifting of double bonds and a certain degree of polymerization occur (Food and Agricultural Organization Report, Brussels 1961, Publ. FAO, Rome, 1962). Especially the formation of many substances containing the reactive carbonyl groups (C=0) is important in the irradiation of fat.

7.4. *Nucleic acids*

Nucleic acids are macromolecules with a very high molecular weight. The most important is deoxyribonucleic acid (DNA) being responsible for the genetic code of an organism. Therefore, the discussion will be confined to DNA. The most readily determined property of solutions of DNA is their high viscocity. Radiation decreases this viscosity and the decrease is proportional to the radiation dose (SPARROW and ROSENFELD, 1946).

With increasing dosage the base constituents are also destroyed. Both WEISS (1959) and HEMS (1960) mentioned that the pyrimidine-ring is somewhat more readily destroyed than the purine-ring. In the presence of dissolved oxygen hydrogenperoxides are formed and the destruction of the bases is enhanced. Irradiation often causes a cleavage between the purine and the sugar moiety and finally there is ring opening and deamination (HEMS, 1960).

The lethal or mutagenic effects of ionizing radiation on living organisms is mainly due to a destruction or a change in the DNA molecules localized in the chromosomes of the cell nuclei of the organisms.

7.5. *Vitamins*

A considerable amount of literature has been accumulated on the effect of radiation on vitamins, especially in relation to foods.

Some basic results comparing the action of heat and that of ionizing radiation on vitamins is summarized in Table 6 (Derived from READ, 1960).

Considerable discrepancy exists, however, between the results of various workers in this field. The differences may be mainly caused by the different environmental conditions in which the vitamins were irradiated.

Table 6. Relative effects of heat and radiation on vitamin destruction in foods (After READ, 1960)

Vitamin	Per cent destruction	
	Heat	Irradiation (2.79 Mrad)
Thiamine	60–70	55–65
Riboflavin	18–22	6–10
Pyridoxine	28–32	24–25
Nicotinic acid	30–35	0–14
Choline	?	0
Folic acid	35	0
Inositol	?	0–5
Vitamin A	20	31–70*
Vitamin E	?	61*

* Dosage: 0.44 Mrads in dairy products

For example, KENNEDY (1965) studying the effect of irradiation on the degradation of B-complex vitamins in eggs and wheat observed that in contrast to the data given in Table 6, especially nicotinic acid and thiamine were reduced in concentration. PROCTOR and GOLDBLITH (1949) exposed pure solutions or mixtures of nicotinic acid, riboflavin and ascorbic acid to soft X-rays and found that ascorbic acid was the most sensitive of the three vitamins and that nicotinic acid was more resistant than riboflavin. Yet a mixture of ascorbic acid and nicotinic acid showed a greater destruction of nicotinic acid and a sparing of ascorbic acid.

DIEHL (1969) studied the contents of vitamin E (α-tocopherol) and B_1 (thiamine) in a number of foods after irradiation and during storage for periods of up to 8 months. He observed a gradual decrease in the vitamin contents of the food caused by irradiation during storage. In experiments with sunflower oil to which vitamin E was added after irradiation, it was found that a vitamin-E-destroying factor was present in the irradiated oil. The production of this factor was favoured by the presence of oxygen, but not completely inhibited by its absence. In contrast to the vitamin-E-destroying property of heated oils, the radiation-induced factor disappeared after 20 days of storage of the irradidated oil in the presence of air. The vitamin E and vitamin B_1 losses were also studied after the combined treatment of irradiation and heat, and they were followed during storage. It was found that in this case the vitamin destruction was higher than expected, if the effects of the individual treatments of irradiation, heat and storage were simply additive. These synergistic effects of vitamin destruction by combined treatments were observed in a number of foods.

8. RADIOSTERILIZATION OF NUTRIENT MEDIA

8.1. *Liquid nutrient media*

Comparatively little work has been done one the sterilization with gamma-irradiation of nutrient media for the growth of bacteria, plant tissues or plant organs.

GOVILA et al. (1963) mentioned the use of gamma-rays for the sterilization of WHITE's (1943) basic medium for the growth of embryos of rye (*Secale cereale*). They compared the influence of autoclaved, irradiated and non-irradiated medium on the growth response of the tissues. They found gamma-irradiation of the medium nearly as effective as autoclaving, but the root length produced in the embryos grown on the irradiated medium was significantly less than that produced on the autoclaved medium. The authors attribute this effect to radiomimetic effects produced by gamma-rays in the nutrient medium, because NATARAJAN and SWAMINATHAN (1958) observed that wheat (*Triticum aestivum*) embryo's cultured in X-ray irradiated White's medium gave chromosome breaks and chromatid breaks in the metaphase of the mitosis of root-tip cells. SWAMINATHAN et al. (1963) and RINEHART (Report of the Working Party on Irradiation of Food, 1964) also mentioned genetic changes in the fruitfly *Drosophila melanogaster*, when this organism was fed on irradiated medium.

HOLSTEN et al. (1965) produced evidence that there is an indirect effect of irradiation on the growth of carrot (*Daucus carota*) tissue in vitro. Analyses demonstrated that this effect was mediated by the sucrose moiety in the nutrient medium. Further experiments with these biologically active substances produced by irradiation showed that they had an antimetabolic effect on the growth of plant tissues. Moreover, both in *Drosophila melanogaster* and in plant tissue (*Tradescantia* and *Vicia*), these substances induced changes in the mitotic and meiotic chromosome processes of the cells. These results were seriously criticized by GOLDBLITH (1966), who stated that the cytotoxic substances studied by HOLSTEN et al. (1965) were most probably 5-(hydroxymethyl)-furfuraldehyde and levulinic acid, which have already been known to be formed from sugar as a result of the heating process.

We have studied the growth of bacteria on irradiated sugar-containing nutrient media. Following irradiation with 250–500 Krads, there was a considerable breakdown of the sugar, making the medium too acid for the growth of bacteria (see radiolysis products of sugars in aqueous solution, section 7.1, p. 73).

In other experiments the growth of bacteria was tested on irradiated proteins.Only isolates from the skin of tropical fish (*Sardinella cameronensis*) were used which could grow on plain gelatin, i.e. pure gelatin (Difco Bacto gelatin (B 143) or Oxoid gelatin, Code No. L8) without supplementing sugar or salts. The bacteria were grown on a gelatin medium and 1 ml of bacteria suspension containing 6.10^5 bacteria per ml was plated on gelatin plates irradiated by gamma-radiation at doses of 100–500 Krads. In this case irradiation did not appreciably change the pH of the medium, since unirradiated controls and irradiated plates showed pH values of 6.2–6.4. The outcome of such an irradiation experiment using three micrococci (*Staphylococcus epidermidis* 4/16, *Micrococcus flavus* 4/6 and *Micrococcus morrhuae* 2/10) as inoculum is presented in Figure 10.

As evident from Figure 10, there is a sharp decline in the number of colonies per plate at the higher doses in *Micrococcus morrhuae*, giving at 500 Krads a reduction of approximately 60 per cent. For the studied *Micrococcus flavus* strain the reduction in colonies was far less pronounced, being only about 20 per cent at 500 Krads, while the *Staphylococcus epidermidis* strain was not affected at all. Thus, there is a difference in response of these bacteria to the irradiated medium. This response is mainly a bacteriostatic effect of the irradiated medium to which some bacteria are more susceptible than others. It was observed that the number of colonies increased and therefore the bacteriostatic effect decreased with prolonged incubation periods. Moreover, if irradiated plates were stored for several days and then inoculated, the inhibitory effect was far less pronounced or even absent. Apparently, the bacteriostatic radiochemical produced in the medium is rather unstable and it is destroyed with the passage of time.

8.2. *Foodstuffs*

Food microbiologists often use foods as a nutrient medium, because it is the closest approach of the actual condition in microbiological spoilage studies of the product.

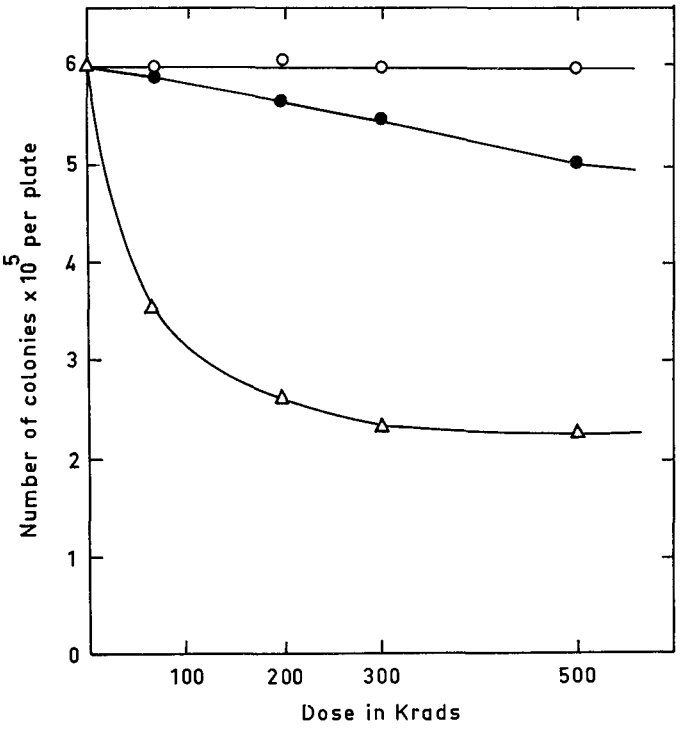


Fig. 10. Growth response of *Staphylococcus epidermidis* 4/16 (○), *Micrococcus flavus* 4/6 (●) and *Micrococcus morrhuae* 2/10 (△) on gamma-irradiated gelatin.

Moreover, these media produce better growth of micro-organisms with complex nutritional requirements. Pathogenic bacteria are often cultured in foodstuffs, because their behaviour in various foods is different and food is the main source of contamination. For example, *Salmonella* species are frequently cultivated in media such as crab, oyster, shrimp, or salmon (see Figure 1, p. 64).

For this reason it would be of considerable interest to know whether by means of irradiation a sterile product can be obtained or at least the number of micro-organisms in the product can be considerably reduced.

We have studied the effect of irradiation on the natural bacteria population of the tropical fish *Sardinella cameronensis* caught at the Ivory Coast, Africa. The main load of bacteria on the fish is to be found in the mucous layer of the skin. The flesh of the fish is completely sterile and the bacteria population of the intestinal tract is remarkably low, because the fish is, when caught, in spawning condition. The initial load of bacteria on the fresh *Sardinella* skin is $1-4.10^5$ bacteria per cm^2, which is more or less comparable with newly-caught Atlantic fish carrying bacterial loads of 10^2-10^5 bacteria per cm^2 of skin. The effect of irradiation (100–600 Krads) on the survival of the mixed bacteria population on the fish skin is presented in Fig. 11.

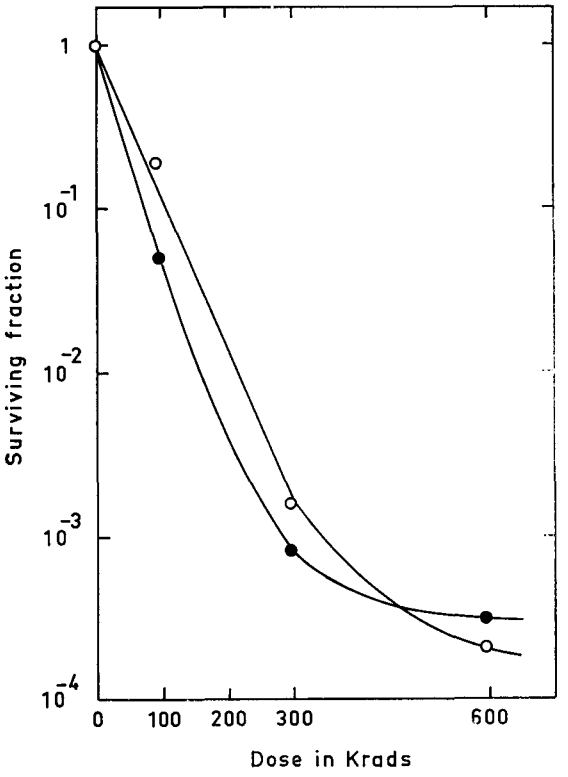


Fig. 11. Effect of radiation on the bacteria numbers of the skin of newly caught fish *(Sardinella cameronensis)* from the Ivory Coast, Africa.

Symbols: ○, bacteria counts at 10°C growth temperature; ●, bacteria counts at 23°C growth temperature.

As evident from this figure the D_{10}-value of the mixed bacteria population of the fish skin is about 100 Krads. Thus, 300 Krads gave a decrease in the bacteria population of the fish skin of 3 decades, i.e. 10^3 times. A sterile product cannot be achieved, since 600 Krads is not enough to kill all the bacteria present and there is already a considerable 'tailing' effect (see p. 63) with higher dosages. Moreover, the application of higher radiation dosages would lead to pronounced radio-chemical changes in the product making it unsuitable for its use as a nutrient medium.

Similar experiments were carried out with a vegetative product such as dried fruitpowders. They consist of fruit solids and drying aids like sucrose or corn sirup in various proportions. A reduction in bacteria numbers in this product is also of considerable technological importance, because it is used for human consumption. Moreover, the ethylene oxide gas-treatment used in practice to reduce bacteria numbers in these fruitpowders is likely to be forbidden by the Public Health authorities, because of the production of toxicants such as chlorohydrins and ethylene glycols. For this reason, radiation treatment may have advantages over other treatments.

Irradiation of a sugar-containing product in aqueous solution will result in radiolysis products of sugar and water (see section 7.1, p. 73). In aqueous solution sugar gave

appreciable deviations of colour and taste with as little as 400 Krads. Therefore, the sugar-containing fruitpowders were irradiated in air-dry condition. The initial number of bacteria in the fruitpowders differed depending their way of processing: vacuum-dried or spray-dried. The vacuum-dried fruitpowder contained significantly less micro-organisms ($2–3.10^2$ bacteria per g) than the spray-dried fruitpowder ($2–5.10^3$ bacteria per g). The bacteria present were mainly spore-forming *Bacillus* species. The relation between bacteria number and dosage is given in Fig. 12. The D_{10}-value for the vacuum-dried sample is 300 Krads and it is a little higher in the spray-dried sample. Only with 800 Krads a 100 times reduction in the initial bacteria flora was obtained. The considerable 'tailing' effect is probably caused by a protecting influence of the sugar surrounding the spores. A bacteria-free product cannot be obtained by irradiation, but the bacteria number can be reduced by the application of about 400–500 Krads, which dosage does not much affect the suitability of this sugar-containing product as a nutrient medium.

Radiation can also be applied to sterilize foodstuffs for agricultural purposes. For example, GROSSBARD et al. (1959) studied with a 4-MeV linear accelerator and a ^{60}Co-source the sterilization of grass samples (ryegrass and cocksfoot) in lacquered tins or polyethylene bags with irradiation dosages of 2.25 to 3 Mrads. It was observed, that irradiation itself did not cause any conspicious change in the overall appearance of the grass. However, a characteristic smell developed, being somewhat reminiscent of that

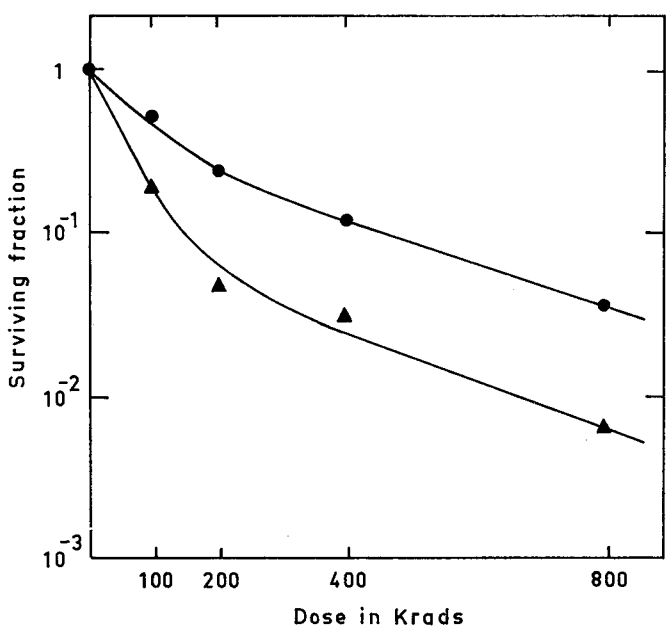


Fig. 12. Effect of radiation on the bacteria numbers in dried strawberry fruitpowder.
Symbols: ●, vacuum-dried fruitpowder; ▲, spray-dried fruitpowder.

of fresh hay. Also the acceptibility of the irradiated grass for sheep was not reduced. Examination showed that a considerable number of these grass samples having received the irradiation treatment had become sterile.

8.3. *Soils*

For the study of bacteria-mediated transformations in soils frequently sterile soils are used. Moreover, sterile soil is used in plant physiological studies to exclude the inference of bacteria. The sterilization or partial sterilization of soils by steam, chemical methods such as gases (e.g. ethylene oxide, carbon disulphide), desinfectants (e.g. formaldehyde, toluene) or fumigants (chloropicrin, 'D-D' (= dichloropropenepropane mixture) and others) all have some disadvantages, because the physical and chemical properties of soil are influenced or residual toxic effects are caused (WARCUP, 1957). Radiosterilization of soils may, therefore, be a new approach.

