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REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

6. General Requirements for the Sterility of Biological Substances

Report of a Study Group

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WORLD HEALTH ORGANIZATION

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GENEVA

1960

**STUDY GROUP ON GENERAL REQUIREMENTS FOR THE STERILITY
OF BIOLOGICAL SUBSTANCES**

Geneva, 20-25 April 1959

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REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

6. General Requirements for the Sterility of Biological Substances

Report of a Study Group

The Study Group on General Requirements for the Sterility of Biological Substances met in Geneva from 20-25 April 1959.

Dr P. Dorolle, Deputy Director-General, opened the meeting on behalf of the Director-General of the World Health Organization and welcomed the members of the Group.

The Deputy Director-General outlined the task of the Study Group which was to draw up an international recommendation on the general requirements that should be fulfilled by biological preparations in order to ensure their sterility. Many biological preparations are produced by methods which must be supervised with great care in order to avoid the presence of harmful micro-organisms in the final product.

In 1958, a study group formulated General Requirements for Manufacturing Establishments and Control Laboratories dealing with general conditions that must be observed in such laboratories, and some of these requirements directly concern the avoidance of contamination of biological products. Other study groups have formulated requirements for poliomyelitis vaccine, yellow fever vaccine, cholera vaccine, and smallpox vaccine; all these requirements contain sections on sterility control.

The document which the present Study Group was asked to formulate was intended to embody requirements on the safeguarding of sterility applicable to all important biological preparations for which sterility is imperative. These requirements would also include general methods for ensuring the highest attainable confidence in the absence of contaminating viruses. Viral sterility is a new and very difficult subject but one which is rapidly increasing in importance and should therefore be included in the considerations of the Study Group.

1. GENERAL CONSIDERATIONS

The Study Group noted the General Requirements for Manufacturing Establishments and for Control Laboratories contained in Requirements for Biological Substances No. 1.¹ It agreed that these recommendations were applicable in part to the safeguarding of the sterility of biological preparations but that additional requirements for this purpose were needed. The Group also noted the sections concerning sterility of poliomyelitis vaccine contained in Requirements for Biological Substances No. 2,¹ as well as the sterility requirements for yellow fever vaccine (Requirements for Biological Substances No. 3),² cholera vaccine (Requirements for Biological Substances No. 4),² and smallpox vaccine (Requirements for Biological Substances No. 5).³

In its discussions of general requirements for the sterility of biological substances that could be internationally recommended, the Study Group considered the preliminary draft of such general requirements,⁴ a number of working documents, and other unpublished data submitted to the Group.⁵

The Study Group surveyed a number of regulations concerning sterility control adopted in some countries,⁶ and discussed the various points that were of importance for ensuring confidence in the sterility of biological preparations. It agreed that this confidence was based partly on enforcing adequate control tests for the sterility of biological preparations in their

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178

² *Wld Hlth Org. techn. Rep. Ser.*, 1959, 179

³ *Wld Hlth Org. techn. Rep. Ser.*, 1959, 180

⁴ Unpublished working document WHO/BS/IR/73

⁵ Bentzon, M. W., unpublished working document WHO/BS/IR/75; Bonnel, P. H., unpublished working document WHO/BS/IR/72; Eissner, G. & Bonin, O., unpublished working document WHO/BS/IR/36; Penso, G., unpublished working document WHO/BS/IR/18; Tobin, J. O'H., unpublished working document WHO/BS/IR/74

⁶ *British Pharmacopoeia*, ninth edition, 1958, London; Bonnefoi, A., Institut Pasteur, Paris—personal communication; Chief of the Control Laboratory, Statens Bakteriologiska Laboratorium, Stockholm—personal communication; Dekking, F., Laboratorium voor de Gezondheidsleer, University of Amsterdam, Netherlands—personal communication; Japan (1958) Minimum Requirements of Biologic Products, Tokyo, p. 207; Lafontaine, A., Institut d'Hygiène et d'Epidémiologie, Brussels—personal communication; Mayer, G., Gex, M. & Mayer, L. S. (1958) In: Maloine, S. A., ed., *Transfusion sanguine*, Paris, p. 245; *Pharmacopoeia of the United States of America*, fifteenth revision, 1955, Washington, D.C., pp. 841-845; *Pharmacopoea Gallica*, seventh edition, 1949, Paris, pp. 547, 1069 (also Supplement I, 1954, p. 20); *Pharmacopoea Helvetica*, fifth edition, second supplement, 1954, Berne, p. 147; *Pharmacopoea Polonica*, third edition, 1954, Warsaw; *Pharmacopoeia of India*, 1955, New Delhi; Prigge, R., Paul-Ehrlich-Institut, Frankfurt-am-Main—personal communication; *Proceedings of the Third International Meeting of Biological Standardization*, 1957, Opatija; *Proceedings of the Fourth International Congress of Biological Standardization*, 1958, Brussels; *Proceedings of the Seventh International Congress of Blood Transfusion*, 1958, Rome, p. 317; *Transfusion*, 1958, 1, 55

final containers, using a sufficient number of random samples, but that an enforcement of precautions with respect to source materials and manufacturing procedures, as well as continued care during the manufacturing process, also contributed greatly to confidence in the sterility of the final biological products.

The Group agreed that it was very difficult to recommend general requirements for the viral sterility of biological preparations but that the increasing importance of this problem made it necessary to include some recommendations of a general nature concerning this aspect of sterility control. Inactivated and live virus vaccines are being manufactured from a variety of animal tissues. For the purpose of the control of viral sterility each of such preparations is usually treated as a separate problem and special tests are designed to detect specific viral contaminants. The Group noted, however, that the Laboratory of Microbiology of the Istituto Superiore di Sanità, Rome, had introduced general tests for viral sterility in its control of human and veterinary preparations.¹

On the basis of the above-mentioned documents and with the above considerations in mind, the Study Group proceeded to formulate the General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6) which are given in the Annex of this report. In its considerations of requirements for sterility, the Group devoted its attention mainly to injectable biological preparations used in human medicine. It agreed, however, that most of the requirements which it formulated would apply equally to veterinary preparations and that the sterility tests recommended by the Group might be found useful by those whose task it was to ensure the sterility of pharmaceutical substances.

