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REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

Manufacturing Establishments and Control Laboratories — Poliomyelitis Vaccine (Inactivated) — Poliomyelitis Vaccine (Oral) — Smallpox Vaccine

Revised 1965

Report of a WHO Expert Group

WORLD HEALTH ORGANIZATION

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WHO EXPERT GROUP ON REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

Revised 1965

Geneva, 16-22 March 1965

Members :

Dr M. P. Čumakov, Director, Institute of Poliomyelitis & Virus Encephalitis, USSR Academy of Medical Sciences, Moscow, USSR (*Vice-Chairman*)

Dr R. Gispen, Director, National Institute of Public Health, Utrecht, Netherlands

Dr J. Kaneko, Chairman, Technical Board of Japan Association of Biological Manufacturers, Hikari Plant, Takeda Chemical Industries Ltd., Hikari, Yamaguchi-ken, Japan

Dr R. L. Kirschstein, Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA

Dr S. S. Marennikova, Deputy Director, Research Institute of Virus Preparations, Moscow, USSR

Dr F. P. Nagler, Chief, Virus Laboratories, Department of National Health and Welfare, Ottawa, Ontario, Canada (*Chairman*)

Dr F. T. Perkins, Head, Division of Immunological Products Control, Medical Research Council Laboratories, London, England (*Rapporteur*)

Dr R. Soemiatno, Director, Pasteur Institute, Bandung, Indonesia

Dr R. Sohier, Professeur à la Faculté de Médecine, Directeur, Section de Virologie, Laboratoire National de la Santé Publique, Lyon, France

Dr P. Tuchinda, Chief, Division of Medical Research, Department of Medical Sciences, Bangkok, Thailand

Secretariat :

Dr W. C. Cockburn, Chief, Virus Diseases, World Health Organization, Geneva

Dr A. S. Outschoorn, Chief, Biological Standardization, World Health Organization, Geneva (*Secretary*)

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REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

Report of a WHO Expert Group [1]

A meeting of a WHO Expert Group on Requirements for Biological Substances was held in Geneva from 16 to 22 March 1965. Dr P. Dorolle, Deputy Director-General, on behalf of the Director-General, welcomed the participants and thanked them for their willingness to come to Geneva for this meeting.

Since this was the first meeting of its kind, he explained the nature of the task it would undertake. Over the past six years, a number of international requirements for biological substances of importance in medicine had been formulated by groups of experts and published by WHO, and these requirements had been found useful in many countries. None of them had, however, been revised, and the task before the present meeting was to suggest the revisions to some of the earlier sets of requirements which were considered to be necessary in the light of advances in knowledge and experience. He also requested the views of the participating experts on this procedure for revising the requirements recommended for international use, and invited them to make any suggestions as to the procedures that should be adopted for this work.

INTRODUCTION

One of the purposes of international requirements for biological substances is to facilitate the exchange of these substances between countries. Frequently WHO is involved in the supply of vaccines either on behalf of or as a gift to a developing country, and in such instances a vaccine is considered to be satisfactory if the national control laboratory of the country of origin certifies it to have satisfied both the national and the international requirements. WHO has arranged with certain laboratories to check the properties of some vaccines but this is not so for others and extension of this facility to all vaccines would strengthen the control by WHO of vaccines used in its own international programmes.

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[1] The text of this report was submitted before publication to the eighteenth WHO Expert Committee on Biological Standardization. The Committee agreed that the revisions were in accordance with the advances in knowledge and experience that had been made since the original requirements were formulated. In preparing the report for publication, certain minor editorial amendments suggested by the Committee have been taken into account. In addition, a few editorial footnotes have been introduced to provide supplementary information that has become available since the meeting of the WHO Expert Group. — ED.

In most instances the international requirements are similar to national requirements, but in those cases where a difference exists it is the responsibility of the national control authority to authorize such deviations and changes. It would be of great help to WHO if all deviations from international requirements were notified to them, with a statement of the reasons why the changes had been made.

During the last six years, the following requirements have been formulated by international groups of experts and published in the WHO Technical Report Series.

No.	*Year*	
178	1959	Report of a Study Group on Requirements for Biological Substances : 1. General Requirements for Manufacturing Establishments and Control Laboratories 2. Requirements for Poliomyelitis Vaccine (Inactivated)
179	1959	Report of a Study Group on Requirements for Biological Substances : 3. Requirements for Yellow Fever Vaccine 4. Requirements for Cholera Vaccine
180	1959	Report of a Study Group on Requirements for Biological Substances : 5. Requirements for Smallpox Vaccine
200	1960	Report of a Study Group on Requirements for Biological Substances : 6. General Requirements for the Sterility of Biological Substances
237	1962	Report of a Study Group on Requirements for Biological Substances : 7. Requirements for Poliomyelitis Vaccine (Oral)
274	1964	WHO Expert Committee on Biological Standardization : 8. Requirements for Pertussis Vaccine 9. Requirements for Procaine Benzylpenicillin in Oil with Aluminium Monostearate
293	1964	WHO Expert Committee on Biological Standardization : 10. Requirements for Diphtheria Toxoid and Tetanus Toxoid.

In addition to these meetings of study groups and of the Expert Committee on Biological Standardization for the formulation of requirements, groups of experts also meet to consider problems concerned with the eradication of a particular disease and the recommendations of such groups often have a bearing upon the requirements for the control of the various preparations of biological substances. It is important that such recommendations should be incorporated into the requirements.