GRIFFITHS and BURNS (1968) observed that gamma-irradiation of soil did not significantly affect the stability of the natural soil aggregates. But when the soil was supplied with synthetic aggregates (i.e. polysaccharides), the stability of these aggregates was significantly reduced. This reduction was found to be negatively correlated with the clay content of the soil.

BOWEN and ROVIRA (1961) studied plant growth in irradiated soil as compared to plant growth in heat-sterilized and propylene-oxide-treated soil. With subterranean clover (*Trifolium subterraneum*), plant growth as indicated by sprout development was in most cases better in irradiated soil than in soil which had received the other treatments. There was, however, some suppression of root-hair formation and the growth response in one of the series suggested that irradiation of the soil is not without some toxic effects on the plants. These phytotoxic effects seemed to increase in the soils during storage, indicating that probably a continued enzyme activity may be responsible for this effect. Indeed, MCLAREN et al. (1957) observed an appreciable enzyme activity in soils after gamma-irradiation, especially with regard to urease activity.

Recently, CAWSE and WHITE (1969) reported a rapid increase in nitrite in gamma-irradiated soils (0.25–2.5 Mrads) and the nitrite formation was found to be correlated with the percentage of soil inorganic carbon and with the incubation time. As is well-known nitrite is very toxic to organisms, including bacteria.

The effect of gamma-irradiation on the sterility of soils was investigated by MONIB and ZAYED (1963). They observed that a dosage of 1 Mrad was not sufficient to sterilize soil completely, because micro-organisms could still be isolated from these soils. A similar result was obtained by ERNST (1965) with a soil from Herrenhausen (near Hannover) in Germany (see Fig. 13) irradiated with 1 Mrad, but a dose of 5 Mrads seemed to kill all micro-organisms present.

It is likely that this high dosage will cause a considerable breakdown of the organic substances present in the soil, but this has not yet been investigated.

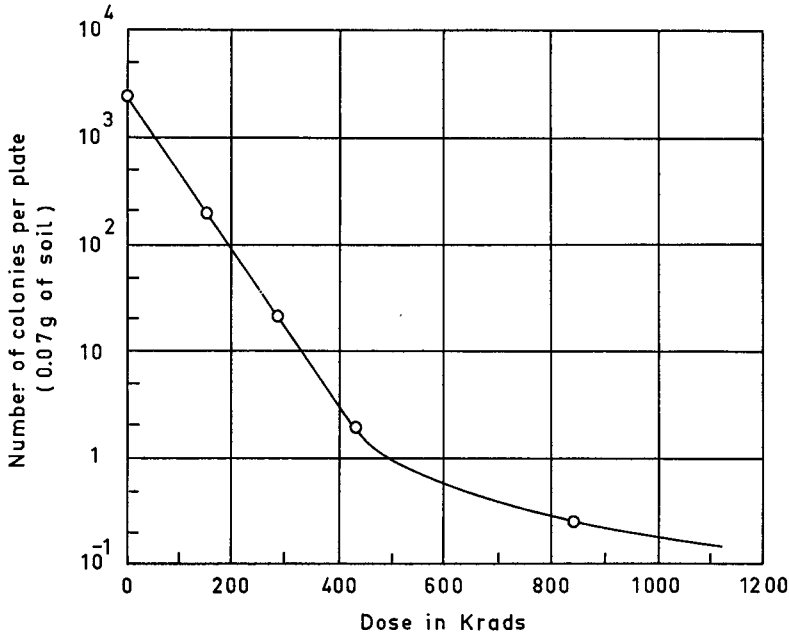


Fig. 13. Effect of radiation on the survival of bacteria in soil. The soil was a loamy sand soil from Herrenhausen near Hannover, Germany (Revised after ERNST, 1965).

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DISCUSSION

F. H. DE HAAS, and R. OKKERS: Which plastics are resistant to gamma-irradiation; e.g. PVC, nylon, PE, and latex rubber.

J. H. BECKING: According to a report of the Isotope Research Division of the AERE (Atomic Energy Research Establishment, Harwell, United Kingdom): 'Radiation Stability of Materials' (1965) and a report of the Instituut TNO voor Verpakkingen (Delft): 'De bestralingsbestendigheid van verpakkingsmaterialen' (1969) polyvinylchloride (PVC) is stable to a dose of 10 Mrads, but some HCl is evolved at the highest doses and it turns brown. The radioresistance of polyethylene (PE) films seems to be much higher. Nylon 6 ('Enkalon') and Nylon 11 ('Rilsan') are very radioresistent. From Nylon 6 it is known that it hardens at high dosages (100 Mrads). In rubbers the stability is influenced by the nature of the antioxidants present. Polyurethane rubber is the radiation-stablest rubber (100 Mrads). Natural rubber, SBR Butadiene styrene rubber and Nitrile rubber have a good stabiltity, but Butyl rubber becomes fluid at comparatively low doses.

It should be borne in mind that radioresistance is not the only factor involved with the application of a polymer film as packaging material. For instance, when preparing packaged, ready-to-use media the gas and moisture permeabilities of the film after irradiation are important.

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CHEMICAL STERILIZATION, WITH SPECIAL REFERENCE TO ETHYLENE OXIDE

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SUMMARY

The use of chemical sterilization methods is mainly restricted to thermolabile objects which cannot be effectively sterilized in any other way, because in many instances either the reactivity of sporicidal compounds prohibits their use, or effective neutralization after sterilization is impossible, or the process is not sufficiently reliable in the given circumstances. These difficulties particularly arise when liquid sterilants are used. Gaseous sterilization by means of ethylene oxide however is increasingly used because the gas has relatively favourable properties in this respect. The sterilizing effect of ethylene oxide depends on concentration, temperature, exposure time and the presence of sufficient humidity. Ethylene oxide sterilization should be adequately monitored which includes a presterilization treatment of the objects to be sterilized, proper bacteriological control and a quarantaine period for those objects that may absorb harmful quantities of the gas. A limited number of substances is inactivated by ethylene oxide, and others tend to form toxic reaction products. Provided sufficient attention is paid to these drawbacks, ethylene oxide sterilization is the method to be preferred for thermolabile instruments and materials.

1. INTRODUCTION

Substances having a killing or growth inhibiting effect on micro-organisms can be classified either by their origin, chemical nature, mode of action or by the purpose for which they are mainly employed. Using the latter criterion as a basis for classification antimicrobial compounds can be divided in groups used for disinfection, sterilization, conservation or therapeutic purposes. A particular agent may be suited for more than one group, depending on the conditions in which the antimicrobial action is required.

A disinfectant can be defined as a chemical compound which under acceptable circumstances will kill the harmfull micro-organisms present on or in an object that has to be safeguarded. The choice of a disinfectant and the concentration-time ratio required to obtain the necessary effect, depend on (1) the type of micro-organism to be killed, (2) the environment in which disinfection will take place and (3) the object to be disinfected.

The sensitivity of different types of micro-organisms to disinfectants widely varies. Furthermore, the variation depends on the type of disinfectant used. In general, spores show the highest degree of resistance against both fysical and chemical adverse

influences. Only a restricted number of disinfectants has reliable sporicidal properties. Chemical sterilization of objects with a bacterial contamination of unknown nature, therefore, requires the use of sporicidal disinfectants. Most of these are only active as liquids, some however are effective in gaseous form.

Environmental factors influencing the activity of disinfectants are: temperature, pH, ion concentration and the presence of protecting or inactivating substances.

The nature of the object to be disinfected usually limits the choice of an appropriate disinfectant, especially when a sporicidal effect is required. Possible disinfectant-residues should be removable.

Sporicidal disinfectants are seriously limited in their usefulness for sterilization purposes of liquids with biologically active components, because of the irreversable changes that may occur in the latter. For the sterilization of media intended for the cultivation of micro-organisms, therefore, they are only incidentally used. Furthermore, adequate neutralization of the disinfectant is usually impossible. Thermal sterilization and sterilization by filtration are the best methods for media and other liquids with biologically active ingredients.

Sterilization of thermolabile objects, however, largely depends on chemical methods. The choice of the disinfectants to be used will have to be based on the considerations outlined above. Some frequently used compounds are formaline, halogens, phenolic derivates, glutaraldehyde, peracetic acid and a number of gaseous agents such as ethylene oxide, formaldehyde and betapropiolacton. Generally, gaseous disinfectants have the advantage that no hazardous aseptic handling is required after sterilization to rid the object of disinfectant residues and their use allows the object to be packaged prior to sterilization, so that sterility is kept for a considerable length of time. Of the gaseous disinfectants only ethylene oxide has reached large scale application in various fields.

2. Ethylene oxide

Ethylene oxide is a gas at room temperature (boiling point 10.7 °C), it is explosive when present in concentrations of 3–80 % in air or oxygen and in this respect has been compared with coalgas and diethylaether. It is toxic to a certain degree, the maximum allowable concentration for prolonged exposure is 50 ppm (Jacobson, Hackley and Feinsilver, 1956). Ethylene oxide reacts with all kinds of chemicals such as amides, organic acids, alcohols and mercaptans. It has a rapid killing effect on all forms of micro-organisms, bacterial spores included. The concentration-time ratio required to kill spores is approximately 5 times the ratio necessary to kill vegetative bacteria, which is exceptionally low as compared to most of the liquid disinfectants. Ethylene oxide has excellent penetrating properties with regard to paper, rubber, leather and most plastics.

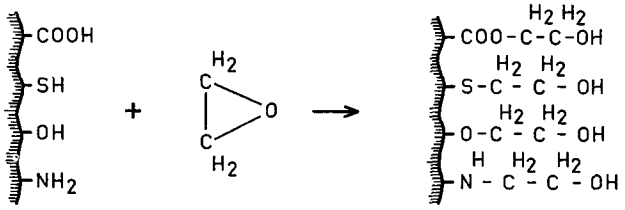

Fig. 1. Probable mode of action of ethylene oxide: alkylization of essential chemical groups within the cell.

2.1. *Mode of action*

The antimicrobial action of ethylene oxide is due to its alkylating effect on essential groups of DNA, enzymes etc. (fig. 1). Desiccation, especially in the presence of salts and/or proteins may render micro-organisms resistant to ethylene oxide. This is probably caused by a lack of free water molecules in the cells and their direct vicinity, which prevents the above mentioned reactions to take place. The inactivation of micro-organisms by ethylene oxide proceeds logarithmically; the speed of the process is dependent on the ethylene oxide concentration, temperature and type of micro-organims present, while sufficient humidity is necessary and desiccation of the micro-organisms must be avoided.

2.2. *Concentration*

Within certain limits the doubling of the gas-concentration reduces the necessary sterilization time by half. The lowest ethylene oxide concentration allowing reliable sterilization to take place is approximately 500 mg/l (OPFELL, HOHMANN and LATHAM, 1959; WEYMES, 1966). Increasing the concentration to more than 900 mgL has little effect at temperatures above 30 °C (WEYMES, 1966). For sterilization procedures in hospitals short cycles (in the order of one hour) are preferred with the use of concentrations of ethylene oxide up to 1500 mg/l at 50–60 °C. Industrial sterilization is usually carried out at lower concentrations with correspondingly longer sterilization periods.

2.3. *Temperature*

Below 30 °C, a 10 °C increase in temperature reduces the necessary sterilization time with a factor 2.8 (ERNST and SCHULL, 1962). In the range of 30–60 °C this factor is 1.8. Above 60 °C a further increase in temperature has relatively little effect and is usually not compatible with the thermolabile nature of the objects to be sterilized. It should be borne in mind that with sterilization at temperatures above ambient, considerable time may be needed for the load to reach the required temperature. Differences in temperature within the load can be avoided by preheating. In order to prevent desiccation of micro-organisms an adequate humidity should be maintained.

2.4. *Time*

The time required for ethylene oxide to obtain a 100 % kill depends, apart from concentration and temperature, on the nature of the objects to be sterilized. Spores on

smooth non-absorbent surfaces exhibit a less steep death curve as the same spores show on porous material (OPFEL, HOHMAN and LATHAM, 1959). The material of which the objects are made is particularly important in the case of hollow objects, where the ethylene oxide must first penetrate the wall of the object before any sterilizing effect can be expected. The sterilization of such objects, e.g. syringes, may require a considerable increase in exposure time (RUBBO and GARDNER, 1968). The same applies with regard to packing material with a low permeability for ethylene oxide. The influence of the different factors can be determined by means of suitably contaminated samples as shown in Table 1 (KAYSER, unpublished).

Table 1. Sterilization by ethylene oxide of *B. subtilis* var. *globigii* spore strips of varying accessability. Ethylene oxide: 1200 mg/l, temperature: 50 °C, exposure time: 60 mins.

Location and method of packaging of strips	Number of spores per strip		
	10^7	10^5	10^3
in open culture tube	10[1]	n.t.[2]	n.t.
in culture tube packed in double p.e.[3]	10	n.t.	n.t.
in 50 cm long capillary glas tube, packed in double p.e.	10	10	n.t.
between barrel and plunger of polypropylene syringe, packed in double p.e.	0	1	4
in hermetically sealed rubber tubing, wall thickness 2 mm	0	0	3

[1] number of strips sterile of 10 strips tested
[2] not tested
[3] polythene foil, thickness 0.03 mm

2.5. *Humidity*

As mentioned earlier, dehydration can render micro-organisms resistant to ethylene oxide. The presence of salt enhances the effect (GILBERT, 1964; ROYCE and BOWLER, 1961). Resistance is restricted to a small part of the micro-organisms present, the majority being killed at the ratio to be expected under normal conditions (fig. 2). Direct moistening of the objects carrying the test organisms immediately restores their normal susceptibility to ethylene oxide. This can also be effected by storage in an atmosphere of high relative humidity but it may take several days. Therefore, care should be taken to avoid dehydration with any pre-sterilization-treatment of objects to be sterilized with ethylene oxide. During the sterilization process a minimum relative humidity of 30% should be maintained.

2.6. *Sterilization cycles*

The forming of explosive ethylene oxide-air mixtures can be avoided either by using ethylene oxide mixed with an inert gas rendering it inexplosive (CO_2 of fluorinated hydrocarbons, Table 2), or by maintaining sub-atmospheric pressure throughout the sterilization process so that any leak that may occur will be inwards. The gas is

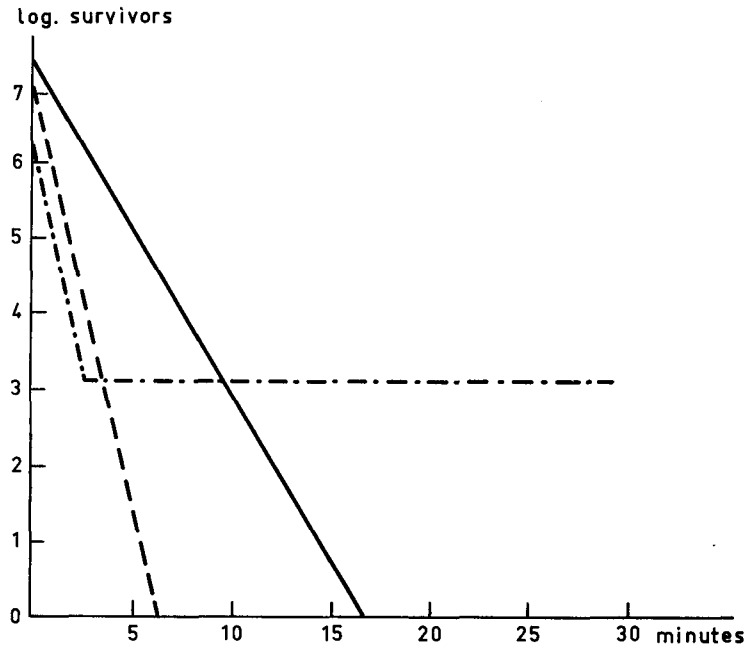

Fig. 2. Influence of desiccation and salts on the survival of *S. aureus* after treatment with ethylene oxide (1200 mg/l, temp. 50 °C).
——— *B. subtilis* var *globigii* spores, methanol suspension dried on aluminium foil.
– – – – *S. aureus*, suspension in saline with 10% calfserum, dried on aluminium foil at 50% rH.
.–.–.–.– *S. aureus*, broth culture, dried on aluminium foil at 10% rH.

Table 2. Some ethylene oxide mixtures used for sterilization purposes

Trade names	% e.o.	% inert gas	sterilization pressure
T-gas, Etox	90	10 (CO_2)	subatmospheric
Oxyfume, Steroxide-20	20	80 (CO_2)	atmospheric
Aeto	15	85 (CO_2)	3–6 atm
Carboxide, Cartox	10	90 (CO_2)	0–2 atm
Cry-oxide	11	79 (Freon 11) 10 (Freon 12)	0.3–1.2 atm
Pennoxide, Steroxide-12	12	88 (Freon 12)	0.3–1.2 atm

supplied in steel cylinders or in single use cartridges for smaller types of equipment designed for hospital or laboratory use. In most countries the storage and handling of ethylene oxide is subject to official regulations.

The sterilization cycle consists of an initial vacuum with or followed by preheating and humidification of the load, subsequent introduction of ethylene oxide to a pre-

Table 3. Some examples of ethylene oxide sterilization cycles used in commercially available sterilizers.

concentration of ethylene oxide (mg/l)	temperature (°C)	time (hours)
1000	25	6–12
1200	50	1–3
600	50	3–6

determined concentration, which is maintained during the actual sterilization phase, after which the sterilizer chamber is evacuated and filtered air is admitted to atmospheric pressure. By repeated evacuation and aeration the elimination of ethylene oxide from sterilized materials is accelerated. Some examples of actual sterilization cycles are shown in Table 3. The choice of the most suitable process will depend on the characteristics of the materials to be sterilized, on the time available to do it and, in industrial sterilization, on considerations of economy.