2. PROBLEMS NEEDING FURTHER INVESTIGATION

2.1 Culture media

The Study Group noted that a large number of different culture media were in use in different countries for testing for the presence of contaminating bacteria, yeasts and fungi in biological preparations. The Group agreed that the recommendations which it had been asked to formulate would be most useful if they included a description of a number of culture media which had been adopted for this purpose in various institutes and control laboratories. It therefore appended the formulae for a number of media to the Requirements but it wished to emphasize that the list of media given was not comprehensive and that certain media not given in the list might be equally suitable for use in sterility control tests.

¹ Penso, G., unpublished working document WHO/BS/IR/18

The Group recommended that a number of laboratories currently engaged in performing sterility control tests be asked to exchange culture media and information concerning their preparation and use, and to perform a series of tests in a systematic way in order to collect data to make it possible to decide which of the media were most satisfactory for their purpose.

2.2 Temperatures and times of incubation

The Group considered the information submitted by its members concerning the most suitable temperatures of incubation for the detection of contaminating bacteria and fungi, as well as the minimum time during which this incubation should be allowed to proceed in order to observe the growth of contaminants. The Group agreed that a high degree of security would be obtained if general tests for sterility were performed concurrently at two different temperatures, one in the range 15°-22°C and the other in the range 35°-37°C, but that the performance of a general test at one temperature only within the intermediate range of about 30°-32°C might be considered adequate. For the detection of contaminating yeasts and fungi, the Group agreed that incubation at one temperature in the range 20°-25°C would be adequate.

The Study Group recommended that further research concerning the optimum temperatures for incubation should be carried out with respect to the detection of bacteria and fungi which present a contamination risk. Such research could profitably be done in conjunction with the comparison of different culture media as recommended in section 2.1, and would also permit the collection of further data on the possible importance of increasing the minimum time of observation required in the general tests for sterility as drafted by the Group.

2.3 Sampling

The Study Group considered various rules for sampling from finished containers which had been proposed or adopted in different countries. It agreed that it was not possible to decide which of these rules for sampling should be preferred and that the Requirements should permit the adoption of any rule, based on the principles of sampling statistics, found to be satisfactory by the national control authorities of individual countries. Though a number of different countries had so far adopted different rules for sampling, the actual number of final containers sampled according to these rules, and the volumes inoculated, did not vary greatly. The Group decided to attach a table to the requirements incorporating some sampling procedures in current use (see Annex, Appendix 2, p. 31).

In its consideration of the principles for sampling among final containers, the Group noted a proposal by one of its members,¹ according to which the number of containers sampled is proportional to the square root of the total number of containers in a given final lot. This rule has been derived from a determination of the optimal distribution of any total number of samples taken among different final lots, such that it permits a minimum of contaminated final containers to be released, assuming the occurrence of contamination to be as "unfavourable" as possible. An "unfavourable" occurrence of contamination is a situation in which the number of contaminated final containers, while not extremely small is not sufficiently large to be detected with a high degree of certainty. The Group observed that the principle of making the number of samples proportional to the square root of the number of containers in each final lot was intermediate between two other principles that have hitherto been adopted elsewhere, namely, that of taking a fixed percentage of final containers from each final lot, and that of taking a fixed number of containers from each final lot independent of the size of a lot.

The Group recommended that further expert opinions be obtained concerning the relative value of these different principles as a basis for an optimal sampling procedure for sterility tests. It agreed that it was important to define clearly and unambiguously what constituted the final bulk material and the final lots of final containers from which sampling should be done, and what minimum volumes from the amount of material sampled should be inoculated for sterility tests.

2.4 Collection of further data

The Group recognized that though very large experience on sterility testing must have accumulated in many laboratories engaged in this work, only few data, concerning the results obtained in the performance of such tests, had so far been published.² It agreed that the availability of more actual data of this kind would make it easier to decide on rules to be adopted and the Group recommended that an effort be made to collect such data in order to obtain a factual basis for revision at a later date of the requirements drawn up by the Group.

Consideration should also be given to the importance of the environment in which biological preparations were produced and tested, since the relative risk of contamination would vary in different climates and under different manufacturing conditions.

¹ Bentzon, M. W., unpublished working document WHO/BS/IR/75

² *Proceedings of the Third International Meeting of Biological Standardization*, 1957, Opatija; *Proceedings of the Fourth International Congress of Biological Standardization*, 1958, Brussels; *Proceedings of the Seventh International Congress of Blood Transfusion*, 1958, Rome, p. 317; *Transfusion*, 1958, 1, 55

2.5 Training

In view of the fact that biological preparations are used on a very large scale in all populations of the world, and having regard to the importance of enforcing the sterility of the products manufactured for this purpose, the Group considered it to be highly recommendable that an opportunity be given to the staff engaged in this control in various countries to obtain adequate training in the techniques employed by expert laboratories. Such training is rapidly becoming increasingly important as the techniques that are required for the detection of viral contaminants are becoming more delicate and exacting. The Group therefore recommended that the World Health Organization study the possibility of providing adequate training in this field of public health laboratory work.

Annex

**GENERAL REQUIREMENTS FOR THE
STERILITY OF BIOLOGICAL SUBSTANCES
(REQUIREMENTS FOR BIOLOGICAL SUBSTANCES No. 6)**

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General Considerations

The present recommendations of general requirements for sterility concern those biological substances for which sterility is essential and which are used currently in the prophylaxis, treatment and diagnosis of human disease. Parts of these recommendations may be applied equally to veterinary preparations and pharmaceutical substances.

Its provisions are based on experience accumulated and techniques currently used in ensuring the sterility of biological preparations. Future revisions of these requirements will therefore be necessary.

The general requirements for viral sterility in the present document represent a first attempt to provide guidance for ensuring the absence of virus contamination in biological preparations at risk.

Each of the following sections constitutes a recommendation. The parts of each section which are printed in large type have been written in

the form of requirements so that, if a health administration so desires, these parts as they appear may be used as definitive national requirements. The parts of each section which are printed in small type concern points on which comments seemed desirable.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning sterility, it is recommended that a clause be included which would permit modifications on the condition that such modifications had been demonstrated, to the satisfaction of the national control authority, to ensure that the degree of confidence in the sterility of a biological preparation is at least equal to that provided by the requirements formulated below. The World Health Organization should then be informed of the action taken.

The terms "national control authorities" and "national control laboratories", as used in these requirements, always refer to the country in which the biological substance is manufactured.

Part A. Requirements for Manufacturing Establishments

1. Definition

This document contains the general requirements applicable to the sterility of all biological preparations intended for injections as well as to other biological preparations which must be sterile.

The word sterility signifies the absence of all micro-organisms capable of multiplying. Wherever a preparation itself consists of living micro-organisms the requirements for sterility refer to a control of purity aiming at the exclusion of micro-organisms of extraneous origin.