The present Group was asked to consider revision of the following :

No.	*Year*	
178	1959	Requirements for Biological Substances : 1. General Requirements for Manufacturing Establishments and Control Laboratories 2. Requirements for Poliomyelitis Vaccine (Inactivated)
180	1959	Requirements for Biological Substances : 5. Requirements for Smallpox Vaccine
237	1962	Requirements for Biological Substances : 7. Requirements for Poliomyelitis Vaccine (Oral).

Publication of a set of amendments would be an impractical way of revising the requirements since the complete set of amended requirements would not be contained in a single document. It was therefore decided to prepare new documents.

No attempt has been made to alter the requirements formulated by the original study groups except where this was indicated. Where necessary new requirements have been added to bring them up to date.

GENERAL CONSIDERATIONS

With the rapid development of techniques required in the production and control of virus vaccines the need for keeping accurate records and for submitting full protocols to the national control authority has been recognized. Moreover, the inspection of production areas and the employment of skilled and experienced scientists, both for producing and for controlling the vaccines, give far greater confidence in the product than can be gained solely by the application of tests to the final material. Indeed, perhaps the greatest safety factor is in consistency of production of successive batches.

The developments in virology have largely been in two directions (*a*) the use of cell cultures known to be free from all detectable extraneous agents and (*b*) the development of more sensitive tests for the detection of contaminant viruses. For example, all national requirements for live measles vaccines produced in chick embryo cultures have demanded either by regulation or implication that the tissue used for the growth of virus shall be produced from embryos derived from a flock known to be free from avian leucosis as well as from all other avian pathogens. This demand has been met by the manufacturers, and vaccines free from all detectable extraneous viruses are freely available. Since live measles vaccine, one of the more recently developed vaccines, is being produced in such tissue it is illogical to continue to allow other vaccines to be produced on tissue known to carry a risk of being contaminated by extraneous viruses. Whilst it is recognized that immediate drastic changes in the requirements may severely interrupt the supply of vaccines, it is recommended that steps should be taken to produce all seed lots and vaccines free from detectable extraneous agents as soon as possible. Cell strains known to be free from such agents are rapidly being made available and manufacturers may wish to gain experience in their handling in the event of such cell strains being approved for vaccine production. Already poliomyelitis vaccine (oral) has been produced on human diploid cell cultures and experimental batches of common cold vaccines are being produced in these cells.[1]

[1] The current revisions of the requirements under consideration have, however, not been extended to include vaccines produced on human diploid cells. No national control laboratory has yet approved such vaccines for general use. If such approval is given and the vaccines become freely available, relevant international requirements will need to be formulated.

Developments in the field of inactivated virus vaccines include the production of purified and concentrated vaccines that give long-lasting immunity. These developments should be encouraged. For some virus vaccines, the technical developments for concentrating and extracting the virus antigen have been achieved. In other cases, the addition of an adjuvant may be necessary but knowledge regarding the safety of some adjuvants is scanty. The whole question of adjuvants in vaccines needs consideration with a view to the formulation of requirements for vaccines containing adjuvants.

In revising these international requirements for biological substances account has been taken of the opinions of consultants, the regulations and requirements for the manufacture and control of biological substances that have been formulated in a number of countries, as well as information from both published and unpublished reports. As far as possible, the views of the participants of the groups of experts who formulated the original requirements were sought. In addition, opinions and data have been received from a number of experts, whose assistance is gratefully acknowledged (see " Acknowledgements ", page 8).

The main modifications to the requirements under current consideration concern the following points:

1. *General requirements for manufacturing establishments and control laboratories*

Requirements have been added specifying that the names of scientists responsible for production should be registered with national control authorities and that separate production processes should be isolated. The need for carefully kept records, for ensuring consistency of production and for submission of protocols is emphasized.

2. *Requirements for poliomyelitis vaccine (inactivated)*

Since the original requirements were written, the simian virus SV40 has been detected in virus harvests grown on monkey kidney tissue, especially on tissue from rhesus monkeys. Steps have been taken to eliminate this agent, both by a recommendation that only monkeys known to be free from this virus shall be used for production of the vaccine and by the introduction of tests for the presence of the virus. Recommendations have also been made to ensure that more potent vaccine is produced. The International Reference Preparation of Poliomyelitis Vaccine (Inactivated) and International Standards for Anti-poliovirus Sera types 1, 2 and 3 are now established and it is desirable that information be obtained on the use of these preparations in the control of poliomyelitis vaccine (inactivated).

3. *Requirements for poliomyelitis vaccine (oral)*

The technique for the detection of simian virus SV40 has required modification and a recommendation that only monkeys known to be free from this virus shall be used for the production of the vaccine has been introduced. A test for mycoplasma has been included and the monkey neurovirulence test has been modified to increase its value. It is suggested that reference preparations for each of the types of poliomyelitis virus be made available for the control of virus titre. Since poliomyelitis vaccine (oral) produced in human diploid cell strains has been given to man without, so far, producing untoward effects, indication of the availability of these cells has been mentioned. However, the requirements are not intended to cover these vaccines.