2.7. *Control procedures*

For the control of ethylene oxide sterilization a variety of chemical indicators has been developed. With the use of impregnated paper strips or selfadhesive tapes which show a colour change after contact with ethylene oxide, no other conclusion can be drawn than that contact has taken place, but it is impossible to say if sterilization conditions have been fulfilled. ROYCE and BOWLER (1959) reported on the use of polyethylene sachets filled with a saturated salt solution containing hydrochloric acid and an indicator. A colour change is indicative of a minimum time-concentration product, but in our experience it has not been found to be a sufficiently reliable method. The ethylene oxide concentration can be determined by means of gas-chromatography and titration but for practical reasons application of these methods is limited to monitoring industrial sterilization. Even an accurate determination of all variables involved does not allow a definite conclusion as to the sterility of the processed load if the characteristics of this load are not taken into account. The best way to achieve this is to distribute strategically throughout the load a number of artificially contaminated samples carrying a known quantity of micro-organisms with known resistance to ethylene oxide. Test objects of this type have been described by BEEBY and WHITEHOUSE (1965) and BREWER (1966). They consist of aluminiumfoil or filter paper strips usually contaminated with 10^5 to 10^6 spores of a *Bacillus* (Table 4). *Bacillus subtilis* var. *globigii* or *Bacillus stearothermophilus* are commonly used. The former is somewhat more resistant to ethylene oxide, the latter has the advantage that it will grow at 60 °C which practically eliminates the possibility of false positive tests due to accidental laboratory contamination. Spore strips of this kind have an almost unlimited shelf life permitting a large number of strips to be prepared at the same time with a known and constant resistance to ethylene oxide.

Table 4. Ethylene oxide sterilization of *B. subtilis* var. *globigii* spore strips with varying numbers of spores

Aluminium foilstrips in culture tube packed in double polythene foil of 0.03 mm thickness. Ethylene oxide: 1200 mg/l, temperature: 50 °C.

number of spores per strip	exposure time		
	5 min	10 min	20 min
10^7	0[1]	5	10
10^6	0	4	10
10^5	2	8	10
10^4	5	10	10
10^3	6	10	10

[1] number of sterile strips of 10 strips tested

The use of *Staphylococcus aureus* for the control of ethylene oxide sterilization should be discouraged as it may easily lead to false positive and false negative results. Earthspores also are unreliable in this respect for the same reasons (STRUPPE 1969).

2.8. *Packing material*

Paper, some types of plastic and combinations of them are suitable to use as packing materials for articles to be sterilized by ethylene oxide. The permeability for ethylene oxide of different types of plastics shows a great variation (Dick an FEAZEL, 1960). Polyethylene of low density has a high permeability for ethylene oxide, it has excellent mechanical properties, it is bacteria resistant and moisture proof, it is readily available in various widths and gauges in the form of bags and tubing, and it is cheap. Other types of plastic should only be used if their permeability for ethylene oxide is known and the sterilizing cycle correspondingly geared. Hermetically sealed polythene tubing or bags tend to burst during sterilization if a low vacuum is reached within a short time. This can be prevented by incorporating a strip of filtering material in the seal such as cotton wadding or tissue paper. The peel-open type of packing commonly employed for disposable hospital products (e.g. polypropylene-polyester foil sealed on paper) is particularly suitable as it allows free passage of air and ethylene oxide and facilitates aseptic handling of the object.

2.9. *Ethylene oxide residues*

Plastics and rubber can retain considerable quantities of ethylene oxide after sterilization which may cause toxic effects at subsequent use (ROYCE and BOWLER, 1959; GIBSON, JOHNSTON and TURKINGTON, 1968). Residual ethylene oxide dissipates into surrounding atmosphere. This can be stimulated by increasing the temperature and by adequate ventilation, thus shortening the necessary quarantaine period for plastic and rubber objects. It is generally advised to apply a quarantaine period of 2–4

days for those plastic or rubber objects which will remain in direct contact with living tissues, e.g. pacemakers, endotracheal tubes, intravenous catheters. It gives a fair safety margin when storage takes place at room temperature. Storage at 50 °C with sufficient ventilation (e.g. in a gas aerator designed for the purpose) allows the quarantaine period to be reduced to less than 24 hrs for most objects.

2.10. *Toxic substances*

Ethylene oxide sterilization of certain materials may lead to the formation of toxic substances such as ethylenechlorhydrin, which has been found to be present in various foodstuffs after ethylene oxide sterilization (WESLEY, 1965). The same phenomenon has occured with polivinylchloride catheters previously sterilized by gamma irradiation. The process supposedly liberates enough reactive chlorine to form chlorhydrin on subsequent resterilization with ethylene oxide (CUNLIFFE and WESLEY, 1967). Toxic substances formed as a result of ethylene oxide sterilization can be detected by animal implantation tests. Tissue cultures are unsuitable for this purpose as they are sensitive to toxic influences which cause no harm in vivo (BAIN and LOWENSTEIN, 1968). Ethylene oxide may hydrolyze to form ethylene glycol. This toxic substance has been found in small amounts in certain foodstuffs sterilized by ethylene oxide. Several vitamins are made inactive by ethylene oxide (riboflavin, nicotinic acid, folic acid). This is also the case with some essential amino acids. Of the antibiotics streptomycin loses 30–40 % of its activity. A wide range of other compounds can safely be sterilized with ethylene oxide without loss of biological activity.

2.11. *Applications of ethylene oxide*

Ethylene oxide has been employed in the sterilization of nearly all sorts of dry substances and objects which are supplied in sterile condition by industry, such as pharmaceutical products, single-use articles for medical application, human and animal foodstuffs. The choice of the sterilization method in those cases is based on economical rather than bacteriological grounds. For the sterilization of thermolabile materials and instruments in hospitals and laboratories ethylene oxide sterilization is the method to be preferred, provided that sufficient knowledge of the practical possibilities and limitations of ethylene oxide sterilization is available.

3. OTHER GASEOUS STERILANTS

Propylene oxide is less active against micro-organisms than ethylene oxide but it has the advantage of having a higher boiling point (34 °C), which makes it more convenient to handle. It is applied on a small scale in the sterilization of foodstuffs, where its use has been encouraged because of the relative non-toxicity of the hydrolytic product propylene glycol as compared with that of ethylene glycol. Propylene oxide requires the same humidity conditions as ethylene oxide to be an effective sterilant.

Formaldehyde is highly active against all forms of micro-organisms, but its penetrating quality is poor, its rapidly polymerizes into the inactive paraformaldehyde, requires at least 70% relative humidity, is toxic and difficult to remove. However, it is succesfully used in combination with steam at subatmospheric pressure of approximately 80 °C to sterilize heat sensitive instruments (ALDER, BROWN and GILLESPIE, 1966).

Beta-propiolactone is a very reliable antimicrobial agent, but it has low penetrating powers. Like formaldehyde it requires a high relative humidity to be effective. Its suggested carcinogenic properties have restricted its use. A number of other gases have strong bactericidal properties but their application is limited to special circumstances because of their corrosiveness, instability or toxicity and they are not suitable for general sterilization purposes.

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DISCUSSION

H. M. DEKHUIJZEN: What is known about the effect of ethylene oxide sterilization on vitamins and plant hormones?

A. M. KAYSER: Inactivation of riboflavin, nicotinic acid and folic acid by ethylene oxide has been reported. Ethylene oxide appears to have an inhibitory effect on the ripening of fruit and flowers contrary to the effects of ethylene (LIEBERMAN, ASEN and MAPSON, Nature **204**, 1964: 756).

R. L. M. PIERIK: The optimal concentration of alcohol employed for surface sterilization is generally taken to be 70%. Can this be confirmed?

A. M. KAYSER: The time required to kill vegetative bacteria decreases with increasing alcohol concentration, provided the bacteria have a normal moisture content. Alcohol 70–80% (W/V) is superior to alcohol 96% in killing dried out bacteria, the difference however is slight. For skin disinfection 70% and 96% alcohol are probably equally effective. It should be remembered that alcohol does not kill spores and some types of viruses.

FILTER STERILIZATION

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SUMMARY

Membrane-filters act essentially as two-dimensional screens and remove all particles,microorganisms and viruses larger than the specific filter pore size from fluids flowing through. They have an absolute particle size rating, no matter how large or how long the filtration run.

As the presence of very small quantities of a wetting agent (present in all existing membrane filters) may influence growth characteristics during the production by tissue culture methods of certain highly sensitive cells, filters free of a wetting agent can be supplied on request.

Retention of viruses by membrane-filters is due to two phenomena:

The first is the retention by physical restriction which occurs when the pore diameter of the membrane is smaller than that of the virus particle.

The second way of retention occurs through adsorption of viruses on the filters. Adsorption can be avoided precoating filters with proteinaceous material. Viruses adsorbed on filters can be eluted by flushing with membrane-coating components.

1. STERILE FILTRATION OF SERUM AND TISSUE CULTURE MEDIUM ON MILLIPORE FILTERS

1.1. *Introduction*

Millipore technology is based on the development of tools and techniques for making precision separations in laboratory and industrial process-fluids.

The technology encompasses basic separation techniques, the manufacturing of products designed for specific uses, application procedures developed at Millipore and also in close cooperation with customers, and finally systems which integrate these techniques, products and procedures.

The use of membrane-filters has been very important in the development of many new products during recent years, and has helped in lowering the cost and improving the quality of a number of others. Before the introduction of membrane-filters, for instance, an economical or reliable method for sterilizing heat-labile biological solutions, such as serum and tissue culture medium containing serum, did not exist.

1.2. *Sterilizing filters – Depth versus screen*

1.2.1. Depth filtration

Depth filters usually consist of fibers, particles or fragmented material of some kind that has been pressed or bonded together to form a tortuous maze of flow passages.

The principal mechanisms of retention, particulary for very small particles as bacteria, are random adsorption or entrapment. Depth filters cannot be relied upon to remove all microorganisms larger than a specific size because it is impossible to set definite limits to the size of particles which may pass. They have a nominal, not an absolute rating. Because depth filters have a non-homogeneous matrix, pieces of filter material can and do break off, which contaminates the filtrate. Media migration is an inherent characteristic of all depth filters.

1.2.2. Screen filtration

Membrane-filters are screen-filters. They are characterized by highly uniform, regularly spaced holes and they have a high capacity for particle retention. A 'sterilizing' screen-filter will retain on its surface all bacteria, without any chance of one passing through. Screen-filters are not prone to media migration because they are homogeneous and continuous.

1.3. *Basic characteristics of millipore filters*

Millipore filters are formed from thin sheets of homogeneous polymers and the precision of pore diameters is closely controlled during manufacturing and through stringent quality control procedures as well. In a given filter, all pores will be exactly the same size.

1.3.1. Porosity – Absorption – Adsorption

Millipore filters are extremely porous, with as much as 84% of their surface area taken up by pores, allowing very high flow rates. These filters are very thin (about 150 microns), so that little fluid is retained within the filter matrix. Adsorption from a solution by a membrane-filter generally is negligible. Potency or titre are unaffected. There are few exceptions which chiefly occur with virological applications.

1.3.2. Purity

Cellulose esters are biologically inert and will not cause tissue reactions during implantation in animals and humans.

All membrane filters from various manufactures contain a detergent. In the case of Millipore filters the detergent is Triton X-100 (iso-octylphenoxypolyethoxyethanol), which is present in an extremely small amount.

For the filtration of the tissue culture media used for the culturing of a few particulary fastidious cell strains, Millipore filters without a wetting agent are preferred. They are available, but only on special request.

1.4. *Sterilizing filtration*

The GS (0.22 microns) filter is recommended for all sterile filtration applications. The GS is known to be capable of removing all bacteria from solutions. The reason why 0.22 microns are necessary is the fact that bacteria up to 0.30 microns (*Pseudomonas*) are often found in blood fractions such as serum, gamma globulin, plasma etc.

The GS filter will be sterilized in place in the filter holder. Sterilizing can be done by autoclaving or by ethylene-oxyde sterilization.

A certain number of precautions should be taken – in particular when filtering tissue culture medium – to avoid negative results in culturing:

When filtering serum or a tissue medium containing serum, a vacuum system should never be used. Vacuum will cause foaming and denaturization of the protein.

All pressure systems should be pressurized with nitrogen. While the choice of gas is optional, air should not be used because nearly all pressurized air contains some pump lubricating oil vapor, which can affect the performance of the medium.

All tubing must be compatible with the filters when autoclaved with the filter-holder. As a general rule, only pure gum rubber hose and no latex or surgical latex hose should be used. Certain other types are also permissible (such as Teflon or silicone rubber) but they should be tested prior to use.

All tubing should be rinsed out, coiled in a pan of water and autoclaved one cycle prior to its first use. This will remove talc and extractibles that could affect media.

Autoclaves have long been known to be a source of toxic materials if they use steam from a central source. Boiler cleaning-compounds appear to be the primary source of the trouble. Autoclaves that produce their own steam from deionized water largely eliminate the problem. It seems that there remains some concern, however, about the type of stainless steel used in the construction of the self-contained autoclave boilers.

Another problem may occur with the installation of new stainless steel pipes which must be acetone-cleaned to remove oil. Pipe-sealing compounds also have been found a source of toxic materials.

Deionize-columns, if not very carefully maintained can produce water which is inferior to tap water. Such columns, used in producing high volumes of water for glassware cleaning, can in fact become excellent nutrient beds for bacterial growth. Bacterial counts of deionized water sometimes show very high figures.

2. Retention of viruses with membrane filters

2.1. *Introduction*

Millipore GS filters will retain all bacteria by physical restriction. Particles smaller than 0.22 microns can pass through the membrane. Untreated membrane filters retain viruses by adsorption as well as by physical restriction which occurs when the pore diameter of the filter is smaller than that of the virus particle. As originally recommended by Elford, membranes have to be pre-treated with proteinaceous material in order to preclude virus adsorption. However, coating materials that prevent adsorption of particular viruses do not necessarily prevent adsorption of others.

In contrast to proteins, salts enhance virus adsorption. Viruses treated with sodium lauryl sulfate to reduce the surface tension, or purified viruses in distilled water, are not adsorbed to membranes. A procedure is recommended in which viruses may be

passed through membranes with a porosity twice the diameter of the virus. Such filtrates which contain 50 to 100% of the initial virus concentration, could be used for sizing viruses by subsequent filtration through smaller pores. The determination of virus size would then be based on the major population of particles in the virus suspension. In the past, as little as 0.1 to 0.001% of the initial virus population was the basis for size determination, because often more than 99.9% of the virus was lost by adsorption to membranes during the filtration process. The following test, executed by Binie illustrates the theory described above.

2.2. *Materials and methods*

Viruses and their assays. Type 1 poliovirus (Mahoney), vaccinia virus (WR), herpesvirus (JES), and rubella virus (WER) are used in these experiments.

Monkey kidney (MK) cells. Kidneys from immature green monkeys were trypsinized and grown in Melnick's medium A and maintained with medium B.

Since the medium in which viruses are suspended plays an important role in the adsorption of viruses to membranes, cell viruses used in this experiment were grown in MK cells maintained with medium B (lactalbumin hydrolisate in Earle's salt solution). Viruses were assayed by counting plaque-forming units (PFU).

Overlay medium consisted of Earle's salt solution 0.4% $NaHCO_3$, 1:60,000 neutral red, 0.1% skim milk, and 1.5% Bacto-agar (Difco). Additives used in overlays for plaque enhancement of poliovirus were 25 mM $MgCl_2$ and, for herpesvirus, 400 μg/ml of protamine. Rubella virus was assayed by the interference method and by using Newcasttle disease virys (NDV) as a challenge. After challenge with NDV, sheep erythrocytes were added to the cultures, and clear plaques, to which erythrocytes did not adsorb, were counted.

Membrane filters. Millipore filters used in this experiment were 25 mm in diameter, and they were set in micro-syringe filter holders.

Membrane coating agents. Materials used to treat membranes were prepared as follows: A 100 ml amount of 10% foetal calf serum in distilled water was filtered at 25 psi through a 90 mm AP 20 micro-fiber pre-filter and then through PH (0.3 μ), GS (0.22 μ), VC (0.1 μ) and VM (0.05 μ) membranes in series. To the final filtrate, 10 × Earle's saline was added in sufficient quantity to restore isotonicity. The filtrate was then used to treat membranes prior to virus filtration. It was capable of coating but not clogging membranes subsequently used for virus filtration. Veal infusion broth (Difco) was constituted as described by the manufacturer and used without dilution. It was clarified as described above. Bovine plasma albumin (Armour) was made up to a 1% suspension in Tris-buffer and used after clarification as described above. Normal monkey kidney cultures were frozen and thawed to simulate a virus harvest, and the material was clarified as above.

Sodium lauryl sulfate (SLS). A 10% suspension of SLS was made in distilled water and adjusted to pH 8.0 with HCL. The stock was then clarified through a GS membrane to remove insoluble particles.

Table 1. Coating agents used for pre-treatment of membrane filters with porosity twice the virus diameter.

Virus	Membrane porosity	Percentage of virus recovered[1] in filtrate from membranes treated with:				
	(mμ)	Tris[2]-buffer	10% Foetal calf serum	Veal Infusion broth	Bovine albumin	Cell extract
Polio	50	0	100	100	100	100
Vaccinia	650	0	40–50	20	40–50	20
Herpes	300	0	100	30	60	0
Rubella	100	0	100	25	100	10

[1] Approximately 100 plaque-forming units of virus were filtered for each test.
[2] Tris(hydroxymethyl)methylamine

2.3. *Results*

It was found in orientating experiments that different coating materials did not always effectively prevent virus adsorption. Therefore, the following experiment was performed to determine the most effective membrane-coating materials for the four types of virus used in the experiment.