The general requirements will have to be supplemented in order to cover biological preparations which pose particular problems in sterility control.

2. Terminology

Contamination : The presence of a live extraneous micro-organism in a biological preparation.

Micro-organisms : Bacteria, yeasts, fungi or viruses.

Final bulk material : The finished biological preparation present in the container from which the final containers are filled.

Final lot : A collection of sealed, final containers that are homogeneous with respect to the risk of contamination during filling or drying. A final lot must, therefore, have been filled in one working session, and (if applicable) have been dried together. The final lot is the collection of sealed,

final containers from which samples are selected for one sterility test, and it shall be discarded if the results of this test fail to satisfy the acceptance criteria set out in the following requirements.

3. General precautions against contamination

The general requirements given in Requirements for Biological Substances No. 1¹ are applicable with the addition of the following.

3.1 *General precautions during production*

All the necessary precautions shall be taken during the production of biological substances to prevent contamination by micro-organisms.

Confidence in the sterility of a biological preparation cannot rest solely on sterility tests because organisms with exacting cultural requirements may not be detected by general tests and because contaminating micro-organisms may not be present in the samples taken.

3.2 *Precautions in establishments producing virus vaccines*

The manufacture of each virus vaccine shall be conducted in separate areas which are isolated from each other.

The air-conditioning and the drainage system should be so designed as to prevent dissemination of micro-organisms from one production area to another.

The efficacy of measures taken to reduce contamination from the air should be controlled periodically by an appropriate method of air sampling, and by careful control of the air filtration.

All material from an area in which virus is being processed shall be autoclaved before being sent to the washing and sterilizing departments, irrespective of whether each area has its own facilities or whether communal facilities are employed.

If the same area is used for the successive production of different vaccines, the area as well as all equipment it contains shall be adequately disinfected after the processing of one vaccine has been completed and before the processing of another vaccine can be commenced.

A common washing and sterilization area for glassware and culture media may be used where two or more virus vaccines are being produced, provided that only heat-sterilized materials

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1959. 178

are issued for use in the production areas and that each production area has its own equipment suitably marked.

All media that are sterilized by filtration alone should be prepared in a separate area with its own equipment for the preparation of such media. Only material sterilized by heat should be introduced into this area from the main washing and sterilizing area. It is preferable that autoclaves used for the sterilization of materials passing in or out of an area be of the "double-ended" type, built into the wall in such a manner that the only communication between areas containing unsterilized and sterilized materials is through the autoclave itself.

Tissue cultures for vaccine production shall be prepared in a special area separated from any area in which virus is being handled.

The removal of monkey kidneys for production of cell cultures shall be carried out in a room which has been set aside for this single purpose and which shall be thoroughly cleaned and disinfected after each group of monkeys has been processed. The operation on each monkey shall be done under aseptic precautions.

Great care is necessary during the removal of monkey kidneys to prevent contamination of the kidney material with extraneous viruses and other micro-organisms from the animal and the environment.

The room and equipment used for filling a live virus vaccine into containers shall be used solely for this particular vaccine, and it shall be disinfected if required for the filling of other biological preparations.

4. Source materials

Precautions shall be taken to avoid contamination from source materials which may affect the sterility of the final product.

4.1 Strains of micro-organisms

All strains of micro-organisms used as source materials for biological preparations shall be maintained in a manner that will assure freedom from contaminating micro-organisms.

4.2 Animals

Animal source materials, such as blood, tissues and embryos used for the preparation of biological substances shall be derived only from healthy animals.

Sections 2.5 and 3.5 of the General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological

Substances No. 1)¹ and section 3.1.2 of the Requirements for Poliomyelitis Vaccine, Inactivated (Requirements for Biological Substances No. 2)¹ shall apply.

Blood from animals shall be taken aseptically.

4.3 *Human donors*

Blood plasma and other substances obtained from human blood or tissues shall not be used as a source material or as a component of biological preparations, except for products consisting essentially of human blood or tissues, or of components of human blood or tissues.

A clinical examination of a human donor of blood must be made and consideration given to the medical history in order to exclude, as far as possible, the presence of hepatitis virus and other transmissible agents. In order further to reduce the risk of hepatitis,² pools of blood from human donors should contain material from as few donors as possible, in any case not more than ten. Specific requirements are necessary to formulate all precautions that should be observed when preparing human plasma and other products of human blood or tissues.

5. Sterility tests

The general precautions against contamination as set out in the General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)¹ shall apply to the performance of sterility tests. The tests shall be done in areas reserved for this purpose and distinct from the production areas. The area should be free from all unnecessary equipment.

The staff performing the sterility tests should have adequate scientific knowledge and experience for the duties they undertake. They should observe all rules and precautions laid down for the testing procedures.

The risk of accidental contamination from the environment during the performance of the sterility tests can be reduced by methods such as the use of germicidal lamps, air filters, and sterilized clothing, by allowing adequate space for the number of people working so that air movement is reduced to a minimum, and by other appropriate measures.

An adequate number of samples from the product shall be taken as laid down in section 5.1 and the sterility of these samples shall be established by the procedures laid down for bacterial sterility in section 5.2 and for

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178

² *Wld Hlth Org. techn. Rep. Ser.*, 1953, 62

mycotic sterility in section 5.3, in accordance with the criteria given in section 5.4.

Tests for viral sterility are outlined in section 5.5.

Though the tests required for bacterial and mycotic sterility concern only the final bulk material and the product in its final containers, it is recommended that samples from earlier stages of production should be tested in order to ensure sterility throughout the manufacturing process.

5.1 *Sampling*

Samples shall be taken from the final bulk material of each batch of a biological substance before filling as well as from each final lot of final containers. Unless otherwise stated samples shall be collected at random.

5.1.1 *Sampling from the final bulk material*

A representative sample shall be taken from each final bulk after thorough mixing of the material. The volume taken shall be sufficient for the performance of the tests set out in sub-sections 5.2.1.2 and 5.3.1.2.

In cases where it is undesirable to hold up the filling into final containers before the completion of the sterility test on the final bulk material, the sample taken from each final bulk should be sufficient to allow tests of sterility to be repeated if necessary.

5.1.2 *Number of final containers to be sampled*

Samples of final containers shall be taken at random from each final lot after filling and sealing, but in such a manner that their selection is spread throughout the filling period.

The number of containers to be sampled from each lot shall be so chosen as to reduce to a minimum the risk of releasing contaminated containers. The rules adopted for this sampling shall be approved by the national control authorities.