4. *Requirements for smallpox vaccine*

A WHO Expert Committee on Smallpox [1] drew attention to the need for a smallpox vaccine known to be successful especially for revaccination. Accordingly, the requirements for virus concentration have been revised. The need to use for vaccine production a virus strain known to give adequate immunity without untoward reaction in man has been emphasized. Although it is not possible to produce in the skin of animals vaccine that is intrinsically free from detectable extraneous agents, the total bacterial count permitted has been decreased. Vaccine produced in eggs or tissue culture, however, should use only tissue free from detectable extraneous agents. It has been recommended that dried vaccine known to be stable at ambient temperatures should be used in hot countries and where transportation and refrigeration are difficult. The International Reference Preparation of Smallpox Vaccine has now been established and it is recommended for use in testing vaccine for virus concentration.

FUTURE REVISIONS OF REQUIREMENTS

Advances in knowledge and development of new techniques will call for future revisions of requirements for various biological substances published by WHO if they are to remain useful working documents. This is the first time that any of these requirements has been revised and the Group was of the opinion that such revisions could only be made following discussion by groups of experts in the particular fields to which the requirements refer. Such groups should make use of all available information on the experience gained in various countries in the application of the

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1964, **283**.

requirements since their original publication and should take into account the difficulties encountered and how they may have been resolved.

Once revisions have been made and approved for distribution they should be circulated to national control authorities and other interested institutions as soon as possible.

Revision of national requirements for biological substances generally results in improvement in the quality of the products and it is equally important that revision of international requirements should have a similar effect.

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Dr E. Krag Andersen, Statens Seruminstitut, Copenhagen, Denmark

Dr O. Bonin, Institute for Chemotherapeutic Research, Paul-Ehrlich Institut, Frankfurt-am-Main, Germany

Dr H. Cohen, Director, Sector Immunology, Rijks Instituut voor de Volksgezondheid, Utrecht, Netherlands

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Professor S. Gard, Department of Virus Research, Karolinska Institutet, Stockholm, Sweden

Dr L. Greenberg, Chief, Biologics Control Laboratories, Laboratory of Hygiene, Ottawa, Ontario, Canada

Dr G. Heymann, Paul-Ehrlich Institut, Frankfurt-am-Main, Germany

Dr J. Johanovsky, Institute of Sera and Vaccines, Prague, Czechoslovakia

Dr C. Kaplan, The Lister Institute of Preventive Medicine, Elstree, Herts., England

Dr H. Koprowski, Director, The Wistar Institute, Philadelphia, Pennsylvania, USA

Dr P. Krag, Director, International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, Denmark

Dr U. Krech, Chief, Virus Department, Swiss Serum and Vaccine Institute, Berne, Switzerland

Dr M. Kurokawa, Chief, Department of General Biological Control, National Institute of Health, Shinagawa-ku, Tokyo, Japan

Dr A. Lafontaine, Director, Institute of Hygiene and Epidemiology, Brussels, Belgium

Dr P. Lemoine, Institute of Hygiene and Epidemiology, Brussels, Belgium

Dr H. von Magnus, Chief, Enterovirus Department, Statens Seruminstitut, Copenhagen

Dr D. McClean, The Lister Institute of Preventive Medicine, Elstree, Herts., England

Dr B. Mirski, State Institute of Hygiene, Warsaw, Poland

Dr J. S. Pagano, The Wistar Institute, Philadelphia, Pennsylvania, USA

Dr G. Penso, Chief, Laboratory of Microbiology, Istituto Superiore di Sanità, Rome, Italy

Dr F. Przesmycki, State Institute of Hygiene, Warsaw, Poland

Dr C. Purannanda, Director, Queen Saovbha Memorial Institute, Bangkok, Thailand

Dr R. S. Rao, Assistant Director, Virus Research Centre, Poona, India

Dr G. Renoux, Laboratory of Microbiology, Institute of Biology, Montpellier-Hérault, France

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Annex 1

GENERAL REQUIREMENTS FOR MANUFACTURING ESTABLISHMENTS AND CONTROL LABORATORIES (REQUIREMENTS FOR BIOLOGICAL SUBSTANCES No. 1)

Revised 1965 *

General Considerations

The procedures required for controlling biological substances during manufacture are different from the control procedures applied to final products by control authorities. Control at the manufacturing level is a matter of national concern, whereas control of final products, including

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* General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1) were first published in *Wld Hlth Org. techn. Rep. Ser.*, 1959, **178**. The original draft of these requirements was prepared by a Study Group on Requirements for Biological Substances which met in Geneva from 7-12 October 1957. The members of this Study Group were: Dr M. L. Ahuja, Medical Adviser to the High Commissioner for India, London, England; Dr J. Desbordes, Service central de la Pharmacie, Bureau des Sérums et Vaccins, Paris, France; Dr G. Eissner, Paul-Ehrlich-Institut, Frankfurt-am-Main, Federal Republic of Germany; Dr J. H. Gaddum, Director, Pharmacological Laboratory, University New Buildings, Edinburgh, Scotland; Dr L. Greenberg, Chief, Biologics Control Laboratories, Laboratory of Hygiene, Ottawa, Canada; Dr D. Ikić, Director, Institute for the Production of Sera and Vaccines, Zagreb, Yugoslavia; Dr M. Kurokawa, Chief, Department of General Assay, National Institute of Health, Tokyo, Japan; Dr A. Lafontaine, Directeur, Institut d'Hygiène et d'Epidémiologie, Brussels, Belgium (Chairman); Dr O. Maaløe, Director, Department of Biological Standards, Statens Seruminstitut, Copenhagen, Denmark; Dr G. Penso, Chief, Laboratory of Microbiology, Istituto Superiore di Sanità, Rome, Italy; Dr W. L. M. Perry, Director, Department of Biological Standards, National Institute for Medical Research, London, England; Dr J. T. Tripp, Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA (Vice-Chairman). Dr N. K. Jerne, Chief, Section of Biological Standardization, WHO, acted as Secretary. This Annex comprises a revised version of these requirements, incorporating additions and amendments made by the WHO Expert Group on Requirements for Biological Substances which met in March 1965.

imported products, by a control authority may have international as well as national implications.