Viruses were diluted to contain 100 PFU/0.1 ml in Tris-buffer, which renders the virus sensitive to adsorption to untreated membranes. The diluted stocks were filtered through treated and untreated membranes with a porosity size twice the diameter of the tested virus. Membranes were first treated with 5 ml volumes of the reagents shown in Table 1, and were then washed with 5 ml of Tris-buffer to remove the residual fluids.

Samples of each virus (5 ml) were than filtered through 25 mm Millipore membranes with 1 psi. Finally unfiltered virus and virus filtrates were assayed simultaneously. The result is shown in Table 1.

No detectable virus was found in filtrates of untreated membranes or of membranes treated with Tris buffer. Poliovirus was effectively passed through all treated membranes without decrease in titer. The results were not as uniform, however, in the case of the other viruses tested. Vaccinia virus, herpesvirus, and rubella virus were not significantly adsorbed to membranes treated with serum or albumin. So the sites that bind these viruses are possibly those coated with albumin or other serum proteins.

It is noteworthy that vaccinia virus could not be quantitatively recovered in the filtrates. The decrease in titer was undoubtedly due to aggregated virus.

2.4. *Conclusion*

In the process of the filtration of a virus suspension through a series of untreated membranes with pore diameters at least twice the diameter of the virus, membrane-coating components adsorb to the membrane-filter.

Membrane-coating components contained in virus harvests are made up of organic, cell-associated materials and serum. When they occupy sites on membranes, they block virus adsorption. When membrane-coating components are added to a membrane containing adsorbed viruses, the virions are rapidly eluted and replaced by membrane-coating components.

Virions suspended in salt solutions, free of organic material adsorb to membranes, whereas, in distilled water, the virions pass through the membrane pores and are found in the filtrate.

If enteroviruses adsorb to membranes during the early phase of a filtration procedure, membrane-coating components in the suspension will subsequently elute the virus into the filtrate, and the adsorption phase may go unnoticed.

However, if suspensions of a lipophilic virus are filtered through a membrane, and the virions are adsorbed, membrane-coating components cannot elute these viruses. Thus, filtration of lipophilic viruses (even through large-pore membranes), under conditions in which the virions adsorb to membranes before the latter are coated with membrane-coating components, yields filtrates with reduced infectivity.

It has been emphasized that efficient filtration of viruses requires that membranes be pre-treated with a proteinaceous diluent that will adsorb to membranes and preclude virus adsorption. However, in the case of viruses which are resistant to the effects of detergents, the virus suspension can be treated with SLS (sodium lauryl sulfate) and can be filtered through untreated membranes without significant virus adsorption to the membrane. Viruses can also be diluted in distilled water in order to lower the salt concentration to below 0.002 M, and then filtered through untreated membranes without adsorbing to them.

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DEGRADATION OF SUGARS AND THEIR REACTIONS WITH AMINO ACIDS

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SUMMARY

The complex of reactions which involve the degradation of sugars and the reaction of sugars with amino compounds is generally called 'non-enzymic browning'. Such browning reactions play an important role in the processing of foods, and though browning is in some cases undesirable, it is usually highly desirable, and may be even essential, because of the formation of compounds with characteristic strong appealing flavours through such processes as frying, baking roasting and storage. It is via the low molecular weight flavour compounds that the high molecular weight colouring materials are formed.

An attempt is made in this paper to describe systematically the most important reactions in the overall browning process.

The relation between the products of caramelization and acid-catalyzed degradation of sugars as well as those resulting from the interaction of sugars with amino compounds (the Maillard reaction) is emphasized.

1. INTRODUCTION

An important but poorly defined aspect of carbohydrate chemistry concerns so called non-enzymic browning. Such browning involves a complex of reactions which take place when sugars are heated alone (caramelization) or with amino compounds (the Maillard reaction); this process plays an exceptionally important role in food technology (ANET, 1964; ELLIS, 1959; HODGE, 1953, 1967; REYNOLDS, 1963, 1965).

The effect of such changes (and I am thinking about the formation of brown materials in foods during processing) is sometimes undesirable. Usually, browning of foods during boiling, frying, baking, drying, and storage is highly desirable; in many cases the early stages of the reaction lead to the formation of low molecular weight compounds with powerful and attractive flavours: it is via these low molecular weight compounds that the brown high molecular weight materials (melanoidins) are formed. Many of the low molecular weight intermediates are well-known taste and odour materials and contribute to the character of the prepared food. These materials are in fact an indispensable part of our foods – consider such flavours as vanilla, chocolate, caramel, coffee, fresh bread, and fried meat. I should at this point just like to mention one of the undesirable effects of browning which is directly related to the theme of this

symposium. At the 19th Congress on Tissue Culture (Mexico, 1968), STEHSEL and CAPLIN (unpublished) reported inhibition of growth of material cultured in an autoclaved medium containing 2% fructose and 15% coconut milk. Growth was not inhibited when cell material was cultured in a medium of the same composition which had been sterilized by filtration. STEHSEL and CAPLIN suggested, without further experimental evidence, that browning reactions were involved. It is not my intention to give a comprehensive survey of the chemistry of browning. I shall restrict myself to the explanation of some of the aspects which fall within the framework of this symposium, and which I hope you will find relevant.

2. BROWNING BY PYROLYSIS OF SUGARS – CARAMELIZATION

It is well known that heating sugars for some time around the melting point (~ 160 °C) leads to the formation of odour and taste materials. As heating progresses the colour of the material changes from yellow-brown, through amber and orangebrown, to dark brown. Formation of caramel-like flavours is especially noticeable when the sugar has been heated to the stage when it has turned amber. The characteristic components are hydroxyacetone, dihydroxyacetone, acetoin, furan, 2-methyl-furan, 2,5-dimethylfuran, and especially the compounds shown in Figure 1.

Maltol and 5-hydroxymethyl-2-furaldehyde (HMF) are often considered as caramelization products of hexoses: in fact they are dehydration products the formation of which possibly proceeds as shown in Figure 2.

I would draw your attention, en passant, to:

The formation of methyl groups.

The formation of a cyclopentenone skeleton.

I shall return to these points later.

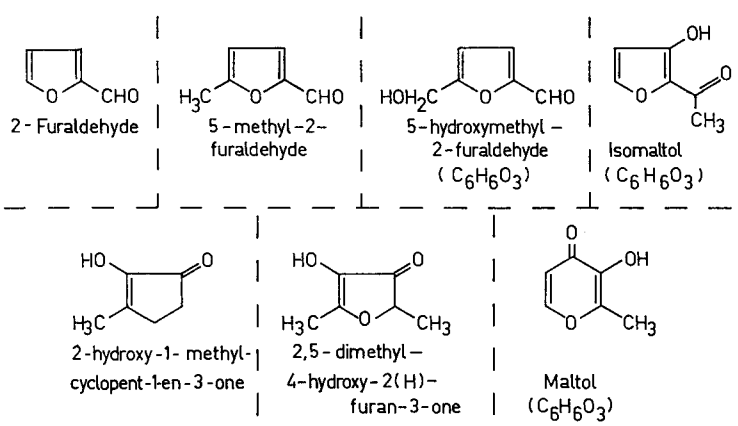

Fig. 1. Characteristic caramelization products.

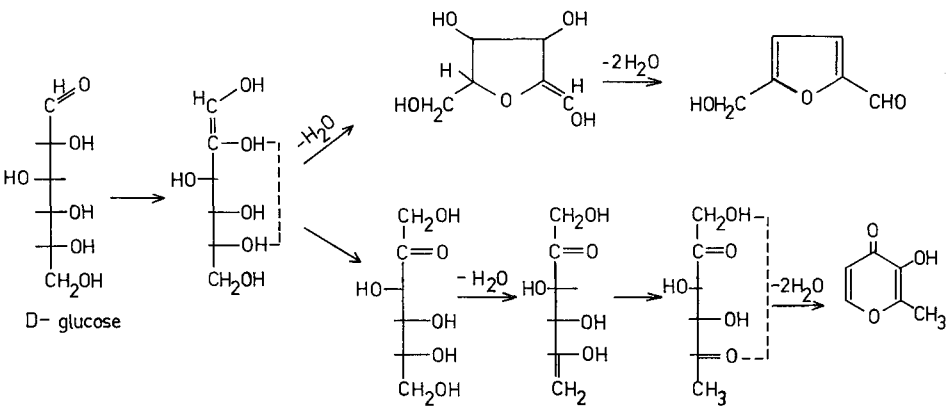

Fig. 2. Possible routes to HMF and maltol by pyrolysis of D-glucose.

3. Acid and base catalyzed degradation of sugars

Caramel flavours are often formed during the heat processing of foods, especially when the heat treatment is too severe (consider the effects of pasteurization of milk and fruit juices). Some foods also form such flavours during storage. BERRY and TATUM (1965) and TATUM et al. (1967) isolated the compounds responsible for the caramel flavour of stored orange powder. A number of these compounds are shown in Figure 3.

SHAW et al. (1967, 1968) assigned a dihydrofuranone structure to the sixth component in the list above, but on the basis of recent work by MILLS et al. (1970) as well as the work of VAN DEN OUWELAND and PEER we have good reason to believe this compound to have a dihydropyrone structure.

As shown in Figure 3, SHAW et al. (1967, 1968) found that many of the compounds were formed when D-fructose was heated in an acidic medium (and to a lesser extent in basic medium). It would thus appear that D-fructose is the precursor of these flavour materials. The formation of these materials probably follows the enolization – dehydration pathway outlined for HMF and maltol in Figure 2 (see also ANET, 1962b; HODGE, 1953; HODGE and RIST, 1953), and the alternative pathways shown in Figure 4.

It is interesting to note that GIANTURCO and FRIEDEL (1963) and GIANTURCO et al. (1964) found furfural, 5-methylfurfural and furfuryl alcohol as well as a group of hydroxycyclopentenones in coffee. The latter compounds have a strong caramel flavour. SHAW et al. (1967, 1968) demonstrated that these materials can be formed by the condenzation of hydroxyketones in alkaline medium as shown in Figure 5.

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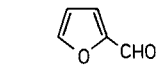
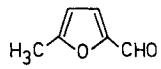
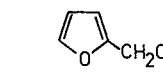
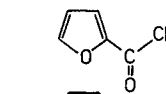
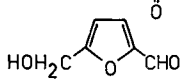
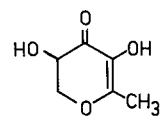
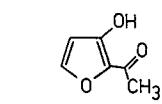
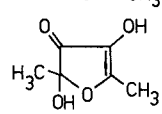
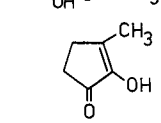
	X	
	X	
		X
	XX	
	XX	
	X	X
	X	
	X	
		X
α-and β-angelica lactone	X	

Fig. 3. Some compounds formed during storage of orange powder.

4. Interaction of sugars with amines – the maillard reaction

The most important reaction which occurs in the processing of carbohydrate-containing foods, for example in the baking of bread, is that between reducing sugars and amino acids, peptides, and proteins which leads to the formation of furaldehydes and further degradation products such as acetaldehyde, pyruvaldehyde and diacetyl. Many of these products, in particular the 1,2-diketones like pyruvaldehyde, in their turn convert amino acids to aldehydes (the Strecker degradation); pyrazines are formed as byproducts as shown in Figure 6.

This whole series of reactions between sugars and amino acids is referred to as the

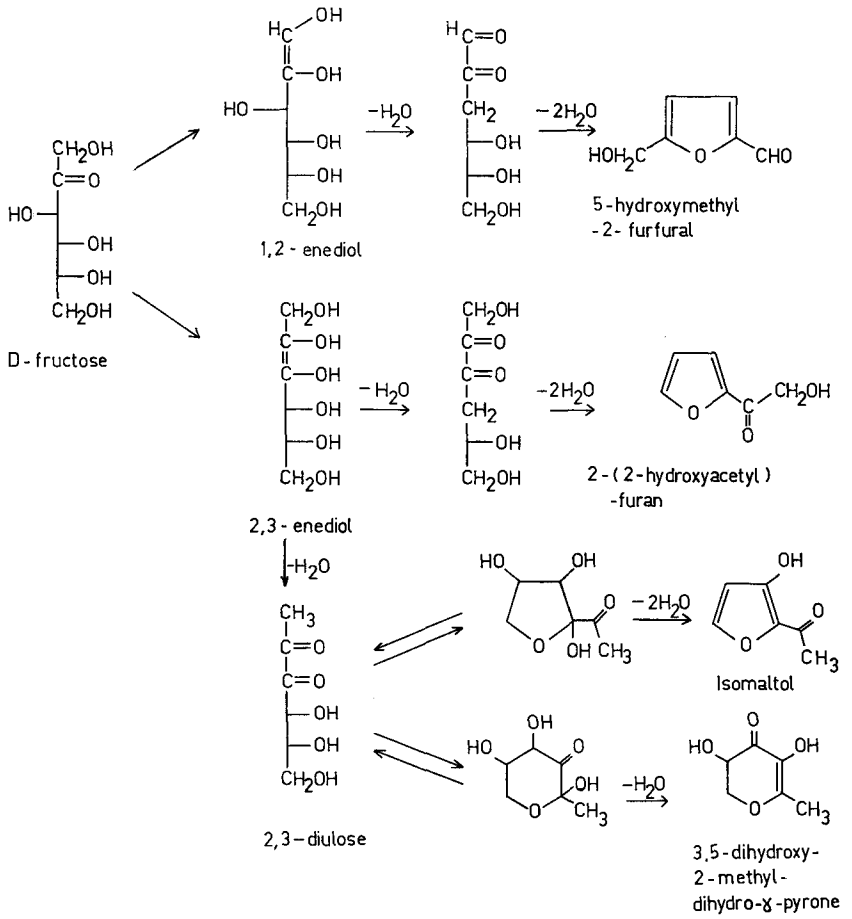


Fig. 4. Acid-catalyzed degradation of D-fructose.

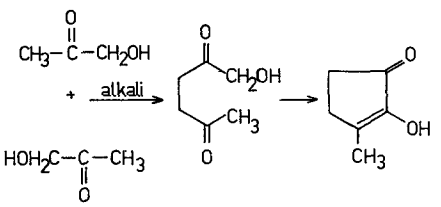


Fig. 5. Formation of hydroxycyclopentenones from keto-aldehydes.

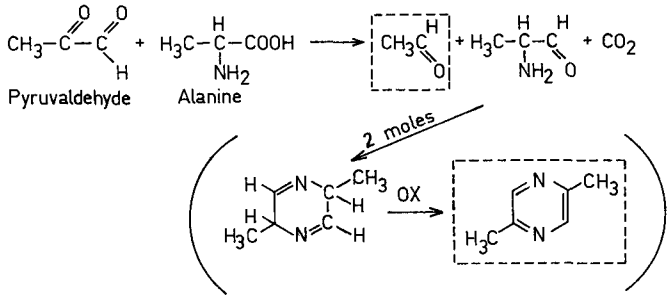


Fig. 6. Strecker degradation of amino acids.

Maillard reaction. It is obvious that multi-component product systems arise from quite simple starting materials. By way of illustration, MORTON and SHARPLES (1959) found that the reaction between glucose and β-phenylalanine give a honey-flavoured syrup; RUCKDESCHEL (1914) showed that heating glucose with leucine led to materials with a bread-like aroma.

Let me now give a stepwise description of the reaction between sugars and amines at a temperature of about 60 °C. The first step in the reaction is the formation of an aldosylamine (ELLIS and HONEYMAN, 1955). Aldosylamines undergo acid-catalyzed rearrangement (the Amadori rearrangement, HODGE and FISHER, 1963b) to amino-ketoses (1-amino-1-deoxy-2-ketoses): the rate of conversion is determined by the basicity of the N-atom. Many investigators have studied the mechanism of this re-arrangement (GOTTSCHALK, 1952; HODGE, 1955; ISBELL and FRUSH, 1958; MICHEEL and DIJONG, 1962; WEYGAND, 1940). The most plausible mechanism is that proposed by WEYGAND with subsequent refinement by MICHEEL and DIJONG (1962). This mechanism is illustrated in Figure 7.

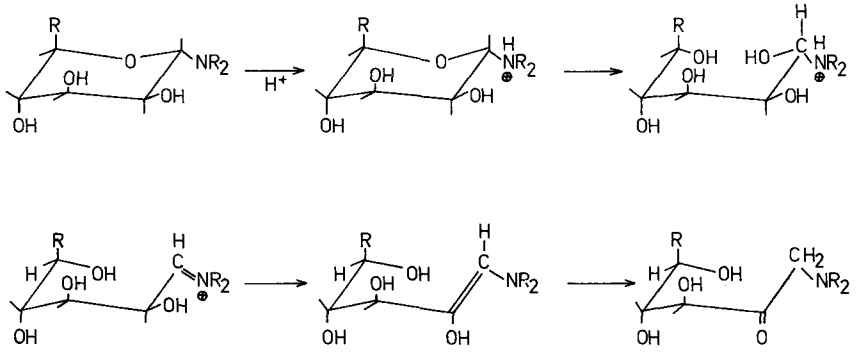


Fig. 7. Amadori rearrangement.