In some countries, a fixed percentage of final containers is sampled from each final lot. In other countries a fixed number of final containers is sampled from each final lot, independent of its size. An intermediate rule might be recommendable.

Sampling procedures in current use are listed in Appendix 2, page 31.

5.2 *Tests for bacterial sterility*

All biological substances for which sterility is required shall pass the tests for bacterial sterility given in section 5.2.1, according to the acceptance criteria laid down in section 5.4.

Special tests may be necessary if the product contains bacteriostatic factors or whenever the product consists of living micro-organisms. In some products the possible presence of specific pathogens may have to be excluded by special tests (see section 5.2.2.).

5.2.1 *General test for bacterial sterility*

5.2.1.1 *Culture media*

The culture medium or media used in this test shall be capable of supporting the growth of contaminating bacteria.

The media selected should be tested for their growth-supporting properties by the inoculation of micro-organisms frequently met as contaminants. There should also be evidence that the media permit the growth of organisms such as *Staphylococcus aureus*, *Streptococcus pyogenes* (Group A) and anaerobic spore bearers.

The formulae and methods of preparation of some media currently used are given in Appendix 1, page 22. The use of a medium capable of supporting the growth of both aerobes and anaerobes is recommended.

5.2.1.2 *Performance of the test*

From the sample taken from each final bulk at least 5 ml shall be inoculated into one or more vessels of each culture medium selected, and for each temperature of incubation.

From each of the final containers sampled from a final lot, an amount of at least 1.0 ml, or if solid at least 25 mg, or at least one-half of the human dose as recommended by the manufacturer, whichever is the larger, shall be inoculated into one or more vessels of each culture medium selected and for each temperature of incubation.

If the amount of material in a final container is insufficient to satisfy this rule additional containers shall be sampled, except in special cases where the requirements for a particular biological substance permit the testing of a smaller amount. Not more than 10 ml, or if solid 500 mg, need be tested from any one container.

The volume of medium used in any vessel shall be sufficiently large in relation to the volume of material inoculated not to impair the growth-supporting properties of the medium.

All vessels shall be incubated at the selected temperature or temperatures. If two temperatures are chosen these shall be in the ranges 15°-22°C and 35°-37°C. If only one temperature is chosen it shall be in the range 30°-32°C.

All vessels shall be incubated for a period of at least seven days and shall be examined at regular intervals and on the last day of incubation for bacterial growth.

Incubation periods exceeding seven days may be necessary, depending on the nature of the preparation and on the bacteriostatic substance that may have been added.

If the substance inoculated has clouded the media to such an extent that it is difficult to recognize whether growth has taken place, subcultures should be made. These should be incubated and observed under the same conditions as the first vessels inoculated.

All inoculated vessels shall be clearly identified by adequate labelling. A record shall be kept of the name and identifying numbers of the product under test, the quantities inoculated, the batch and type of culture media used, the temperatures of incubation, the dates of inoculation and the test results.

5.2.2 *Other types of test*

5.2.2.1 *Bacteriostatic factors*

In order not to invalidate the test for bacterial sterility, the growth-inhibitory effect of a preparation that kills or prevents the growth of bacteria, or of a preparation containing added bactericides or bacteriostatics, shall be neutralized, or the volume of medium into which the sample of such a preparation is inoculated for the sterility test shall be sufficiently large to make the growth inhibitors ineffective.

A substance may be added which destroys or neutralizes the inhibitor provided it does not, in the concentration employed, interfere with the growth of bacteria; or the sample may be tested in a medium which itself neutralizes the growth inhibitors without interfering with the growth of bacteria.

Media containing sodium thioglycollate or hydrosulfite have an inhibitory effect on certain preservatives and bacteriostatics but the degree of this effect varies considerably.

The amount of dilution required should be controlled for each bacteriostatic or bactericide by seeding the medium selected—already containing the product under test—with a relevant test bacterium.

For the sterility control of certain antibiotics special methods are needed to overcome their inhibitory effect.

5.2.2.2 *Tests for specific bacteria*

In the case of vaccines consisting of killed bacteria a test shall be done designed to exclude the possibility that any of these bacteria have survived the killing procedure. The general sterility test described in section 5.2.1

shall suffice unless the bacteria have special growth requirements, in which case an additional test shall be performed using an adequate medium.

In certain cases it will also be necessary to supplement the general test for bacterial sterility by tests designed to detect specific bacteria which present a special risk for certain biological substances.

Modifications of the general sterility test will be necessary for preparations of living bacteria (e.g., B.C.G.). Such tests will be described in the requirements for particular biological substances.

5.3 *Tests for mycotic sterility*

All biological substances for which sterility is required shall pass the tests for mycotic sterility given in section 5.3.1, according to the acceptance criteria laid down in section 5.4.

Special tests may be necessary if the product contains fungistatic factors (see section 5.3.2).

5.3.1 *General test for mycotic sterility*

5.3.1.1 *Culture media*

The culture medium used in this test shall be capable of supporting the growth of contaminating yeasts and fungi.

The medium selected should be tested for its growth-supporting properties.

The formulae and methods of preparation of some media currently used are given in Appendix 1, page 22.

5.3.1.2 *Performance of the test*

From the sample taken from each final bulk at least 5 ml shall be inoculated into one or more vessels of the culture medium selected.

From each of the final containers sampled from a final lot, an amount of at least 1.0 ml, or if solid at least 25 mg, shall be inoculated into one or more vessels of the culture medium selected. If the amount of material in a final container is insufficient for this purpose, additional containers shall be sampled, except in special cases where the requirements for a particular biological substance permit the testing of a smaller amount.

The volume of medium used in any vessel shall be sufficiently large in relation to the volume of material inoculated not to impair the growth-supporting properties of the medium.

All vessels shall be incubated at a temperature in the range 20°-25°C for a period of at least 14 days and shall be observed for mycotic growth at regular intervals and on the last day of incubation.

All inoculated vessels shall be clearly identified by adequate labelling. A record shall be kept of the name and identifying numbers of the product

under test, the quantities inoculated, the batch and type of culture media used, the temperature of incubation, the dates of inoculation and the test results.

5.3.2 *Fungistatic factors*

In order not to invalidate the test for mycotic sterility, the growth-inhibitory effect of a preparation that kills or prevents the growth of yeasts and fungi, or of a preparation containing added fungicides or fungistatics, shall be neutralized, or the volume of medium into which the sample of such a preparation is inoculated for the sterility test shall be sufficiently large to make the growth inhibitors ineffective.