The most important information concerning the safety of a biological substance is given by consistency of production, which is complementary to the tests applied to the final filled material. The approval of manufacturing methods, the maintenance of accurate records, and the inspection of production by the national control authority play a major part in creating confidence in the safety of the product.

The general requirements given in Part A are applicable to all manufacturing situations.

In an ideal situation the same control measures would be exercised by the governments of all countries. In such circumstances there would be no problem in the free exchange of biological substances between countries, and the control authority in any one would be faced only with the problem of controlling substances manufactured within its own jurisdiction. It is, however, essential to realize that it will be many years before such an ideal situation can possibly be brought about ; in the interim it will continue to be necessary for the national control authority to deal not only with the substances manufactured within its own jurisdiction, but also with substances imported from other countries.

The general requirements given in Part B should apply to all control laboratories operating under present conditions. These general requirements should operate, regardless of the number or kind of biological substances being controlled, and whether these substances have been manufactured within the country or imported.

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 included in definitive national requirements. The parts of each section which are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis for their national regulations concerning general requirements for the manufacture and control of biological products, it is recommended that a clause be included which would permit modifications, on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of safety and a potency of the products at least equal to those provided by the requirements formulated below. It is desirable that the World Health Organization should then be informed of the action taken.

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

Part A. General Requirements for Manufacturing Establishments

1. Personnel

Manufacturing shall be in charge of a person who has been trained in the techniques used in manufacturing biological substances and the scientific knowledge upon which the manufacture of these products is based. This person shall have sufficient authority to enforce discipline among employees, who shall include specialists with training appropriate to the products made in the establishment. The names and qualifications of such specialists, especially those responsible for signing a protocol, shall be registered with the national control authority.

Thus, in dealing with the problems of manufacture, a training is needed in some or all of the following fields: bacteriology, biometry, chemistry, medicine, pharmacy, pharmacology, veterinary medicine and virology.

The staff making control tests should be separate from the manufacturing unit and not responsible to the person in charge of production.

All staff engaged in manufacture, testing, and animal care should be vaccinated with appropriate specific vaccines, and should submit to a regular tuberculosis control.

2. Buildings and equipment

2.1 *Buildings*

Laboratories, operating rooms, animal rooms and all other rooms and buildings used for the manufacture of biological products shall be so designed and constructed of such materials that the highest standards of cleanliness and sanitation can be maintained and freedom from dust, insects and vermin ensured. All such buildings shall be equipped with hot and cold running water and drainage. Adequate precautions shall be taken to avoid contamination of the drainage system with dangerous effluents and also to avoid airborne dissemination of pathogenic microbes and viruses. Staff changing rooms, etc., shall be provided as needed. All buildings and rooms shall be clean and sanitary at all times. If rooms intended for the manufacture of biological substances are used for other purposes, they shall be cleaned thoroughly and, if necessary, sterilized prior to resumption of manufacture of biological substances in them. Any persons not concerned with the production process who enter the production area for the purposes of inspection shall be supplied with sterile protective clothing.

Technical library facilities, including both books and journals, should always be available.

2.2 *Constant temperature rooms*

Adequate refrigerator space, as well as incubators or warm rooms, capable of being maintained at a uniform temperature within any required range shall be provided.

> Refrigerators and incubators should maintain a uniform temperature in all parts of the interior and should preferably be equipped with recording thermometers and with appropriately placed alarm signals to ensure that an early repair can be effected in case of breakdown.

2.3 *Sterile rooms*

Sterile transfer and processing rooms shall be of minimum size for their function and have low ceilings and smooth surfaces to permit thorough cleaning before each use.

> These rooms should be essentially dust free and preferably supplied with filtered air at a pressure higher than that in adjacent rooms.

Staff working in these rooms shall be provided with a special changing room and wear sterile gowns.

2.4 *Washing and sterilization equipment*

Adequate facilities shall be available for washing apparatus. Steam autoclaves, dry-heat sterilizers, and bacterial retaining filters shall be available for sterilizing supplies, media and apparatus.

> Autoclaves should preferably be equipped with recording thermometers. Other means of sterilization, including ultraviolet irradiation and chemical sterilization, have special applications and when appropriate should be used with proper controls.

2.5 *Animal quarters*

Quarters for animals shall be designed in a manner and constructed of materials that permit maintenance in a clean and sanitary condition free from insects and vermin. Facilities for animal care shall include isolation units for quarantine of incoming animals, and vermin-free food storage. Provision shall be made for animal inoculation rooms which shall be separate from the post-mortem rooms.

> There should be provision for the disinfection of cages, if possible by steam, and an incinerator for disposing of waste and of dead animals.

2.6 *Apparatus*

Instruments and apparatus shall be of high precision.

All instruments and apparatus should be calibrated and checked at regular intervals.

3. Production control

3.1 *Production methods*

Production methods shall be approved by the national control authority and written procedures shall be prepared for each product, describing each step in production and testing. Proposals for modifications shall be submitted for approval to the national control authority before their implementation. At any one time, manufacture of each biological product shall take place in a separate area using separate equipment. Only strains of micro-organisms or viruses used for the production of the particular biological product shall be permitted in the manufacturing area.