Aldosylamino acids undergo autocatalyzed Amadori rearrangement, and N-carboxylaminoketoses are thus found to occur as such in nature. Aminoketoses, however, are unstable in acid medium and undergo degradation at higher temperatures. In this respect the aminoketoses derived from secondary amines appear to differ from those derived from primary amines and amino acids. The latter group of compounds naturally plays an important role in food processing. The main reaction between aldoses and primary amines or amino acids leads to 3-deoxyosuloses (I, see Figure 8), osuloses (II), and furaldehydes (III) probably as shown in Figure 8.

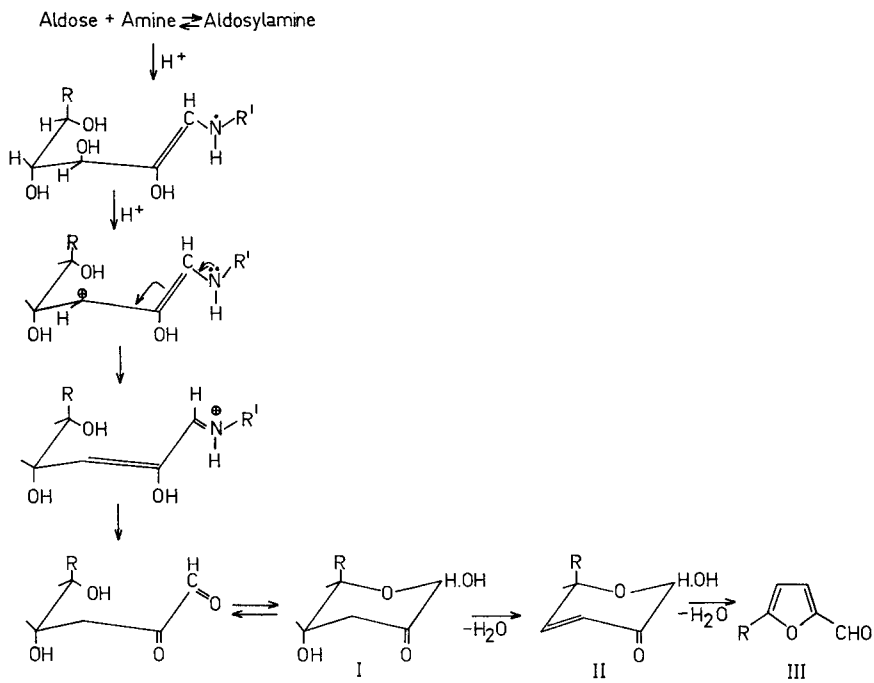


Fig. 8. Reaction of aldose with salt of primary amine.

Apparently hydrogen ion abstracts the hydroxyl group on C_3; the resulting carbonium ion rearranges to an immonium ion which is hydrolyzed to a 2-ketoaldehyde: formation of compounds I, II, and III from the ketoaldehyde is then quite plausible. ANET (1960) found that 3-deoxyglucosulose (I, R = CH_2OH) was formed when an aqueous solution of fructose paratoluidine at pH 4.5 was heated for five minutes at 100 °C. KATO (1960) and KATO and SAKURAI (1962) isolated a pentosulose (I, R = H) from pasteurized soy sauce, and found that addition of xylose enhanced the formation of I. KATO (1961, 1962) also prepared compound I with R = H and R = CH_2OH by reaction of aldosyl-butylamine with acetic acid in methanol. ANET obtained compounds I, II, and III with R = CH_2OH by heating an aqueous solution of difructose-glycine

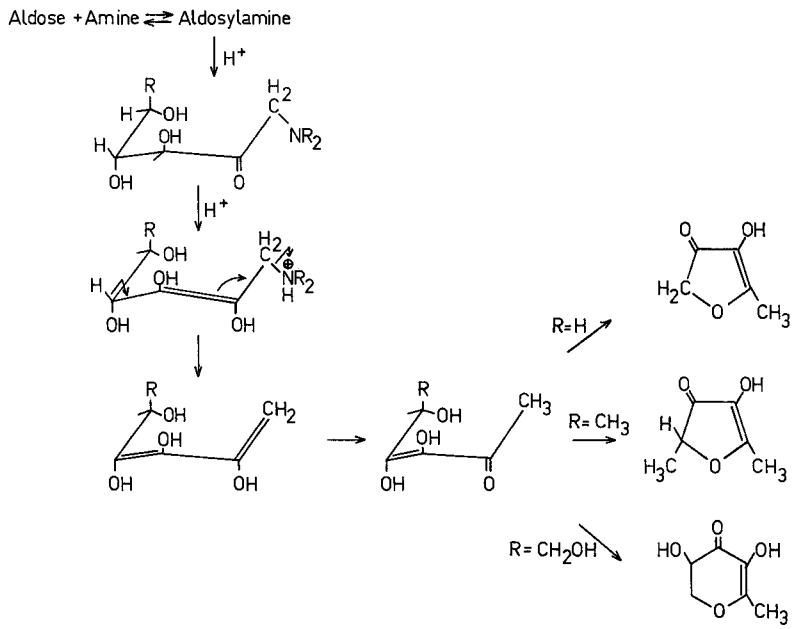


Fig. 9. Reaction of aldose with salt of secondary amine.

for four minutes at 100 °C (ANET, 1960, 1962a). In acidic media compounds I and II were rapidly converted to furaldehydes.

The reaction between aldoses and secondary amines proceeds somewhat differently, as illustrated in Figure 9.

Probably as a result of its stronger proton affinity, the N-atom of the aldosylamine is protonated: amine is subsequently split off (via a 1,4-elimination) leading to a 2,3-diketo compound. The diketone cyclises via a hemiketal configuration to a dihydro-γ-pyrone, or to a dihydrofuranone in the case of pentoses. Much work in this area has been done by HODGE and collaborators (HODGE, 1955, 1960; HODGE and FISHER, 1963a, 1963b; HODGE and MOSER, 1961; HODGE and MOY, 1963; HODGE and NELSON, 1961; HODGE and RIST, 1953; HODGE et al., 1963), who found that 6-deoxy sugars, such as L-rhamnose, are converted by the above mechanism to 2,5-dimethyl-4-hydroxy-2,3-dihydrofuran-3-one, a material which has since been found in a wide range of natural materials. We have already encountered this compound as a product of caramelization (see Figure 1). RODIN et al. (1965) isolated this furanone from burnt pineapple, and the material (together with a monomethylfuranone) has been isolated from beef broth (bouillon) by TONSBEEK et al. (1969). The dimethylfuranone in particular has a very powerful caramel odour. We synthesized the monomethyl analogue in reasonable yield by reaction of pentoses with secondary amines (PEER et al., 1968). When we carried out the reaction with glucose, however, we obtained not the expected furanone but 3,5-dihydroxy-2-methyldihydro-γ-pyrone (VAN DEN OUWELAND and

PEER). Steric factors presumably prevent $C_2 \rightarrow C_5$ ring closure, and the six-membered ring ($C_2 \rightarrow C_6$ closure) results. The pyrone above was one of the materials which I remarked earlier as being isolated by SHAW from stored orange powder.

5. INHIBITION OF BROWNING

I would like to make a few remarks about the ways in which browning may be prevented. In principle we can apply the brake to browning at two distinct stages:

In the formation of aminoketoses.

In the degradation of aminoketoses.

The formation of aminoketoses is inhibited by sulfur dioxide and by bisulfite ion, and degradation of aminoketoses is inhibited by mercaptoacetic acid and thiophenol. As yet, extraordinarily little is known about the manner in which these compounds function. It is interesting to note that both mercaptoacetic acid and thiophenol increase the rate of formation of aminoketoses yet inhibit their degradation (HODGE, 1955; HODGE and FISHER, 1963b). INGLES (1963) found that D-glucosyl-morpholine heated in the presence of thiophenol was converted to 1-morpholino-1-deoxy-D-fructose in 85% yield: browning (i.e. further reaction of the latter compound) was negligible.

I have attempted to give some idea of the great variety of reactions in which sugars are involved – both under mild conditions (Maillard) and rather drastic conditions (pyrolysis). In retrospect you will surely agree that the growth inhibition observed by STEHSEL and CAPLIN (unpublished) in autoclaved fructose-containing medium is an interesting (and as yet unexplored) territory.

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THE pH AND STERILIZATION

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SUMMARY

Interactions between pH and sterilization in the course of the preparation of culture media are described.

The influence of sterilization on the pH of the medium is very difficult to reproduce, which necessitates measurement of the pH before and after sterilization. This is especially important because the growth promoting properties and the selectivity of culture media are pH-dependent.

1. INTRODUCTION

The 'pH and Sterilization' in the context of the preparation of culture media comprises three different subjects.

The influence of the pH on the effectiveness of the sterilization method.

The influence of the sterilization method on the value of the pH of the culture medium after sterilization.

The influence of both pH and sterilization method on the quality of the culture medium after sterilization.

It is surprising that a survey of the literature (LAWRENCE and BLOCK, 1968; SYKES, 1967) on these interactions yields only specific data scattered as details of other subjects.

2. INFLUENCE OF pH ON STERILIZATION

2.1. *General considerations*

The slope of the logarithmic part of a decimal reduction curve at a given temperature is among other things generally dependent on the value of the pH. At extreme pH values the slope at room temperature may be steep enough to result in a reasonable sterilizing effect.

This influence of the pH can be understood to be an acceleration of the denaturation of proteins.

In the presence of potentially toxic substances the pH plays an especially important

role when the toxicity of the substance depends on the degree of dissociation. Many substances are toxic only in the un-dissociated state.

Hypochlorite has negligible activity at pH over 8, where it is completely dissociated, below 8 the activity increases rapidly parallel to the decrease in dissociation.

Glutaraldehyde and quaternary ammonium compounds are inactive below pH 7 and very active at a higher pH.

2.2. *Sensitization of bacterial spores*

ALDERTON and SNELL (1969a, 1969b) reported that *Bacillus* spores that had been subjected to pH 3 at 70 °C for one hour, were much more sensitive to heat at pH 7 than untreated spores were. The explanation for this phenomenon is supposed to be ion-exchange on the surface or in the wall of the spore; Ca^{++} and Mg^{++} ions being exchanged for H^{+} ions, resulting in increased heat sensitivity.

In special cases when one or more of the medium components are heavily contaminated with bacterial spores, the phenomenon might be useful to shorten the required sterilization time.

3. THE INFLUENCE OF STERILIZATION ON THE pH OF THE MEDIUM AFTER STERILIZATION

It would be very practical if there were rules, even empirical ones, to describe the behaviour of the pH during sterilization. Such is not the case; the behaviour of the pH is irregular. It is, therefore, important to find the reasons why the pH behaves irregularly and to see which conclusions must be drawn from the facts.

First, however, we should regard the reasons why the pH of a culture medium after sterilization must have a given value.

The growth rate of the micro-organism for which the medium is intended depends on the pH.

In selective media the pH plays a role in suppressing the growth of undesirable micro-organisms.

The detection of growth of a specific micro-organism can be based on the colour change of a pH-indicator.

The fact that the pH of a medium also changes during the growth of the micro-organism must of course be considered in the choice of the initial pH value.

To understand the behaviour of the pH of a culture-medium during heat-sterilization it is practical to describe the composition of the medium as a heterogeneous mixture of acids, bases and polymers.

The course of temperature versus time of a sterilization shows that the medium passes through many temperature conditions (fig. 1).

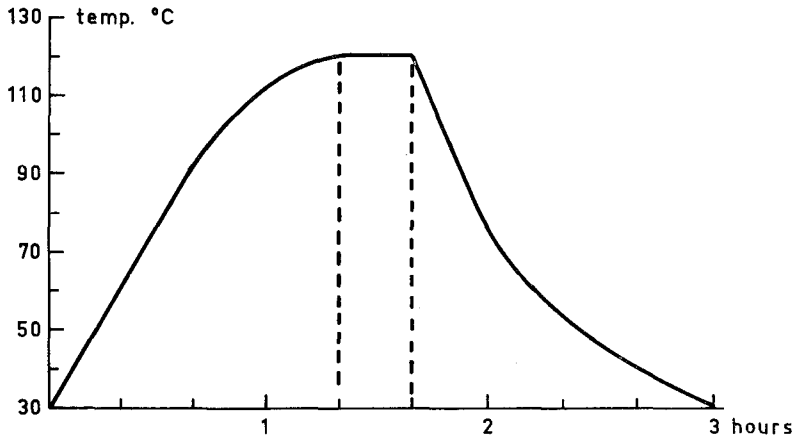

Fig. 1. The course of temperature versus time of a sterilization.

For each of those conditions a distinct equilibrium and a set of specific temperature-dependent reaction rates apply.

The reactions concerned are:

precipitation or dissolution of salts.
neutralization or esterification of acids.
hydrolysis of proteins, polysaccharides and esters.
reactions between carboxyl- and aminogroups.
polymerization or depolymerization of alcohols, aldehydes and saccharides.

These reactions are all pH-dependent and result in a change of the pH value.

The sum of what has happened at the end of the sterilization is therefore dependent on the course of the temperature.

The rate of heating and cooling and the exact value of the sterilization temperature play an important role.

We should bear in mind that the temperature conditions inside an autoclave are not homogeneous, especially during heating and cooling, which means that two bottles of the same autoclave-load do not receive quite the same temperature course or heat-treatment. The temperature course or heat-treatment for two different autoclave-loads will result in larger differences, the more so if two different autoclaves are used.

Differences in the course of the pH are therefore unavoidable. Besides, there are also factors influencing the pH prior to sterilization like the quality of the materials and the water.

The use of buffers to avoid this problem is sometimes possible, but can also lead to complications. Acetate, phosphate, ethanolamine and TRIS will all participate in case of heatactivated reactions, which implies that only relatively high buffer concentra-

tions are usable. On the other hand high concentrations of these substances are directly or indirectly toxic to some micro-organisms.

Fifty subsequent batches of 60 l of nutrient broth without sugar, prepared and sterilized according to a well standardized procedure in a 70 l fermenter, which is heated from 20 °C to 120 °C in 7–10 minutes, then kept 20 minutes at 120 °C, after which it is cooled down to 60 °C in 7–10 minutes, show a variation in pH after sterilization of 0.15 (7.25–7.40). The result seems to be independent of the pH prior to sterilization, which shows a variation of 0.3 (7.10–7.40).

The same broth with 3% starch shows a variation in pH of 0.60 (7.30–7.90) after sterilization under the same circumstances.

From these considerations it is evident that the pH should be measured and registered before and after sterilization to avoid doubts about the usefulnes of the medium. The information received from these measurements is also useful in that it provides a means of controlling the composition of the medium because deviations of the prescription nearly always result in larger variations in pH.

For other sterilization methods than heat-sterilization similar considerations are valid.

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VITAMINS

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SUMMARY

On the basis of the chemo-physical properties of the common vitamins it is considered which general factors are to be taken into consideration when these nutrients are added to culture media of varying composition. Generally, the addition of vitamins to culture media before sterilization by heating is not advisable; filter-sterilization should be preferred.

1. INTRODUCTION

The primary objective of this lecture is to come to an attractive composition of facts, which, in principle, will be well-known.

As a matter of fact, this procedure resembles very much a cook's method of working and this leads to the principal field of application of vitamins, viz. nourishment (for man and animal). For a chemist it is strange that such a chemically heterogeneous group of compounds as the vitamins keeps functioning as a group, not only historically but also during its further development, and therefore a mini case history will be presented. Thereafter the chemo-physical properties will be shown in a table from which some general conclusions concerning the application of vitamins in culture media will be drawn.

2. 'CASE HISTORY' VITAMINS

The common denomination of vitamins as a group is based on their physiological action, and thus, briefly stated, vitamins are chemical substances, which, in very small quantities, from a part of the food of man and animal, essential to their health.

In order to fix the attention a little longer on this fact it should be repeated that even in small quantities, vitamins
show their activity
are essential to the health of man and animal
are usually consumed through the food.

This 'case history' can be divided into three parts, viz. the confrontation with the effects of a vitamin deficiency, the scientific study, and the industrial manufacture of the vitamins.

2.1. *Vitamin deficiency*

Although since ancient times mankind has been confronted with the consequences of an insufficient vitamin supply, and especially the seafaring nations, among which the Dutch, met with evident operation difficulties, notably in the 17th and the 18th century, for instance by the occurence of scurvy, the scientific research leading to the aforementioned insight was not started before the beginning of the present century.

2.2. *Scientific research*

As is well-known the initiative for scientific research was taken in the former Dutch East Indies round about 1900 by the Dutchmen Eykman and Grijns. After their basical discovery it took another few decennia before things really developed more rapidly. This fact is to be attributed to two causes:

In the first place the above mentioned great activity of the substances in question made it very difficult to get sufficient supplies for further investigation by means of the then current chemical and analytical techniques.

In the second place the fragile structure of most of the vitamins could not stand the rather rigorous chemical treatments applied in those times during the examination of material from natural sources, for instance cooking with acid or lye, etc.

Then in the period from 1926 to broadly 1960, especially about 1940, the secrets of vitamins were revealed at a rather high rate according to the pattern classic for natural substances: isolation, structure determination and synthesis. Particularly in the 1930s the scientific avant-garde was highly interested and very active in this field. Thus more than twenty vitamins could be identified and, quite apart from a physiological point of view, because later on it became clear that most of the vitamins perform their function in the organism as a prosthetic group of an enzymatic system, one gets the impression that the period of the classic vitamin research is over now.

The great structural specificity of the vitamin action is remarkable. In spite of a lot of research only in a few cases other substances were found, of which the action equalled that of the natural products.