If the dilution method is used it should be controlled by seeding the medium—already containing the product under test—with a very dilute suspension of *Candida albicans*. The duplicate medium inoculated should show growth within 48 hours.

5.4 *Acceptance criteria*

All biological substances shall comply with the criteria given in subsections 5.4.1 and 5.4.2, except when a manufacturer submits data showing to the satisfaction of the national control authorities that another method of testing for sterility is as good as or superior to that set out in these Requirements; or when the national control authorities decide that a sterility test is unnecessary, impracticable, or precluded; or when they permit a certain level of contamination with nonpathogenic micro-organisms because of the nature of a particular biological substance.

5.4.1 *Acceptance criteria for the final bulk*

If no growth occurs in any of the vessels inoculated for the tests of sterility set out in sections 5.2 and 5.3 the final bulk material shall be deemed to have passed the sterility test.

If growth occurs in any of the vessels inoculated, either the final bulk material shall be deemed to have failed the sterility test, or a second test shall be performed in the culture media and at the temperatures of incubation where growth occurred. If no growth occurs in this second test, the final bulk material shall be deemed to have passed the sterility test; if growth again occurs, the material shall be deemed to have failed the test.

If there is conclusive evidence that contamination encountered in culture vessels during a sterility test is due to a source other than the samples themselves, the test should be considered invalid and should be repeated.

5.4.2 *Acceptance criteria for a final lot of final containers*

If no growth occurs in any of the vessels inoculated for the tests of sterility set out in sections 5.2 and 5.3 the final lot of final containers shall be deemed to have passed the test for sterility.

If growth occurs in the vessels inoculated from three or more final containers of one final lot, this final lot shall be deemed to have failed the test for sterility.

If growth occurs in the vessels inoculated from one or two final containers of one final lot, either this final lot shall be deemed to have failed the sterility test, or a second test shall be performed in the culture media, and at the temperatures where growth occurred using samples from the same number of final containers as in the first test. If no growth occurs in this second test, the final lot shall be deemed to have passed the test for sterility. If growth occurs in more than one of the vessels inoculated for this second test, the final lot shall be deemed to have failed the sterility test.

If growth occurs in only one vessel in the second test, either the final lot shall be deemed to have failed the sterility test or a third test shall be permitted, provided that the organism detected in the second test has been shown to be different from any organisms found in the first test. If no growth occurs in the third test, the final lot shall be deemed to have passed the test for sterility. If growth again occurs, the final lot shall be deemed to have failed the test.

If there is conclusive evidence that contamination encountered in culture vessels during a sterility test is due to a source other than the samples themselves, the test should be considered invalid and should be repeated.

5.5 *Tests for the presence of viruses*

The tests for viral sterility considered in the following are applicable mainly to viral and rickettsial vaccines. They are in general unnecessary for biological substances made from materials incapable of supporting the multiplication of these agents.

The problems presented by the control for viral sterility arise in a large part from the source material used in the manufacture and processing of the product. Tests can only be applied to those virus agents which produce some detectable effect in animals, embryonated eggs, tissue cultures or other diagnostic systems. The pathogenicity for man of some of the contaminating agents encountered in the production of viral vaccines is not known. The preclusion of viruses that are at present unknown and undetectable by the methods now available may become of importance at any time.

General principles of tests for viral sterility apply mainly to vaccines, the actual techniques used being dependent on the vaccine concerned.

When there is a possibility that the source material of vaccines is infected, special testing procedures may be necessary.

5.5.1 *General tests applicable to viral vaccines*

5.5.1.1 *Seed virus*

Tests shall be done to ascertain that the seed lots of virus used for vaccine production are free from extraneous viruses.

Examples of the use of seed lot systems have been set out in Requirements for Yellow Fever Vaccine (Requirements for Biological Substances No. 3)¹ and in Requirements for Smallpox Vaccine (Requirements for Biological Substances No. 5).² Aliquots of such lots should be tested in the same *in vivo* or *in vitro* culture systems as were used for the production of these lots—after neutralization of seed lot virus by homotypic serum, free from inhibitors to any extraneous virus likely to be present—or in other systems, such as systems resistant to the seed virus but not to the contaminating viruses for which such a test is designed.

5.5.1.2 *Growth tissue*

Where applicable, tests shall be done to exclude the presence of contaminating viruses in the systems employed for growing the virus incorporated in the vaccine.

Such tests should be done whenever there is doubt that viruses capable of transmission are absent from the animal tissue in which the virus to be incorporated in the vaccine is to be grown.

5.5.1.3 *Final vaccine*

Where applicable, tests for detecting contaminating viruses shall be done on samples of the final product, or at the last stage of production at which adequate tests can be done.

These should include tests in the same system as employed for the production of the virus material incorporated in the vaccines, whether or not the inactivation of the virus incorporated in the vaccine is tested in another cell system. The search for extraneous virus contaminants in live vaccines should be done by methods similar to those given in sub-section 5.5.1.1.

All test systems shall be free from inhibitors to the viruses under test.

Serum should not be used in tissue culture systems unless their sensitivity is impaired by its absence. All sera should be tested for inhibitors before use. Where possible, hyperimmune

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1959, 179

² *Wld Hlth Org. techn. Rep. Ser.*, 1959, 180

monotypic neutralizing sera should be prepared in hosts different from those used for supplying culture material for virus production.

5.5.2 *Additional tests applicable to vaccines made from monkey tissue cultures*

Before inoculation of virus, all cell cultures shall be examined macroscopically for signs of gross contamination and those infected discarded.

If possible material for inactivation should be free from all micro-organisms other than that from which vaccine is to be prepared.

Samples of the original medium used for cell culture initiation should be taken from each culture vessel at the time the medium is changed prior to their inoculation with virus. A suitable number of aliquots from each vessel should then be combined and tested for fungal and bacterial sterility.

Depending on the method of manufacture the presence of extraneous viruses should be excluded by microscopical observation of vessels or by subculture into fresh monkey kidney cell cultures of an aliquot of a pool of these samples. Fluids from infected bottles should not be included in pools for inactivation. These pools should, if necessary, be tested for B virus before inactivation.

The procedures recommended above will not be adequate for the control of sterility of tissues or cells used for live virus vaccine production. Stringent control of cultures will be necessary, together with tests on adequate volumes for the detection of possible viral contaminants.

5.5.3 *Mixed vaccines*

In mixed or combined vaccines each component shall be tested for sterility by an appropriate method, before blending.