3.2 *Cleanliness*

Apparatus, equipment and materials used in manufacturing shall be clean and, if necessary, sterile and free from pyrogenic contamination.

3.3 *Orderliness*

All containers of biological substances, regardless of the stage of manufacture, shall be identified by securely attached labels.

3.4 *Precautions against contamination*

All procedures with spore-forming micro-organisms or viruses shall be confined to separate areas with complete equipment used exclusively in those areas.

Separate facilities shall be provided for work with each virus and care shall be taken to prevent aerosol formation (especially by centrifugation and blending), which might lead to transfer of virus from one production unit to another.

Adequate staff shall be provided to avoid the necessity for staff to work in any one working day in areas in which different biological products are being manufactured.

Sequential manufacture of different products in the same area shall be allowed provided that the method of sterilization of the area between manufacture of the different products is shown to be satisfactory and has the approval of the national control authority.

Pathological specimens sent in for diagnosis shall be permitted only in separate areas not used for manufacturing biological substances.

Employees should stay in their own work areas, and wear protective clothing, including shoes, caps etc., which should remain in the area. Employees suffering from an infective illness should not be permitted to work until completely recovered.

Visitors should be as few as possible and they should not normally be permitted to enter sterile rooms.

3.5 *Animal care*

Animals used for production purposes, or for test purposes, shall show no signs of communicable disease, and shall be adequately housed at all times. They shall be provided with a well-balanced diet, and be kept clean and sanitary. If the production process or test necessitates the use of animals of a particular species or strain, the animals used shall be approved by the national control authority.

Animals intended for use in production or in tests should be observed daily during a quarantine period of not less than one week. In some instances it is desirable to maintain the animal rooms constantly at the optimum temperature for the particular species and test, and it may also be necessary to maintain pure strains of test animals.

It is desirable to use specific pathogen-free (S.P.F.) animals for both production and testing of certain biological substances.

Animals or animal carcasses shall not be removed from the establishment if capable of transmitting disease.

Animals that die from infection are destroyed, preferably in an incinerator.

4. Filling and containers

4.1 *Filling rooms*

Filling shall be performed in rooms reserved for this purpose. These shall be sterile rooms equipped specifically for transferring measured quantities of finished biological substances from bulk containers to the final containers. Strict dust control measures and aseptic techniques shall be enforced to ensure that the product is not contaminated during the filling process.

These measures include, for instance, laying of dust by steam or spray, proper protective clothing for workers etc.

4.2 *Filling procedures*

Filling operations shall be conducted in such a way as to avoid any contamination or alteration of the product. They shall take place in areas that are completely separate from those in which living micro-organisms, including viruses, are handled.

The filling process should be checked at least twice each year at the end of a working day by filling not less than 500 ampoules with a nutrient medium containing no antibiotics or bacteriostatic substances and incubating the complete batch of filled ampoules. Not more than 1% of the ampoules filled in this way should show signs of contamination and all contaminants should be identified.

4.3 *Containers*

The final container shall be sealed as soon as possible after filling. Closures shall be of material that does not have a deleterious effect upon the biological substances, and shall be designed to maintain a hermetic seal throughout the dating period.

5. Tests

All tests of a specific biological substance, requiring the use of living micro-organisms, shall be carried out in rooms separate from those used for production.

Preferably, all tests should be carried out in such separate rooms.

The descriptions of the tests necessary to establish the safety, purity and potency of each lot of a biological substance will be given in the requirements to be formulated for the particular biological substance.

6. Records

6.1 *Production protocols and distribution records*

Records shall be permanent and clearly indicate all steps in processing, testing, filling and distribution. Written records shall be kept of all tests irrespective of their results. The records shall be of a type approved by the national control authority. They shall be retained throughout the dating period of a lot or batch of a biological product and be available at all times for inspection by the national control authority.

Records must make it possible to trace all steps in the manufacture and testing of a batch, and should include records of sterilization of all apparatus and materials used in its manufacture. Distribution records must be kept in a manner that permits rapid recall of any particular batch, if necessary.

6.2 *Records of cultures*

Records shall be maintained of the complete passage history of all cultures kept in the establishment. Cultures shall be labelled and stored in a safe, orderly manner.

7. Samples

Samples from each lot shall be taken in a sufficient amount to satisfy the requirements for samples of the national control laboratory. Additional samples shall be retained throughout the dating period as reference material, in a manner which ensures the identity of the lot.

Manufacturers should retain sufficient additional samples to permit repetition of the control tests.

8. Labelling

All products shall be clearly identified by labels. The information given on the label on the container or the label on the package shall be determined by the national control authority.

The label on the container shall show at least :

the name of the product (i.e., the international name and/or the proper name) ;
the name of the manufacturer (and address if required) ;
the number of the final lot ;
the recommended human dose and route of administration ;
the condition of storage and expiry date.

The label on the package shall, in addition to the information shown on the label on the container, show at least :

the nature and amount of any preservative or added substance present in the product ;
a description of any substance likely to cause any adverse reaction ;
any contra-indications to the use of the product.

In addition, the label on the package or the leaflet in the package should indicate the stability under different storage conditions, contain instructions for the use of the product and give information about reactions that may follow administration of the product.

It is desirable to have an indication that the product fulfils the relevant requirements published in the WHO technical report series.