2.3. *Industrial manufacture of vitamins*

On the basis of these scientific data and stimulated by the possibilities of application for man and animal, industrial processes have been developed for all the important vitamins particularly in the last few decennia, through which these scientific curiosities from the 1930s can be supplied in practically any quantity desired. Their quality and prices allow an extensive application. In the last few years the manufacture of the components that are most important for application has even exceeded the manufacturing scale of fine-chemicals and can be ranged among the activities of the big chemical industries. It is remarkable that in general the coherence of the group is maintained, in spite of the highly varying chemical structures.

3. CHEMO-PHYSICAL PROPERTIES

The chemo-physical properties are summarized in Table 1, in which the classification into fatsoluble and watersoluble vitamins is maintained.

Table 1. Some chemo-physical properties of common vitamins, as related to their stability.
Stability: ++ = very good; + = good; 0 = reasonable; − = moderate; −− = bad.

	pH		Ultraviolet light	Oxidation (peroxydes)	Reducing substances	Heavy metals	Heat
	<7	>7					
FATSOLUBLE VITAMINS							
Vitamin A esters	−−	−−	−−	−−	−−	−−	−−
Vitamin D (D_2 and D_3)	−−	−	−−	−−	−	−−	0
Vitamin E (tocopherol)	0	0	−−	−−	−	−−	0
Vitamin A acetate	+	−	0	0	−	−−	+
Vitamin K_3 (menadione)	−−	−−	−−	−	−−	−−	0
Vitamin K_3 bisulfite	−−	−−	−−	−	−−	−−	0
WATERSOLUBLE VITAMINS							
Vitamin B_1 HCl	+	−−	−−	−−	−−	−−	0
Vitamin B_2	+	−−	−−	0	−−	−−	0
Vitamin B_6 HCl	+	−−	−−	−−	−	−−	0
Vitamin B_{12}	+	−−	−−	−−	−−	−−	0
Vitamin C	+	−−	−−	−−	−	−−	0
Nicotinic acid	0	0	0	0	0	0	0
Nicotinamide	−	−−	0	0	0	0	0
Calcium pantothenate	−−	−−	0	0	0	0	−
Biotine	−−	−−	−−	−−	0	0	0
Folium acid	−	−	−−	−−	−−	−−	0
p-Aminobenzoic acid	0	0	0	−−	0	−−	0
Choline	0	−	0	0	0	+	0

It will be clear that particularly for combination products for pharmaceutical application it may be advisable to modify this solubility. In fact there are workable solutions for this purpose, although they often have a pragmatic character, to which the above mentioned great structural specificity may frequently be a handicap. In order to overcome the practical obstacles of the poor stability special coatings have been developed for a number of vitamins. They are notably applied in cattle feed.

Table 1 also shows that, generally, the addition of vitamins to culture media before sterilization by heating is not advisable; filter-sterilization is to be preferred.

AUXINS

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SUMMARY

Auxins, as plant growth regulators, are normally added to plant culture media in concentrations varying from 10^{-8} to 10^{-5} g/ml. Most commonly used for this purpose are the natural auxin indole-3-acetic acid (= IAA) and the synthetic auxins naphthalene-1-acetic acid (= NAA) and 2,4-dichloros phenoxyacetic acid (= 2,4-D). They are frequently used in the form of K-salts because of their higher solubility in water. Auxins are mostly added to the culture media before sterilization from higher concentrated stock solutions.

Sterilization by autoclaving at 110°–120°C during 15–60 min. does not destroy the auxins, as they are all rather thermostable. IAA is destructed under the influence of a low pH (especially of mineral acids), light, oxygen and peroxides, oxidation katalysts and special plant enzymes (for example peroxidases). These factors may be present during preparation and/or handling of culture media. In this respect the use of IAA in culture media must be dissuaded.

In most cases it is advisable to use NAA or 2,4-D which are less sensitive to destruction. When IAA has to be used, special control of the auxin concentrations in the media before, during and after use is advisable.

Plant growth regulators like auxins are added to most of the media on which normal plant material is cultured, as they are normally necessary for cell division and cell growth. For the cultivation of plant tumor tissue, for example crown-gall, the addition of an auxin is not required. In some cases the auxins may also influence, in a special balance with the cytokinins, the differentiation of tissues. Auxins are applied in concentrations varying from 10^{-8} to 10^{-5} g/ml, depending on the purpose of the cultivation and the kind of material. Indole-3-acetic acid (= IAA), naphthalene-1-acetic acid (= NAA) and 2,4-dichlorophenoxyacetic acid (= 2,4-D) are the auxins mostly used for plant cell, tissue, and organ culture (fig. 1) These auxins are frequently used as K-salts because of their higher solubility in water. Sometimes the acids are dissolved in 96% ethanol, and subsequently diluted with an aqueous culture solution. In this case however, attention should be paid to the possible effects of ethanol on the plant material. Auxins usually are added to the culture media from concentrated stock solutions. As a rule this is done before sterilization of the medium.

Sterilization by filtration of nutritive solutions by means of Seitz or Millipore filters gives no problems, as far as the auxins are concerned. Sterilization by U.V.-light is disastrous for IAA, as it is rapidly destroyed by radiation. Auxins are not destructed by

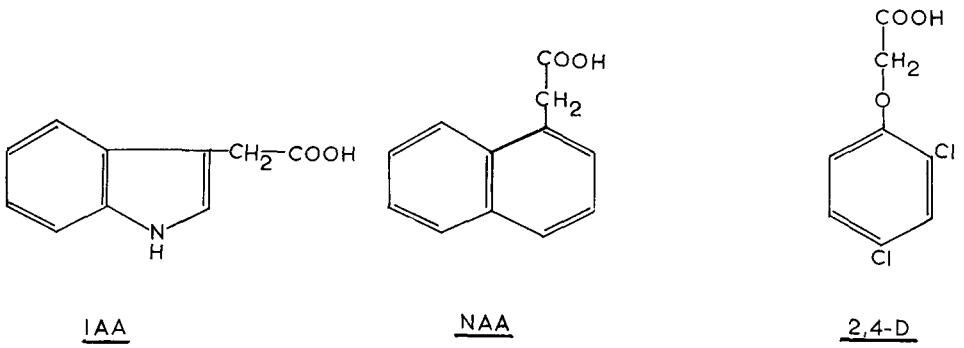

Fig. 1. Structural-formulas of the most commonly used auxins.
IAA = indole-3-acetic acid.
NAA = naphthalene-1-acetic acid.
2,4-D = 2,4-dichlorophenoxyacetic acid.

autoclaving at 110°–120°C during 15–30 min., or even one hour, and it is the most commonly used sterilization technique. This result might be expected as most of the auxins are rather thermostable, if they are not autoclaved in strong alkaline or acid media. There are, however, some other IAA-degradation stimulating factors like light, oxygen and peroxides, oxidation-stimulating substances and certain plant enzymes, for example peroxidases. For all these reasons it is advisable to use NAA and 2,4-D, which are much less destruction-sensitive than IAA is.

Moreover, radioactive labelled IAA is more sensitive to destruction by self-radiation than radioactive NAA.

Sometimes, however, it is necessary to use IAA, which naturally occurs in many plants, in stead of the synthetic auxins NAA and 2,4-D. Some literature references will be cited here, illustrating the risk in using IAA in culture media.

BITANCOURT (1963, 1966) found that IAA solutions are rapidly inactivated by spontaneous oxidation of IAA into polymerized deuterauxin (a dimer of IAA). Deuterauxin was depolymerized into active IAA by heating the solution at 100°C. During storage IAA solutions become rose coloured, presumably by the formation of deuterauxin, in which process light may play a stimulating role.

DEVERALL (1965) warned against the use of glassware which has been chemically contaminated and cleaned with chromate, as IAA is destructed in the presence of chromate at a pH of 4.5–5.3. Moreover, he prefers glass-distilled water to deionized water, as it is possible that spores of some substances may act as stimulators of the oxidative breakdown of IAA.

LOEWENBERG (1965) found that IAA was destructed during autoclaving, if Mn^{++}, citrate and oxygen were also present in the medium. Aside from the fact that citrate stimulated the breakdown of IAA during the preparation of the media, the author also noticed a stimulation of the activity of the IAA-destroying exoenzyme secreted into

the medium by the cultured plant material. Furthermore, according to Loewenberg, L-alanine stimulated the IAA destruction, but only when plant tissue was present as well.

POSTHUMUS (1967) showed that at the partitioning of aqueous IAA solutions with ether at different pH's twice the amount of IAA was lost at pH 2.5–4.0 than the amount lost at pH 7.0–8.5. Low pH, especially of mineral acids, clearly promoted the oxidative destruction of IAA, which was also evident when comparing chromotography of IAA in alkaline and acid solutions. During chromatography the unfavourable influence of light on IAA was also noticed.

It was observed in the course of the research on enzymatic IAA destruction that the non-enzymatic IAA destruction was promoted also by aeration and the addition of the cofactors Mn^{++} and 2,4-dichlorophenol. Presumably both forms of degradation take place by means of the same mechanism, at which free radicals play a role and 3-methylene-oxindole is an important product (fig. 2).

WICHNER and LIBBERT (1964) indicated that the so-called IAA oxidase could be an exoenzyme that can be secreted by plant tissue in the culture medium. Consequently, the use of another auxin than IAA is preferred to be sure about a more or less constant supply of auxin in a medium.

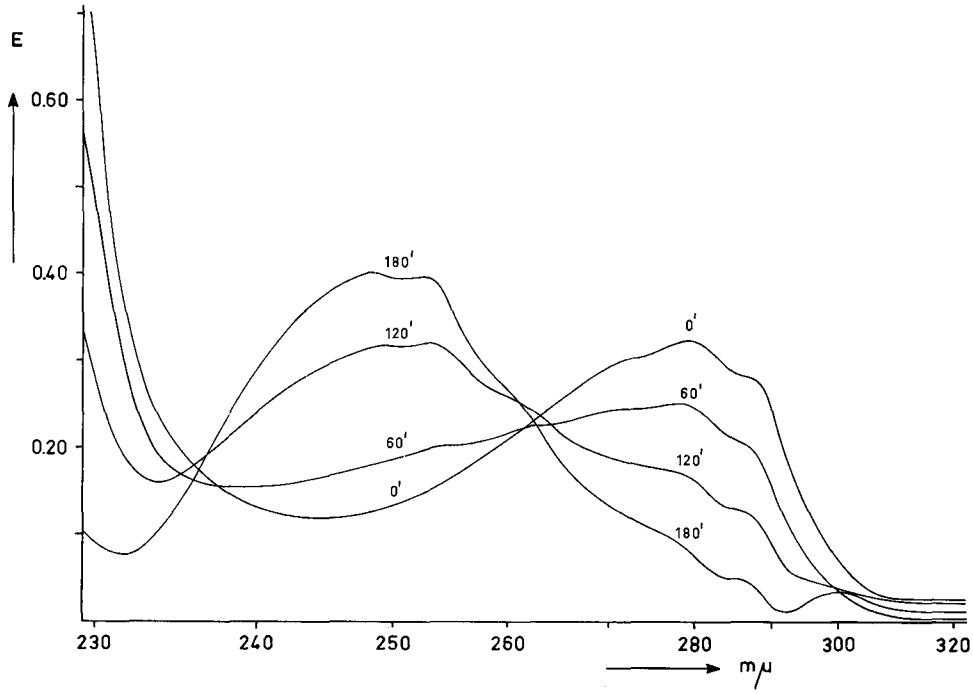

Fig. 2. Example ofspect rophotometrically followed breakdown of IAA by enzymes from plant material, at which 3-methyleneoxindole is formed (abs. max. at 247 and 253 mμ).

Finally, there is a chance that tryptophane, present in the medium, will be converted into IAA. This may occur during the process of autoclaving under favourable conditions of pH and other factors, but it is especially brought about by processes in the plant tissue itself. Non-enzymatic and enzymatic conversion of tryptophane into IAA are both stimulated by the same cofactors (compare WHITMORE and ZAHNER, 1964).

From the preceeding it is clear that the control of autoclaved media with and without IAA as an added auxin is desirable. However, it is not simple to isolate and determine exactly the very low quantities of IAA possibly present in the media. Extraction of an aqueous homogenate of the medium with ether at low pH, chromatographic purification and fluorimetric determination possibly are useful methods, in which the loss of IAA during manipulations will be a fact to be reckoned with (compare POSTHUMUS, 1967).

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DISCUSSION

R. L. M. PIERIK: Do you believe what is said in the publication of Bitancourt about the conversion of IAA to deuterauxin?

A. C. POSTHUMUS: Undoubtedly IAA is converted to some other products, but the real chemical identities of the compounds formed, may be different from what is suggested as the structure of deuterauxin.

R. L. M. PIERIK: After long storage (below 0 °C) coconutmilk becomes rose. Does coconutmilk contain IAA, which also becomes rose coloured at storage?

A. C. POSTHUMUS: I suppose that coconutmilk contains, besides the natural cytokinins, IAA as a natural auxin, but whether it is coloured rose by IAA degradation products or by other compounds I do not know.

R. KOKKE: Is there a possibility for a microbiological test for plant hormones, and if so, could it be a simple method to control media on degradation during sterilization etc.?

A. C. POSTHUMUS: As far as I know, microorganisms have never been successfully used in a quantitative estimation of plant hormones, but it would be a welcome technique for rapid control of plant culture media.

STERILIZATION OF CYTOKININS

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SUMMARY

Aqueous solutions of kinetin, zeatin and isopentenyladenosine have been chromatographed on thin layer silicagel chromatograms before and after they had been autoclaved for 1 hour at 120 °C in water. The compounds on the chromatograms were located by means of ultraviolet light. From these experiments no breakdown products of the cytokinins could be detected.

However, non-active 1, 3 or 9 substituted purine molecules can be converted into callus growth stimulating N^6-substituted purines during autoclaving at 120 °C. Crude extracts can also be sterilized by Seitz or Millipore filtration before addition to an autoclaved medium. This method, however, is time consuming, especially when extracts are chromatographed on paper first. Parts of paperchromatograms can also be sterilized by adding first a mixture of ethanol and water (1:1) to the paper. Subsequently the wet paper is brought into tubes with an autoclaved and solidified agar medium. The ethanol evaporates when the tubes, which are covered with cotton or plastic caps, are exposed to heating at 110 °C for 2 minutes in a steam heated autoclave. A gas heated autoclave is not suitable because it heats up to slowly and the ethanol does not evaporate completely before the agar melts.

Media together with extracts can also be sterilized by boiling them twice for 30 minutes with a period of 24 hours between the two treatments.

When light is required in long-term experiments with kinetin, attention should be paid to photochemical degradation.

Cytokinins are a group of plant hormones which are defined as compounds that induce cell division in plant cells in cooperation with an auxin (SRIVASTAVA, 1967).

Most naturally occurring cytokinins are N^6-substituted purines (fig. 1). The destructive effect of autoclaving on compounds with a purine structure has been discovered in an early stage of research.

In this respect it is worthwhile to mention that kinetin (6-furfurylaminopurine), which is often used in media for tissue cultures, is not a naturally occurring compound but is formed from deoxyadenosine which is split from DNA during autoclaving. Furthermore, from extensive work by HAMZI and SKOOG (1964), SKOOG et al. (1967), SKOOG and LEONARD (1968) it appears that non-active 1, 3 or 9 substituted purine molecules can be converted into callus growth stimulating N^6-substituted purines during autoclaving.

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[2] The work has been carried out at the Laboratory of Fytopathology, Agricultural University, Wageningen, the Netherlands.

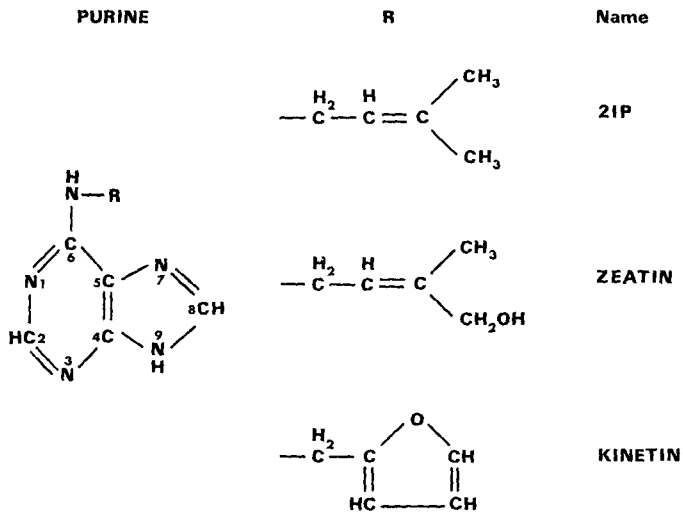

Fig. 1. N^6-substituted purines with cytokinin activity. Zeatin and 2IP (N^6-(Δ^2-isopentenylamino) purine) are naturally occurring compounds.

Crude extracts can also be sterilized by Seitz or Millipore filtration before addition to autoclaved media. This method, however, is time consuming, especially when many extracts from paper chromatograms have to be assayed on growth stimulating activity in tobacco pith or soybean callus tissue culture. Parts of paper chromatograms can also be sterilized by using a combination of two different sterilization techniques. First the paper is wetted with a mixture of ethanol and water (1:1 v/v). Subsequently the paper is brought into tubes with an autoclaved and solidified agar medium. The ethanol evaporates from the paper when the tubes, which are covered with plastic Bellco caps (Technomara, Zürich), are exposed to a 2 minutes period of heating at 110 °C in a steam heated autoclave (Fig. 2). The ethanol evaporates rapidly from the paper before the agar liquefies. Yield of tobacco callus appeared not to differ from filter sterilized extracts of paper chromatograms. Pretreatment with ethanol makes it possible to keep the period of exposure to high temperatures as short as possible. However, a gas heated autoclave is not suitable for this procedure because it heats up too slowly and the ethanol does not evaporate completely before the agar melts. Callus of tobacco and soybean are highly sensitive to small amounts of ethanol (fig. 3).