After blending, the tests for sterility of the final containers can be limited to bacterial and fungal sterility if this is approved by the national control authority.

6. Protocols

The general requirements concerning protocols given in Part A, section 6, of the General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances, No. 1)¹ shall apply.

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178

7. Additional samples

The general requirements concerning additional sampling given in Part A, section 7, of the General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)¹ shall apply.

Part. B. National Control Requirements

1. General

The general requirements for control laboratories given in Part B of the General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)¹ shall apply.

Appendix 1

Formulae and Methods of Preparation of a Number of Media currently used for Sterility Tests in various Control Laboratories

Some of the media listed are available commercially in a dried form which requires only the addition of distilled water, distribution into the appropriate vessels, and sterilization, before being ready for use. This avoids the necessity of preparing these media from the individual ingredients.

Each batch of a medium should be tested for its growth-supporting properties as indicated in Part A, sections 5.2.1.1 and 5.3.1.1 of this document.

A. Media for the Detection of both Aerobic and Anaerobic Bacteria

A.1 Fluid thioglycollate medium^{2, 3, 4}

Formula :

| | |
|--|-------|
| L-Cystine | 0.5 g |
| Sodium chloride | 2.5 g |
| Dextrose (C ₆ H ₁₂ O ₆ ·H ₂ O) | 5.5 g |

¹ *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178

² Pharmacopeia of the United States of America, fifteenth revision, 1955, Washington, D.C., p. 841

³ American Pharmaceutical Association (1955) *National Formulary*, 10th ed., Washington, D.C., p. 720

⁴ National Institutes of Health (1955) *Memorandum on culture media for the sterility test*, fourth revision, Bethesda, Md.

| | |
|--|---------|
| Granular agar (less than 15% moisture by weight) | 0.75 g |
| Yeast extract (water-soluble). | 5.0 g |
| Pancreatic digest of casein | 15.0 g |
| Distilled water | 1000 ml |
| Sodium thioglycollate | 0.5 g |
| or thioglycollic acid | 0.3 ml |
| Resazurin (0.10% fresh solution). | 1.0 ml |

Final pH = 7.1 ± 0.1

Preparation :

Mix all the ingredients except the thioglycollate and the resazurin in the order given above in a mortar, thoroughly grinding each as it is added. Stir in a portion of the water, previously heated, transfer to a suitable container, add the remainder of the water and complete the solution by heating in a boiling water bath. Add the sodium thioglycollate. Adjust the reaction with 1 N sodium hydroxide so that the pH of the completed and sterilized medium will be 7.1 ± 0.1 . Reheat the solution, but do not boil, filter (if necessary) through a moistened filter paper and add the resazurin solution. Distribute the medium into suitable vessels and sterilize in an autoclave for 18-20 minutes at 121° - 123° C (15-17 lb. pressure). Cool promptly to 25° C and store at 20° - 30° C, avoiding excessive light. If more than 30% of the uppermost portion of the medium has changed to a pinkish colour it is unsuitable for use. The medium may be restored once by heating in a boiling water bath until the pink colour disappears. Medium more than three weeks old should not be used unless kept under seal.

A.2 Semi-fluid sodium hydrosulfite medium ¹

Formula :

| | |
|--|---------|
| Sodium chloride. | 2.5 g |
| Dextrose ($C_6H_{12}O_6 \cdot H_2O$). | 5.5 g |
| Yeast extract | 5.0 g |
| Peptone | 15.0 g |
| Sodium hydrosulfite ($Na_2S_2O_4 \cdot 2H_2O$) | 0.5 g |
| Shredded agar | 1.3 g |
| Resazurin. | 0.001 g |
| Distilled water | 1000 ml |

Final pH = 7.1 ± 0.1

¹ Bonnel, P. H. & Raby, C. (1957) In : *Proceedings. Third International Meeting of Biological Standardization. 2-6 September 1957, Opatija*, p. 327

Preparation :

To 1000 ml of distilled water add sodium chloride, peptone, yeast extract and then the agar which has first been allowed to soak for 12 hours. Complete the solution by heating in a boiling water bath. Adjust to pH 7.3-7.5 with 40% sodium hydroxide, autoclave at 120° for 30 minutes, filter while hot, and add dextrose and sodium hydrosulfite (a technical grade of sodium hydrosulfite is satisfactory). If necessary heat at 80°C to dissolve, but do not boil. Check pH and adjust the reaction with 40% sodium hydroxide so that the pH of the completed and sterilized medium will be 7.1 ± 0.1 . Add resazurin and mix carefully for 10-15 minutes. Distribute about 15 ml to tubes with internal diameters of 15-17 mm. Sterilize at 110°C for 30 minutes and then cool the tubes rapidly by placing in cold water.

Store medium at 20°-30°C. Provided that less than 30% of the medium is pink, it is suitable for use (see medium A.1). It can be regenerated once by boiling for 15 minutes in a water bath. Cool rapidly after heating.

A.3 Corn-steep-liquor/sodium thioglycollate medium¹**Formula :**

| | | |
|--|-------|----|
| Corn-steep-liquor (10% solution) | 300 | ml |
| Meat extract | 5.0 | g |
| Dextrose (C ₆ H ₁₂ O ₆ ·H ₂ O) | 15.0 | g |
| Sodium chloride | 7.0 | g |
| Sodium thioglycollate | 1.0 | g |
| Sodium hydrosulfite (Na ₂ S ₂ O ₄ ·2H ₂ O) | 0.5 | g |
| Granular agar | 0.7 | g |
| Resazurin | 0.002 | g |
| Distilled water | 700 | ml |

Sterilize in autoclave for 20 minutes at 115°C
Final pH = 7.0 ± 0.1

Preparation :

Prepare a 10% corn-steep-liquor solution, adjust the reaction to pH 7.2 with sodium hydroxide and heat at 120°C for 10 minutes. Filter while hot through moistened filter paper. Dissolve the other substances in the distilled water and add the corn-steep-liquor ; sterilize in an autoclave for 20 minutes at 115°C.

The addition of meat extract improves the growth-promoting qualities for proteolytic *Clostridia* without affecting the growth of the most exacting strains like *Corynebacterium diphtheriae*.