It is also desirable that the labels used remain permanently attached to the containers under all storage conditions and that an area of the container be left uncovered to allow inspection of the contents.

If the final container is not suitable for labelling (for example, a capillary tube), it should be in a labelled package.

9. Distribution and shipping

9.1 *Release for distribution*

A lot of a biological substance shall not be released until all the required tests have been performed, summarized and reviewed and until any other

official control requirement is satisfied. These tests shall always include an identity test performed on the contents of a finished package from each filling, to confirm the accuracy of the labelling.

No new biological substance shall be released until consistency of production has been established.[1] In routine production, failure of a single batch to meet the requirements for safety shall be considered as a breakdown in production, and consistency shall be re-established to the satisfaction of the national control authority before any further batches are released.

9.2 *Shipping*

Biological substances shall be shipped with precautions to ensure that the product retains its potency upon arrival at its destination.

Rules cannot be laid down to cover all situations; this requires the continuous exercise of judgement.

10. Storage and expiry date

The statements concerning storage temperatures and expiry dates appearing on the label and the packing leaflet, as required in Part A, section 8, shall be based on experimental evidence and shall be submitted for approval to the national control authority.

10.1 *Storage conditions*

Biological products shall be stored at all times at controlled temperatures within a range which ensures optimal stability.

During distribution, short periods at ambient temperatures may have to be permitted.

10.2 *Expiry date*

The expiry date of a biological product shall be defined and fixed with the approval of the national control authority.

Part B. General Requirements for Control Laboratories

1. Administration and personnel

The control laboratory shall be administered by or on behalf of the national control authority. In the event of manufacture and national control being done in the same establishment, the control laboratory shall be an independent unit directly responsible to the national control authority.

Authority for taking measures designed to ensure that biological substances used in a country are safe, potent and

[1] In some countries, consistency of production has to be established for the first five production batches.

biologically pure, normally rests upon the health department of the government of that country. This authority must, however, be delegated to the expert in charge of the control laboratory, who should have full authority and full responsibility.

The head of the control laboratory shall be a person qualified and experienced in the control of biological substances.

The staff of the control laboratory shall include experts in all disciplines required to cover the biological substances which the laboratory must control, both those that are manufactured in the country and those imported for use.

It will therefore usually be necessary for the staff to include persons trained in some or all of the following fields : bacteriology, biometry, chemistry, medicine, pharmacy, pharmacology, veterinary medicine, and virology.

2. Buildings and equipment

The requirements in respect of buildings and equipment described in Part A, section 2, shall apply in a general way to a control laboratory.

3. Scope of activities

The system of control shall include licensing of manufacturers and routine inspection of their establishments. In addition, the national control authority shall determine the extent of control testing of individual products.

All these methods of control should be under the direction of the control laboratory. The requirements that should be met by manufacturing establishments have been outlined in detail in Part A of these requirements and should be enforced by the control laboratory.

3.1 *Licensing and inspection*

Manufacturers shall be licensed in respect of each individual biological substance which they manufacture and methods shall exist for withdrawing the licence for that substance in the event of failure to meet the appropriate general and special requirements. The manufacturing area and process shall be made accessible to the national control authority for inspection at all reasonable times.

Routine inspection of all manufacturing establishments should be carried out by the expert staff of the control laboratory, preferably at intervals of not more than one year.

3.2 *Tests by control laboratory*

The control laboratory shall be staffed and equipped in such a way as to be able to carry out effectively all the required tests on samples of the

finished products, as well as on samples taken at an intermediate stage of manufacture.

The tests carried out by the control laboratory on the final products will usually be identical with those which are required of the manufacturer, but the control laboratory should have discretionary power to vary the tests applied and to decide whether to apply tests to all or only to selected batches.

Control tests on the final product are sometimes closely similar to those applied during manufacture; but this is not always the case, since the marketed forms of biological substances, such as mixtures with other active ingredients or with adjuvants and preservatives, may greatly complicate the problem of carrying out the necessary tests. It will, in general, be impracticable to give guidance on the ways in which the numerous marketed preparations of any biological substance should be treated in order to make the tests proposed for the parent substance applicable. Control laboratories should therefore develop their own technique for this purpose.

The control laboratory shall devise effective internal control measures to permit objective interpretation of tests and evaluation of its own reliability in performing all tests.

The inclusion of replicate coded samples in products to be tested, the simultaneous independent testing of the same batch of substance, and routine checks on sensitivity and calibration of instruments are measures that may be applied as self-imposed "controls" for the control laboratory.

Healthy animals of various species and unquestioned strains shall be available in adequate numbers for an effective performance of the tests to be undertaken.

Test animals must conform to stricter requirements than those used in manufacturing control because the number of samples is limited and maximum reliability of the tests is demanded. Animals should be maintained under optimum nutritional and environmental conditions before and during tests. It is essential for test animals to be kept free from infectious diseases. This is best accomplished by breeding and maintenance of animals primarily free from specific pathogens and protected against infections by contaminated food, air and water, or by contact with vectors, animals and man.

The national control authority should investigate the use of specific pathogen-free (S.P.F.) animals for control tests.

Control authorities should be familiar with international standards for the assay of potency and, where national standards exist, they should be calibrated in terms of the international standards.

3.3 *Release and certification*

A lot of a biological substance shall be released only if it fulfils the requirements adopted by the national control laboratory.

In certain circumstances, the official in charge of the national control laboratory shall provide a statement, at the request of the manufacturing laboratory, certifying whether or not a given lot of a biological substance meets all appropriate requirements.