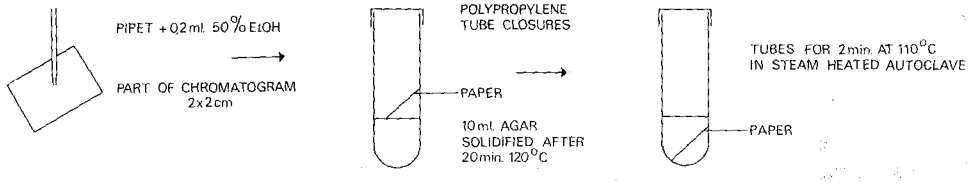

Fig. 2. Scheme for the sterilization of paper chromatograms with ethanol.

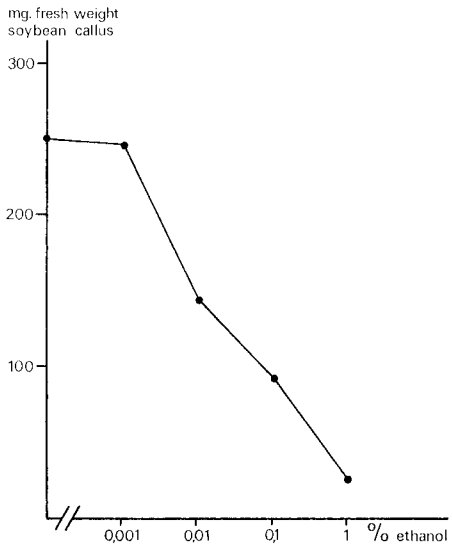

Fig. 3. Effect of ethanol on yield of soybean callus after 20 days. Medium (MILLER, 1965) with ethanol has been sterilized by boiling it twice for 30 minutes with a period of 24 hours between the two treatments.

The possible breakdown of zeatin (Calbiochem), kinetin and isopentenyladenosine (2IP with ribose in the N^9-position, fig. 1) during autoclaving has been investigated. The latter compound was a generous gift of Mrs. BEZEMER-SYBRANDY (Laboratory of Biochemistry, Leiden, the Netherlands). Aqueous solutions of these compounds at pH 5.6 (5 ppm) have been chromatographed in ethylacetate: n-propanol: water = 4:1:2 on thin layer silicagel chromatograms (F_{254}, Merck) before and after they had been autoclaved for 1 hour at 120 °C. The compounds (100 μg) on the chromatograms were located by means of ultraviolet light. No difference between the spots before and after autoclaving could be detected. The R_f value of kinetin, zeatin and isopentenyladenosine are, respectively 0.8, 0.49 and 0.76. Furthermore, the extinction of the UV absorption spectra at 268 nm was not changed after autoclaving. The results indicate no serious breakdown of these cytokinins during autoclaving.

Similar results have been obtained by BEZEMER-SYBRANDY (personal communication) with 2IP, isopentenyladenosine, 6-benzyladenine and 6-benzyladenosine after autoclaving at pH 4.4–4.8 for 20 minutes at 110 °C. N^6-aminopurine cytokinins appear to be fairly resistant against autoclaving. This view is supported by work of KENDE (1965, 1967) who found that zeatin in root exudates after Seitz filtration yielded as much callus tissue as they did after autoclaving. Similar results have been obtained by SKOOG et al. (1967) with 6-benzyladenine.

To avoid the chance of a prolonged heating period, it is recommended to attach an electric time clock which shuts off gas supply on gas heated autoclaves after 20 minutes.

Media together with extracts can also be sterilized by boiling them twice for 30 minutes with a period of 24 hours between the two treatments.

Tissue culture bioassays are at present the most reliable method for testing crude extracts. However, to be sure that no conversion of non-active purine molecules into cytokinin active molecules occurs during autoclaving of crude extracts, other assays which do not require sterilization can also be used as the mobilization bioassay (DEKHUIJZEN, 1968).

The factor light does not affect decomposition of cytokinins during autoclaving but may play a role in long-term experiments with kinetin solutions. BEZEMER-SYBRANDY et al. (1968) demonstrated the degradation of kinetin into adenine and some unknown compounds after irradiation with light of a wavelength between 300–800 nm.

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DISCUSSION

E. C. WASSINK: Would it be possible to sterilize heat sensitive cytokinins separately from the other compounds in the medium in order to minimize decomposition of cytokinins.

H. M. DEKHUIJZEN: It is not possible to autoclave heat sensitive N_1, N_3 or N_9 substituted purines apart from the medium. It can be recommended to sterilize these compounds by filtration or be using a combination of ethanol and a short heating period as described in the text.

THE EFFECT OF AUTOCLAVING ON THE GIBBERELLIN ACTIVITY OF AQUEOUS SOLUTIONS CONTAINING GIBBERELLIN A_3

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Publication 336

SUMMARY

The biological activity of freshly prepared GA_3 solutions of pH 4.8, 5.3 and 6.0 was compared with the activity of solutions autoclaved at 114 °C during 20 minutes. Three experiments were carried out, each with another bioassay: growth of dwarf peas, release of α-amylase by barley half seeds and inhibition of auxin-induced adventitious root formation of bean epicotyls cultured in vitro. These experiments demonstrated that autoclaving reduced the gibberellin activity of the GA_3 solutions by more than 90%.

1. INTRODUCTION

Hormonal effects on processes like growth and regeneration of plants are often studied with the aid of sterile culture techniques. This implies the sterilization of the plant material and the media. When the investigations require the application of a hormone to the media, the stability of this compound needs special attention. This paper is confined to the stability of gibberellin A_3 (GA_3), a naturally occurring plant hormone widely used in plant research. For practical reasons – and in agreement with the empirical approach of the problems conditioned by the organizing committee – this paper deals only with the stability of GA_3 as observed from its biological activity.

2. SURVEY OF LITERATURE

GA_3 is stable when dry but slowly hydrolyzed by water and rapidly decomposed by heat (ANONYMOUS; HILLMAN, 1960). The rate of decomposition also depends on the pH of the solution (ANONYMUS; HENDERSON, 1960; HENDERSON and GRAHAM, 1962; HILLMAN, 1960). In alkaline solution GA_3 is converted to an isomer that is biologically inactive. In acid solution and at elevated temperatures gibberellenic acid and allogibberellenic acid are formed. At 100 °C and pH 2 the latter is changed to gibberic acid. These compounds have only little biological activity.

The pH of an aqueous solution of GA_3 is 3 to 4. The biological activity of such a solution is reduced by about 50% when left at 20 °C during 14 days.

HILLMAN (1960) found that the gibberellin activity of a GA_3 containing medium (pH 6.3 to 6.5, Hutner's minerals) was reduced by 90% when autoclaved at 121 °C during 20 minutes. The activity of the medium was tested with a modified dwarf ('Progress') pea assay.

These findings do not agree with the data obtained by HENDERSON (1960) and HENDERSON and GRAHAM (1962) from investigations on the influence of pH and autoclaving on GA_3. These authors autoclaved a solution of GA_3 in a phtalate-sodium hydroxide buffer of pH 6.0 at 126 °C during 10 minutes. Bioassays with dwarf maize d_1 and dwarf pea ('Variety 9', Ferry Morse) showed that the activity of the solution was reduced by 25% only. In general, the results of their experiments demonstrate that aqueous solutions of GA_3 are rather stable.

In view of the data mentioned above, we decided to reinvestigate the stability of GA_3 solutions by comparing the biological activity of autoclaved solutions with freshly prepared ones. Three experiments were carried out; each experiment was based on a different bioassay.

3. EXPERIMENTS

Experiment 1

A concentration series was made with GA_3 dissolved in a 0.07 M phosphate buffer of pH 5.3. The solutions were autoclaved at 114 °C during 20 minutes. The biological activity of the solutions was than determined with a modified dwarf pea assay (VAN BRAGT, 1968). For comparison, the activity of freshly prepared solutions was also determined. The results are presented in fig. 1 which shows that autoclaving reduced the activity of the solutions by 90 to 100%.

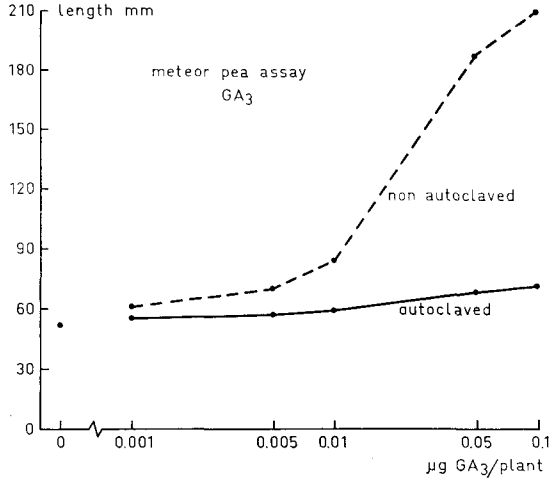

Fig. 1. The effect of autoclaved and non-autoclaved GA_3 solutions on the growth of dwarf 'Meteor' peas.

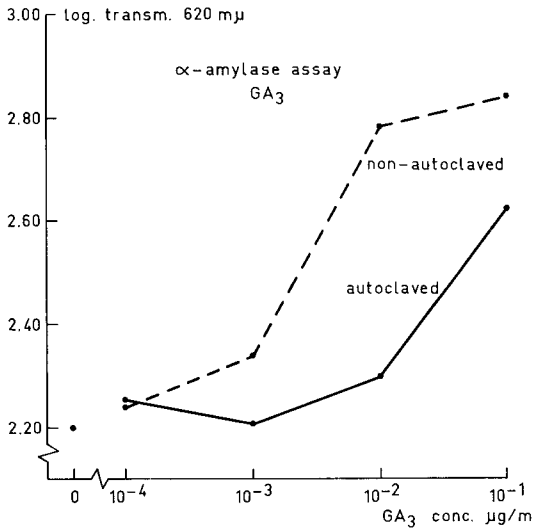

Fig. 2. The effect of autoclaved and non-autoclaved GA_3 solutions on the activity of α-amylase (parameter: transmission of starch-iodine complex) released from barley ('Himalaya') half seeds.

Experiment 2

Another concentration series was made with GA_3 dissolved in Knop's minerals. The pH of the solutions was adjusted to 4.8 using 0.002 M sodium acetate acetic acid buffer. The solutions were autoclaved at 114 °C during 20 minutes. The biological activity of the autoclaved solutions was determined with the α-amylase assay (JONES and VARNER, 1967). For comparison the activities of freshly prepared solutions were also determined. The result of this experiment is presented in fig. 2. This figure shows that autoclaving reduced the activity of the solutions by 90 to 100 %.

Experiment 3

In this experiment the inhibitory effect of GA_3 on auxin-induced adventitious root formation of bean epicotyls cultivated in vitro was investigated. This bean epicotyl assay is based on the observation that the mean number of root primordia per explant is decreased by increasing the GA_3 concentration in the media.

Material and methods

The sterile culture techniques were similar to those described earlier (PIERIK, 1967). Bean (*Phaseolus vulgaris* L. cv. 'Vroege Wagenaar') epicotyls, 4 cm in length were cut from 10 days old plants growing at 25 °C in continuous fluorescent light (Philips TL 55). The explants were grown aseptically, with their basal ends up. Epicotyls were sterilized by immersion in 70 % ethanol for a few seconds, followed by sterilization in 1 % NaOCl for 10 minutes and subsequent washing during 40 minutes in sterile tap water. The basic media contained pyrex-distilled water, Difco-Bacto agar 0.6 %, Heller's macroelements, Heller's microelements (except $FeCl_3$) at 1/4 strength,

NaFeEDTA 25 mg/l, glucose 2%, H_3BO_4 10^{-5} g/ml and K-IAA 10^{-7} g/ml. The pH of the media was adjusted to 6 (but not buffered) before autoclaving at 114°C during 20 minutes. GA_3 was dissolved in a few drops of ethanol. This solution was diluted with water and sterilized by filtration or by autoclaving at 114°C during 20 minutes. The number of explants per treatment was 24. Explants were grown in darkness at 25°C. Ten days after isolation the number of rooted epicotyls and the mean number of root primordia per epicotyl were recorded.

Results

After autoclaving, the pH of the media had changed to 5.3. Therefore the influence of the pH on root formation was examined first. No effect was found with media buffered with KH_2PO_4-Na_2HPO_4 at pH 5, 6 or 7.

Table 1. The effect of autoclaved and filter sterilized GA_3 solutions on auxin-induced adventitious root formation of in vitro cultivated bean epicotyls.

GA_3 concentration g/ml	sterilization by autoclaving		sterilization by filtration	
	% rooting	mean number of roots per explant	% rooting	mean number of roots per explant
0	100	14.7	100	14.7
10–8	100	12.4	100	6.5
10–7	100	9.5	79	1.8
10–6	79	2.9	0	0.0
10–5	13	0.5	0	0.0

Table 1 shows the results of an experiment in which the effect of GA_3 (heat- or filter-sterilized) was examined. This table shows that adventitious root formation has strongly decreased with increasing GA_3 concentrations. Heat sterilized GA_3 was more than 90% less effective than filter-sterilized GA_3.

4. Discussion

The results of the experiments show that autoclaving at 114°C during 20 minutes reduces the activity of GA_3 solutions at pH 4.8, 5.3 or 6.0 by 90 to 100%. These findings are in agreement with the results of Hillman (1960) but contradict the results of Henderson (1960) and Henderson and Graham (1962), who found reductions of about 25%. Hillman (1960) concluded that the loss in activity was in the order of one magnitude. A similar conclusion might be drawn from the results of our experiments. However, the residual activity of autoclaved GA_3 solutions might be due to the effect of degradation products of GA_3.

No experiments were carried out on the effect of the pH on the stability of GA_3 solutions. Comparison of our findings at pH 4.8, 5.3 and 6.0 with the results of HENDERSON (1960) and HENDERSON and GRAHAM (1962) indicates that further research in this field may be worthwile.

Nor did we investigate the influence of light on the stability of GA_3 solutions. Literature research provided no information on this subject, except the work of KUCHEROV (1969) on transformation by UV radiation.

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STABILITY OF ABSCISIC ACID ISOMERS TO HEAT STERILIZATION AND LIGHT

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SUMMARY

The plant hormone abscisic acid (ABA) occurs naturally in plant material as the dextrorotatory enantiomers of its 2-*cis* and 2-*trans* isomers. In connection with a study of morphogenesis in plant tissue cultures, both the 2-*cis* and 2-*trans* isomers of (±)-ABA were synthesized.

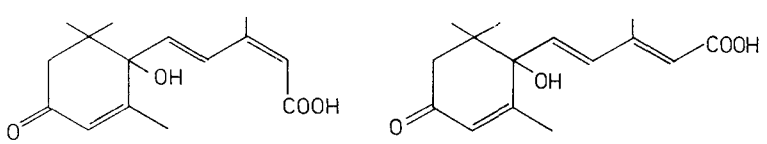
Dilute solutions of the two isomers at various pH values were subjected to heat sterilization, and exposed to light and aeration. The effect of these treatments on the ABA isomers was monitored by means of the *Avena* cylinder test, thin-layer and gas chromatography, and ultraviolet spectroscopy.

Neither isomer is affected by heat sterilization. Light, however, brings about a number of changes, including the partial conversion of the 2-*cis* isomer to the 2-*trans* isomer, and vice versa. The biological activity of 2-*trans*-ABA is lower than that of the 2-*cis* form.

1. INTRODUCTION

Abscisic acid (ABA) is a natural plant hormone which, in contrast to the auxins, gibberellins and cytokinins, has a growth-retarding function. It manifests itself in physiological effects such as the retardation of germination, cell elongation, cell division, and bud development, and the stimulation of the ageing and dropping of leaves. Both the 2-*cis* and 2-*trans* forms are found in plants, each as the dextrorotatory enantiomer.

In connection with an investigation into morphogenesis in plant tissue culture, (±)-abscisic acid was synthesized (fig. 1) by the methods of CORNFORTH et al. (1965), MOUSSERON-CANET et al. (1966) and ROBERTS et al. (1968). The 2-*cis* isomer was obtained from the mixture of (±)-2-*cis*-ABA and (±)-2-*trans*-ABA by fractional



I II

Fig. 1. (±)-2-*cis*-Abscisic acid (I) and (±)-2-*trans*-abscisic acid (II).

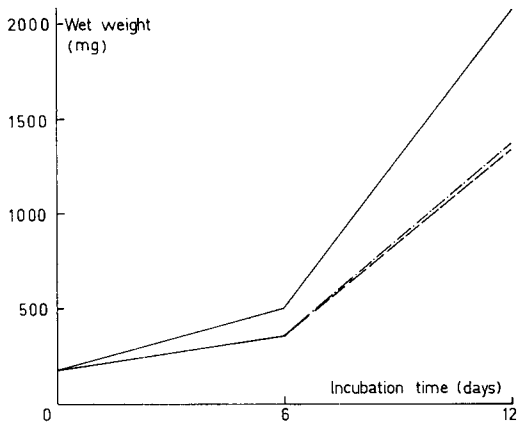


Fig. 2. Effect of the method of sterilization on the activity of ABA (1:1 mixture of 2-*cis* and 2-*trans* isomers) in *Daucus* suspension cultures.
———, Murashige and Skoog's (MS) medium; – . – .– MS + 1 mg/l ABA, autoclaved; – – – –, MS + 1 mg/l ABA, sterilized by filtration.

crystallization. Crystallization of a mixture of their respective methyl esters gave the *trans* methyl ester, which was converted into (±)-2-*trans*-ABA by saponification.