¹ Penso, G., unpublished working document WHO/BS/IR/18

A.4 Romanov-D-hydrosulfite medium¹*Formula :*

| | | |
|--|-------|----|
| Meat water 1 : 2 | 750 | ml |
| D-peptone | 250 | ml |
| Shredded agar | 0.75 | g |
| Dextrose (C ₆ H ₁₂ O ₆ ·H ₂ O) | 5.5 | g |
| Sodium hydrosulfite (Na ₂ S ₂ O ₄ ·2H ₂ O) | 1.0 | g |
| Methylene blue | 0.002 | g |

Sterilize in autoclave

Final pH = 7.2 ± 0.1

D-peptone is an autoprotoeolytic product of minced pig stomach and of young live yeasts obtained in the presence of free hydrochloric acid at 50°C.

This medium was designed for sterility control of preparations containing merthiolate.

A.5 Modified Brewer medium²*Formula :*

| | | |
|--|-------|----|
| Infusion from beef | 500.0 | g |
| Peptone | 10.0 | g |
| Dextrose (C ₆ H ₁₂ O ₆ ·H ₂ O) | 5.5 | g |
| Sodium chloride | 5.0 | g |
| Dipotassium phosphate | 2.0 | g |
| Sodium thioglycollate | 0.5 | g |
| Shredded agar | 0.5 | g |
| Methylene blue | 0.002 | g |
| Distilled water | 1000 | ml |

Sterilize in autoclave for 20 minutes at 121°C

Final pH = 7.2 ± 0.1

A.6 Brain heart infusion medium³*Formula :*

| | | |
|-------------------------------------|-------|---|
| Calf brain, infusion from | 200.0 | g |
| Beef heart, infusion from | 250.0 | g |
| Peptone | 10.0 | g |

¹ Didenko, S. I. (1957) In: *Proceedings. Third International Meeting of Biological Standardization, 2-6 September 1957, Opatija*, p. 359

² Pittman, M., National Institutes of Health, Bethesda, Md.—personal communication

³ American Pharmaceutical Association (1950) *National Formulary*, 9th ed., Washington, D.C., p. 619 (This reference gives the formula without the addition of agar.)

| | | |
|--|------|----|
| Dextrose ($C_6H_{12}O_6 \cdot H_2O$) | 2.0 | g |
| Sodium chloride. | 5.0 | g |
| Disodium phosphate. | 2.5 | g |
| Shredded agar | 1.2 | g |
| Distilled water | 1000 | ml |

Sterilize in autoclave for 15 minutes at 121°C

Final pH = 7.4 ± 0.1

A.7 Thioglycollate broth medium¹

Formula :

| | | |
|--|------|----|
| L-Cystine | 0.5 | g |
| Sodium chloride. | 2.5 | g |
| Dextrose ($C_6H_{12}O_6 \cdot H_2O$) | 5.5 | g |
| Yeast extract (water soluble) | 5.0 | g |
| Pancreatic digest of casein | 15.0 | g |
| Distilled water | 1000 | ml |
| Sodium thioglycollate | 0.5 | g |
| or thioglycollic acid | 0.3 | ml |

Final pH = 7.1 ± 0.1

Preparation :

Prepare as for medium A.1., except that no agar or resazurin is added. Adjust the solution with 1 N sodium hydroxide so that, after sterilization, it will have a pH of 7.1 ± 0.1 . Filter if necessary and distribute to Smith's fermentation tubes. Sterilize in the autoclave at 120°C for 20 minutes. Within four hours before use, heat the tubes in a boiling water bath to drive off the oxygen in the deep part of the medium.

This medium can be used for viscous and semi-fluid preparations.

B. Media for the Detection of Aerobic Bacteria

B.8 Peptone broth²

Formula :

| | | |
|---------------------------|------|----|
| Beef extract. | 5.0 | g |
| Peptone | 10.0 | g |
| Sodium chloride. | 5.0 | g |
| Distilled water | 1000 | ml |

Final pH = 6.9 ± 0.1

¹ National Institutes of Health (1955) *Memorandum on culture media for the sterility test*, fourth revision, Bethesda, Md.

² Formula based on common laboratory practice

Preparation :

Heat the ingredients gently with shaking until solution is effected. Filter the hot mixture through good quality filter paper. Adjust the reaction with 1 N sodium hydroxide so that after sterilization the pH will be 6.8-7.0. Autoclave at 120°C for five minutes to precipitate phosphates. Filter hot through a muslin filter. Distribute 15 ml of the medium into suitable test-tubes and autoclave at 115°C for 20 minutes (20 minutes from the time when the temperature reaches 115°C).

B.9 Glucose peptone broth¹*Formula :*

| | |
|--|---------|
| Beef extract. | 5.0 g |
| Peptone | 10.0 g |
| Sodium chloride. | 5.0 g |
| Dextrose (C ₆ H ₁₂ O ₆ ·H ₂ O) | 5.0 g |
| Distilled water | 1000 ml |

Preparation :

Prepare peptone broth (medium B.8. above). Add dextrose before distribution to culture vessels and sterilize at 115°C for 20 minutes.

C. Media for the Detection of Anaerobic Bacteria ***C.10 Cooked meat medium**¹

Place 500 g of fresh minced ox heart in 500 ml of boiling distilled water and add 1.5 ml of 1 N NaOH. Simmer for 20 minutes. The neutralization of the lactic acid will be complete by the end of this time. Drain off the liquid through a muslin filter and, whilst still hot, press the minced meat in a cloth and dry partially by spreading it on a cloth or filter paper. In this condition it can be introduced into tubes without soiling them. Take enough dried cooked meat to occupy about 1 cm of a 30-ml tube and cover with 10 ml of peptone-infusion broth made as follows : Add peptone 0.5% and sodium chloride 0.25% to the liquid filtered from the meat. Steam for 20 minutes. Add 1.0 ml of pure hydrochloric acid and filter. Bring the reaction of the filtrate to pH 8.2, steam for 30 minutes, and adjust reaction to pH 7.7-7.8. Distribute to tubes, preferably with screw caps, and autoclave at 120°C for 20 minutes. After sterilization, the pH of the medium

* These media will also support the growth of aerobes

¹ Formula based on common laboratory practice

over the meat should be 7.4-7.5. Before inoculation, heat for 15 minutes in boiling water and cool.

C.11 Semi-fluid meat medium ¹

Prepare peptone broth (medium B.8. above) with the inclusion of 0.2% shredded agar.