It is in general impracticable and may in the future become unnecessary for a control laboratory adequately to fulfil the requirements for licensing and inspection of manufacturing establishments outside its own jurisdiction. For the control of imported products it is therefore primarily dependent upon tests on the final products themselves, supplemented by protocols of tests carried out by the manufacturer and, in certain circumstances, by a certificate to the effect that the control authority of the country of origin has found the product to fulfil specified requirements.

3.4 *Research and training*

It is desirable so to organize the control laboratory that opportunity is provided for research in addition to routine testing. Encouragement of research activities will not only lead to the development of better methods of control, but will also help the laboratory to retain an interested, efficient, and highly qualified staff. The number of specialists in the control of biological substances needed by any country is too small to justify specific university courses in this field. It is therefore necessary for the control laboratory itself to adopt a vigorous training programme, covering both the technical and the administrative aspects of control procedures. In general, this will be best accomplished by direct supervision of junior staff during the actual performance of duties, but such supervision may be supplemented where conditions permit by more formal instruction.

Annex 2

REQUIREMENTS FOR POLIOMYELITIS VACCINE (INACTIVATED) (REQUIREMENTS FOR BIOLOGICAL SUBSTANCES No. 2)

Revised 1965 *

General Considerations

Since the Requirements for Poliomyelitis Vaccine (Inactivated) [1] were first formulated, a number of changes in production and control have occurred which makes a revision of the requirements necessary. Whilst it is still true that international requirements are complicated by the fact that

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* Requirements for Poliomyelitis Vaccine (Inactivated) (Requirements for Biological Substances No. 2) were first published in *Wld Hlth Org. techn. Rep. Ser.*, 1959, **178**. The original draft of these requirements was prepared by a Study Group on Requirements for Biological Substances which met in Geneva from 2-7 June 1958. The members of this Study Group were: Dr O. Bonin, Scientific Member, Institute for Chemotherapeutic Research, Paul-Ehrlich-Institut, Frankfurt-am-Main, Federal Republic of Germany; Dr D. G. Evans, Director, Biological Standards Control Laboratories, Medical Research Council, Hampstead, London, England; Dr S. Gard, Professor of Virus Research, School of Medicine, Karolinska Institutet, Stockholm, Sweden (Rapporteur); Dr J. H. S. Gear, Director of Research, Poliomyelitis Research Foundation Laboratories, Johannesburg, Union of South Africa (Chairman); Dr A. Lafontaine, Directeur, Institut d'Hygiène et d'Epidémiologie, Brussels, Belgium; Dr P. Lépine, Chef du Service des Virus, Institut Pasteur, Paris, France (Vice-Chairman); Dr H. von Magnus, Chief, Department of Poliovirus, Statens Seruminstitut, Copenhagen, Denmark; Dr R. Murray, Director, Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA; Dr G. Penso, Chief, Laboratory of Microbiology, Istituto Superiore di Sanità, Rome, Italy; Dr V. Soloviev, Scientific Director, Moscow Institute for Poliomyelitis Prophylactics, Moscow, USSR. Dr N. K. Jerne, Chief Medical Officer, Biological Standardization, WHO, acted as Secretary. This Annex comprises a revised version of these requirements, incorporating additions and amendments made by the WHO Expert Group on Requirements for Biological Substances which met in March 1965.

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

a number of different manufacturing and testing procedures are in use in various countries, much more experience has been gained of the different procedures. The International Reference Preparation of Poliomyelitis Vaccine (Inactivated) and International Standards for Antipoliovirus Sera types 1, 2 and 3 have been established.

A hitherto undetected contaminating simian virus SV40 was detected by the use of kidney cell cultures from different monkey species; many manufacturers now avoid the use of the rhesus monkey for vaccine production and tests for the detection of SV40 virus have been established. Continuous studies to reveal further contaminating viruses in all monkey species should be undertaken. Tests should be developed for the detection of such viruses and measures taken for their elimination. Finally, much effort has been put into the production of concentrated inactivated vaccine which gives better and longer-lasting immunity. More precise methods of measuring potency have been investigated and these should be considered.

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 included in definitive national requirements. The parts of each section which are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis for their national regulations concerning poliomyelitis vaccine (inactivated), it is recommended that a clause be included which would permit modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of safety and a potency of the vaccine at least equal to those provided by the requirements formulated below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms " national control authority " and " national control laboratory ", as used in these requirements, always refer to the country in which the vaccine is manufactured.

Part A. Manufacturing Requirements

1. Definitions

1.1 *International name and proper name*

The international name shall be " Vaccinum poliomyelitidis inactivatum ". The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 *Descriptive definition*

Vaccinum poliomyelitidis inactivatum shall consist of an aqueous suspension of poliovirus types 1, 2 and 3 grown in monkey-kidney tissue cultures and inactivated by a suitable method. The preparation shall satisfy all the requirements formulated below.

1.3 *International standards or reference preparations and international units*

The International Reference Preparation of Poliomyelitis Vaccine (Inactivated) (established in 1962) is stored frozen in ampoules containing 10 ml of trivalent inactivated poliomyelitis vaccine. This reference preparation is intended for the determination of the relative potencies of preparations of poliomyelitis vaccines (inactivated).

The International Standards for Antipoliovirus Sera type 1, type 2 and type 3 (established in 1962) are stored in ampoules each containing 10 IU of dried hyperimmune monovalent serum per ampoule. These standards are intended for calibration of national standards for antipoliovirus sera.