To get an initial impression about the stability of ABA, a solution of a 1:1 mixture of *cis* and *trans* was divided into two parts, of which one was sterilized by filtration (millipore) and the other autoclaved for 20 minutes at 120 °C. These solutions were each added to a cell-suspension culture of *Daucus carota*, grown in Murashige and Skoog's synthetic medium (MS medium, LINSMAIER and SKOOG, 1965). The final concentration of the abscisic acid was 1 mg per litre of medium. At this concentration the hormone was found to have a marked influence on growth (fig. 2). There was no difference between the effects of the solutions sterilized by the two methods. It must, however, be taken into account that the cultures were incubated on a shaking table (and therefore aerated) in a lighted growth chamber at about 26 °C during the 12-day growth period. In addition, the pH decreased during growth. The effects of pH, aeration and light on the abscisic acid might therefore have masked any small influences of the sterilization method.

After this initial experiment, we investigated by UV spectrometry, thin-layer and gas chromatography whether the abscisic acid is in any way converted by heat sterilization, aeration or exposure to light, and what the effect of each of these factors is on the biological activity.

2. METHODS

Solutions of both the 2-*cis* and 2-*trans* isomers were made up in each of the following:

1. MS medium, pH 5.8
2. Phosphate-citrate buffer, pH 4.4
3. Phosphate-citrate buffer, pH 6.3

The abscisic acid concentration was 1.5 mg per 100 ml. The volumes of the solutions were measured and their UV spectra recorded on a Hitachi/Perkin-Elmer 124. The

solutions were then sterilized for 20 minutes at 120 °C, brought to their original volume with sterile water and their UV spectra again recorded. They were then incubated for 10 days at 26 ± 1 °C on a New Brunswick gyrotory shaker (100 rev/min), half of them in the dark and half under light. The light was provided by a combination of Philips TL 34 fluorescent tubes and Philips 400 W HLRG lamps. The total light intensity at the height of the flasks containing the solutions was 16–19 mcal.cm^{-2}.min.$^{-1}$ The UV spectra were recorded again after the incubation period.

Thin-layer chromatography on silica gel (Merck DC-Fertigplatten F 254) was performed with the solutions after acidification and extraction with chloroform. Benzene: ethyl acetate : acetic acid (20:5:1) was used as the solvent system. The spots were made visible by irradiation at 254 nm. For gas chromatography, the two isomers (and the internal standard) were esterified with diazomethane. Good separation of the two

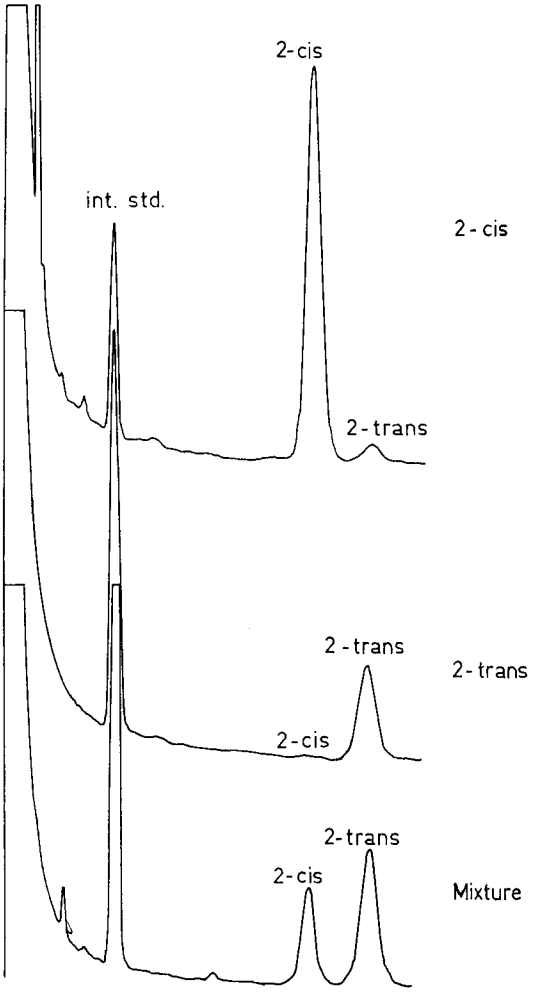


Fig. 3. Gas-liquid chromatography of methyl esters of 2-*cis* and 2-*trans* isomers of (±)-ABA.

esters could be achieved with an Aerograph 1200 gas chromatograph at 210 °C (stationary phase: 10% OV-17 on Diaport S). Heptadecanoic acid was used as the internal standard, which enabled the amount of each of the isomers to be determined quantitatively (fig. 3).

The *Avena* cylinder test was used to determine the biological activity of the solutions. This test was chosen because the incubation period is relatively short and in the dark, which reduces the chance of artefacts. The test solutions were added in amounts of 0.7 ml to 6.3 ml of 0.01 M phosphate buffer containing 0.1 mg naphthaleneacetic acid per litre buffer.

3. Results

The results of the UV spectrometric measurements are summarized in Table 1. If the changes in optical density (OD) are taken as a measure of the stability of abscisic acid, then heat sterilization causes no detectable conversion. Incubation in the dark, together with aeration, caused only a slight reduction ($< 5\%$) in OD. Light, on the other hand, was responsible for a sharp decrease in OD and the absorption maxima shifted to lower wavelengths. ABA appeared to be much less stable at low pH. Likewise, in the MS medium at pH 5.8, the OD was only lowered by light; autoclaving and incubation in the dark had no effect.

The *Avena* cylinder test showed clearly that the untreated solutions of the 2-*cis* isomer were biologically more active than those of the 2-*trans* isomer. This ties up with the findings of Cornforth et al. (1965), Sondheimer and Galson (1966) and Milborrow (1966). There were no changes in biological activity after autoclaving or incubation in the dark. After exposure to light, however, the difference between the biological activities of the 2-*cis*-isomer solutions and of the 2-*trans*-isomer solutions

Table 1. Effect of heat sterilization and incubation in light or in darkness on the absorption maxima (λ_{max}) and maximum optical density (OD) of the 2-*cis* and 2-*trans* isomers of ABA.

	λ_{max} (nm) and OD	2-*cis*		2-*trans*	
		pH 4.4	pH 6.3	pH 4.4	pH 6.3
Starting material	λ_{max}	248	242	251	245
	OD	1.03	1.18	1.27	1.37
After heat sterilization	λ_{max}	248	244	252	246
	OD	1.03	1.12	1.26	1.35
After incubation in the dark	λ_{max}	246	248	252	245
	OD	0.99	1.10	1.24	1.34
After incubation in the light	λ_{max}	238	243	244	245
	OD	0.54	0.77	0.74	1.12

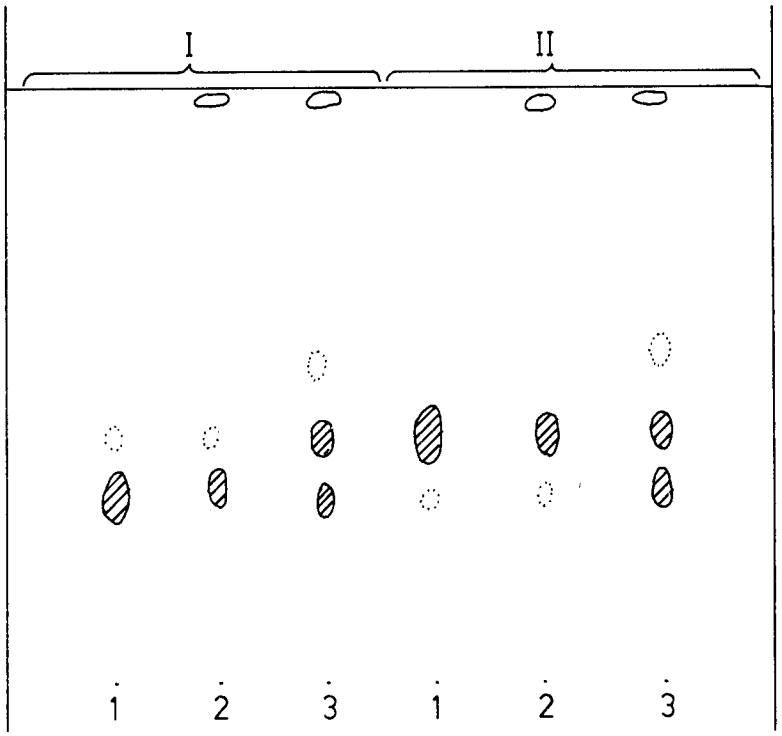


Fig. 4. Thin-layer chromatogram of (±)-2-*cis*-ABA (I) and (±)-2-*trans* ABA (II). 1. Starting material 2. After incubation in darkness. 3. After incubation in light.

was less. The most plausible explanation for this is that isomerization had occurred during incubation in the light and that, as a result, the 2-*cis*-isomer solutions had become less active and those of the 2-*trans* isomer more active.

This was confirmed by thin-layer chromatography of the solutions. The chromatograms of the two isomers in MS medium are given in fig. 4. A similar picture was obtained when the isomers were dissolved in buffer at pH 4.4 or 6.3. The extent of *cis-trans* isomerization was estimated visually to be about 1:1. There was hardly any evidence of decomposition or conversion products on the thin-layer plates.

More quantitative data were obtained from gas chromatography. Figures 5 and 6 and Table 2 show that isomerization had occurred to a slight degree even in the starting material. After autoclaving and incubation in the dark, only the 2-*cis*-ABA solution at pH 4.4 showed any conversion to other products; all the other samples remained pretty well unchanged. The extent of conversion in the light was in approximate agreement with that indicated by the reduction in OD in the UV spectra. The most marked conversion was again found at pH 4.4. The ratio of *cis*:*trans* was indeed about 1:1.

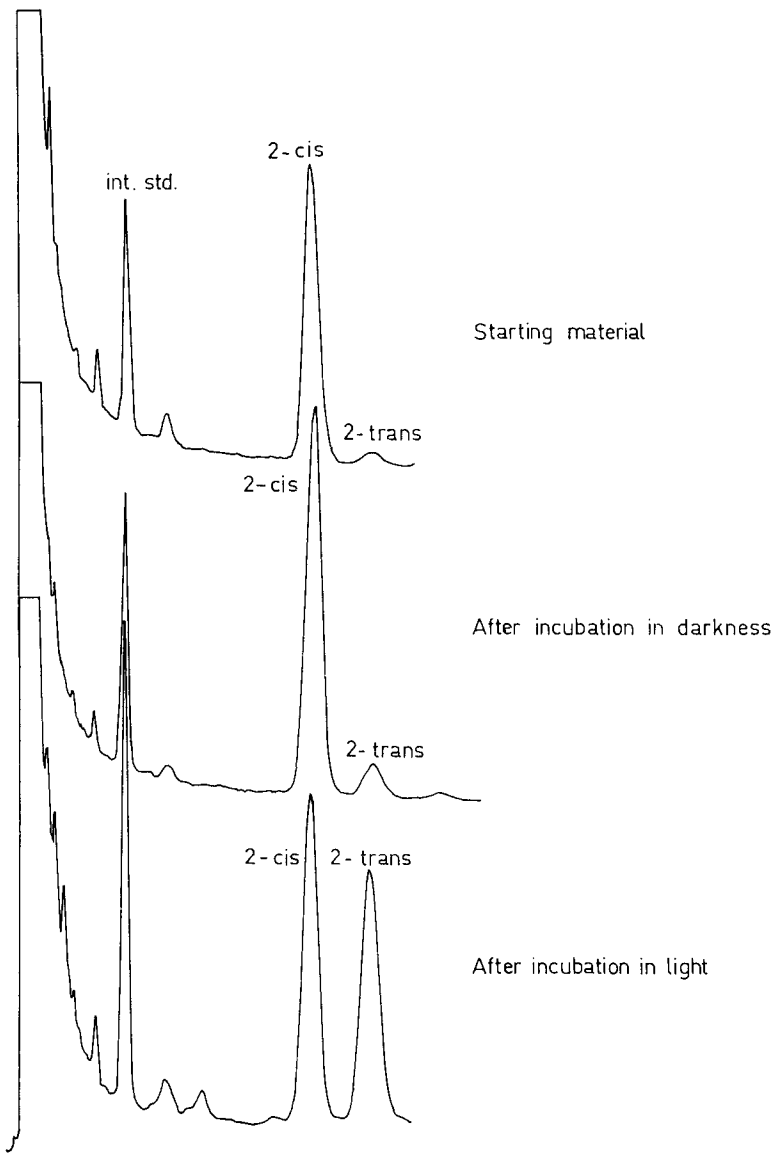


Fig. 5. Incubation of (±)-2-*cis*-ABA in Murashige and Skoog's medium. Gas-liquid chromatography of methyl esters.

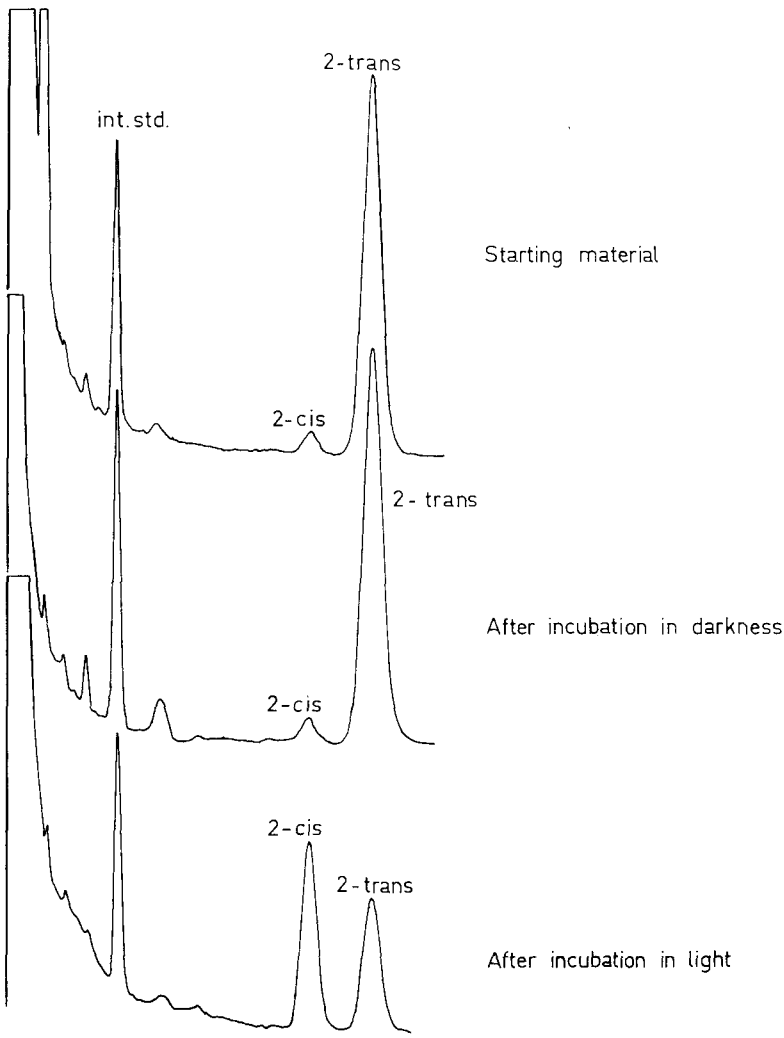


Fig. 6. Incubation of (±)-2-*trans*-ABA in Murashige and Skoog's medium. Gas-liquid chromatography of methyl esters.

Table 2. Effect of aeration, light and pH on the stability of the 2-*cis* and 2-*trans* isomers of absicisic acid. The amounts of each isomer are expressed as % of the total original amount of ABA (approx. 1.5 mg/100 ml).

	Composition	2-*cis*			2-*trans*		
		Buffer, pH 4.4	Buffer, pH 6.3	MS medium pH 5.8	Buffer, pH 4.4	Buffer, pH 6.3	MS medium pH 5.8
Starting material	*cis*	94	95	–	3	4	–
	trans	6	5	–	97	96	–
After incubation in the dark	*cis*	75	94	92	11	6	7
	trans	6	6	8	89	94	93
	lost*	19	0	0	0	0	0
After incubation in the light	*cis*	7	37	44	20	37	47
	trans	9	33	42	24	31	43
	lost*	84	30	14	56	32	10

* converted to other products

4. Conclusions

From the data obtained, we may draw the following conclusions:

1. Both (±)-2-*cis*-ABA and its (±)-2-*trans* isomer are stable to autoclaving for 20 min at 120 °C.
2. They are also stable during storage and aeration in the dark, certainly at pH 5.8 and 6.3.
3. During storage in the light (white light, 16–19 mcal.cm^{-2}.min^{-1}), *cis-trans* isomerization occurs, the final ratio of the two isomers being about 1:1.
4. In addition, light causes chemical conversion; this is more marked at pH 4.4 than at pH 5.8 and 6.3.
5. Since 2-*trans*-ABA is biologically less active than the 2-*cis* isomer, lighting causes an increase in the activity of the former and a decrease in that of the latter.

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