C.12 Tarrozzi liver broth medium ¹

Formula :

| | |
|---------------------------------|---------|
| Ground beef liver | 500 g |
| Water | 1000 ml |
| Peptone | 10 g |
| Dipotassium phosphate | 1 g |

Final pH = 9.0 ± 0.1

Preparation :

Soak liver in 1 litre of water overnight in refrigerator. Skim off the fat and autoclave for 10 minutes at 15 lb. pressure. Strain through cheese cloth and set aside the meat. Add peptone and dipotassium phosphate to broth and heat to 100°C. Adjust pH to 9.0, filter through paper, and make up the final volume to 1 litre with water. Place a small amount of calcium carbonate in each test-tube; add meat to a depth of 1 cm. Cover with broth to a total depth of 5 cm. Sterilize the tubes by autoclaving at 121°C for 20 minutes.

D. Media for the Detection of Yeasts and Fungi

The inclusion of malt extract in the following media facilitates the growth of yeasts and fungi because of the action of the group B vitamins.

D.13 Sabouraud medium ¹

Formula :

| | |
|---------------------------|---------|
| Maltose | 40 g |
| Peptone | 10 g |
| Shredded agar | 20 g |
| Distilled water | 1000 ml |

Final pH = 5.25 ± 0.25

Instead of maltose, malt extract or pure dextrose can be used.

¹ Formula based on common laboratory practice

Preparation :

Mix all the ingredients in a container with the agar suspension prepared 24 hours in advance. Place in the autoclave and heat slowly until 120°C is reached. Cool to about 40°C. Shake the container. Filter hot through a good quality filter paper. Place the medium in suitable culture tubes and sterilize at 115°C for 20 minutes. Allow the tubes to set in a slanting position.

D.14 Malt medium¹*Formula :*

| | |
|--|---------|
| Malt (ground barley) | 200 g |
| Dextrose (C ₆ H ₁₂ O ₆ ·H ₂ O) | 10 g |
| Shredded agar | 20 g |
| Distilled water | 1000 ml |

Final pH = 6.0 ± 0.2

Instead of 200 g malt, 20 g malt extract can be used.

Preparation :

Heat the malt in a portion of the distilled water in a water bath to 45°C. Increase the temperature by 1° per minute to 70°C and leave at 70°C for about 10 minutes until all the starch has disappeared. Check the end-point of the reaction with iodine. This part of the preparation is unnecessary if malt extract is used. Add dextrose and agar and the remaining distilled water to make a total volume of 1000 ml. Adjust the reaction so that after sterilization the pH will be 5.8-6.2. Filter hot through a good quality filter paper. Distribute 15 ml to tubes and autoclave at 115°C for 20 minutes. Allow the medium to set in a slanting position.

D.15 Malt extract agar²*Formula :*

| | |
|--|---------|
| Malt extract | 20 g |
| Peptone | 1 g |
| Dextrose (C ₆ H ₁₂ O ₆ ·H ₂ O) | 20 g |
| Granular agar | 25 g |
| Distilled water | 1000 ml |

Final pH = 5.65 ± 0.15

¹ Bonnel, P. H., Centre de Transfusion Réanimation de l'Armée, Clamart, Seine ; Direction centrale des Services de la Santé des Armées, Paris—personal communication

² Penso, G., unpublished working document WHO/BS/IR/18

Preparation :

Dissolve the ingredients by heating on a steam bath. Adjust the reaction so that the pH after sterilization will be 5.5-5.8. Filter if necessary. Place 20 ml of the medium in culture tubes. Sterilize at 121°C for 20 minutes and allow the medium to solidify in a slanting position in such a manner as to give a generous butt.

Appendix 2
Number of Samples required in some Countries from a Final Lot of N Final Containers

| Number of final containers (N) in final lot | Belgium ² | Brazil ³ | Italy ⁴ | Japan ⁵ | Switzerland ⁶ | UK ⁷ | USA ⁸ | Yugoslavia ⁹ | Calculated according to suggested rule ¹⁰ |
|---|----------------------|---------------------|--------------------|--------------------|--------------------------|-----------------|------------------|---------------------------|--|
| < 100 ¹ | — | — | — | 3 | — | — | — | 2 | — |
| 100 | 3 | 3 | — | 3 | 5 | 2 | 10 | 2 | 4 |
| 200 | 6 | 4 | — | 5 | 7 | 4 | 20 | 4 | 6 |
| 500 | 15 | 10 | — | 10 | 13 | 10 | 20 | 10 | 9 |
| 1 000 | 20 | 12 | 30 | 10 | 20 | 20 | 20 | 10 | 13 |
| 2 000 | 20 | 12 | 30 | 12 | 20 | 20 | 20 | 11 | 20 |
| 5 000 | 20 | 12 | 30 | 12 | 20 | 20 | 20 | 14 | 28 |
| 10 000 | 20 | 12 | 30 | 12 | 20 | 20 | 20 | 19 | 40 |
| 50 000 | 20 | 12 | 30 | 12 | 20 | 20 | 20 | 59 | 89 |
| 50 000 | 20 | 12 | $\frac{N}{1 000}$ | 12 | 20 | 20 | 20 | $9 \cdot \frac{N}{1 000}$ | $\frac{4}{10} \sqrt{N}$ |

¹ Special provisions obtain for testing final lots of less than 100 final containers

² *Pharmacopoea Belgica*, Edition IV, 1931, Brussels, p. 606 (if the product is heat sterilized the maximum number of samples required is 10.)

³ de Góes, P., Instituto de Microbiologia, Universidade do Brazil, Rio de Janeiro—personal communication

⁴ Penso, G., *Sterility test* (unpublished working document WHO/BS/IR/18)

⁵ *Minimum Requirements of Biologic Products*, Japan, Tokyo, 1958, p. 217

⁶ *Pharmacopoea Helvetica V*, Supplement II, 1955, Berne (The numbers of samples given apply if the volume of material in each final container is 1 ml. Other numbers apply if 0.5 ml, 2 ml and more than 2 ml volumes are present in each final container.)

⁷ United Kingdom (1953) *The Therapeutic Substances Regulations, 1952, Statutory Instruments, No. 1937*, London

⁸ United States of America (1960) *Federal Register, 25, 77, 3397* (Federal Regulations, Title 42, Chapter I, Part 73)

⁹ Ilic, D., Institute for the Control of Serum & Vaccine, Zagreb—personal communication; Benkovic, J. & Higy-Mandic, L. J. (1957). In: *Proceedings, Third International Meeting of Biological Standardization, 2-5 September 1957*, Opatija, p. 373

¹⁰ Formula: $0.4\sqrt{N}$ (see section 2.3, p. 6 of this Report)—Bentzon, M. W., *Note on sampling for sterility control* (unpublished working document WHO/BS/IR/75)