The above standards and reference preparation are in the custody of the International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen. Samples are distributed free of charge on request to national control laboratories.

1.4 *Terminology*

Seed lot : A quantity of virus processed together and of uniform composition. In each manufacturing establishment a *primary seed lot* is that from which *further seed lots* are prepared. Vaccine is one passage removed from the seed lot.

Monovalent pool : A virus suspension of a single virus type processed at the same time.

Trivalent bulk : A pool of a number of monovalent pools and containing all three virus types.

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

Filling lot (final lot) : A collection of sealed final containers that are homogeneous with respect to the risk of contamination during filling. A filling lot must, therefore, have been filled in one working session.

Tissue culture infective dose 50% ($TCID_{50}$) : The smallest quantity of a virus suspension that will infect 50% of inoculated cell cultures.

2. General manufacturing requirements

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for

Manufacturing Establishments and Control Laboratories) [1] shall apply to establishments manufacturing poliomyelitis vaccine (inactivated), with the addition of the following :

The areas where processing of inactivated poliomyelitis vaccine takes place shall be separate from those where work with active virus is performed.

It is recognized that staff members working with active poliovirus may be exposed to the danger of infection. Accordingly, it is recommended that all susceptible personnel be immunized against poliomyelitis.

3. Production control

3.1 *Control of source materials*

3.1.1. *Virus strains and seed lot system*

Strains of poliovirus used in the production of vaccine shall be identified by historical records, infectivity tests, and by immunological methods. Any strain which will yield a vaccine meeting the requirements set forth in the present document may be used. Production of vaccine shall be based on the seed lot system ; the seed virus used for vaccine production shall not have passed more than ten subcultures, counted from a seed lot used for the production of the vaccine on which the original laboratory and field tests were done.

Samples of the strains used shall be deposited in the national control laboratory.

3.1.2 *Monkeys*

Suitable species of monkeys, in good health, shall be used as the source of kidney tissue for the production of poliovirus. Monkeys known to be carrying viruses that would affect the safety of the product shall not be used. Each animal shall be examined at necropsy for signs of disease and if there is any pathological lesion of significance with regard to their use in the preparation of the vaccine the kidneys shall be discarded. Kidney tissue from monkeys that have been used previously for experimental purposes involving infectious agents shall not be used.

An exception can be made in the case of monkeys used for the safety or potency tests if these have given negative clinical findings.

It is recommended that monkeys be kept in as small groups as possible in order to reduce dissemination of infections within the colony.

A further hazard exists because of the occurrence of B virus infection in some monkeys, and it is recommended that the handling of animals be reduced to a minimum. Workers should

[1] See Annex 1 of this report.

use protective clothing and other protective devices where possible. It is suggested that no monkey should be handled unless anaesthetized. It is further recommended that research be conducted towards developing prophylactic agents and protective sera against this infection.

Past experience has shown that monkeys may be infected with *Mycobacterium tuberculosis*. Adequate measures should therefore be taken to protect the personnel.

3.1.3 *Tissue culture for virus production*

Virus for the preparation of vaccine shall be grown by aseptic methods in cultures of monkey-kidney cells that have not been propagated in series. The maintenance medium shall contain no protein. If animal serum is used in the propagation of cells, the final vaccine shall not contain more animal serum than one part per million. Penicillin shall not be used.

Suitable other antibiotics in minimum concentrations required for sterility may be used. Non-toxic pH indicators may be added, e.g., phenol red in a concentration of 0.002%.

3.2 *Production precautions*

The general production precautions as formulated in Part A, section 3 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply to the manufacture of poliomyelitis vaccine (inactivated).

3.3 *Control at the monovalent stages of the product*

3.3.1 *Treatment before inactivation*

Prior to inactivation each monovalent virus pool shall be filtered or clarified.

The importance of filtration or clarification of the crude virus suspensions as a means of improving the regularity of the inactivation process has been clearly established. Generally, filters are used in series or filtration is performed stepwise through filters of decreasing porosity. Seitz S1 pads are most widely used as final filters, but satisfactory results have also been reported with other filter types, as well as with clarification procedures not involving filtration.

3.3.2 *Virus titration*

After filtration or clarification and before the initiation of inactivation a sample shall be taken of each monovalent pool for titration of infective poliovirus using tissue culture methods. This titration shall be carried out in tenfold dilution steps using ten tubes per dilution, or any other arrangement of tubes and dilutions yielding equal precision.

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[1] See Annex 1 of this report.

Each monovalent pool should show a titre of not less than 10^7 $TCID_{50}$ per ml using a batch of tissue culture of normal sensitivity.

Other methods for virus titration, such as the enumeration of plaque-forming units, may be used. If this is done, the monovalent pool should show a titre which is on a comparable level.

The main purpose of determining the titre of virus pools destined for inactivation is to select pools which can be expected to meet potency requirements after inactivation.

3.3.3 *Tests for adventitious agents prior to inactivation*

3.3.3.1 *Test for* Mycobacterium tuberculosis

Adequate samples of each monovalent pool shall be withdrawn for tests in guinea-pigs and in suitable culture media for the absence of *Mycobacterium tuberculosis*. If the presence of *Mycobacterium tuberculosis* is demonstrated, any product made from the pool shall be discarded.

3.3.3.2 *Test for SV40 virus*

Each virus pool shall be tested for the presence of SV40 virus by a method approved by the national control authority.

The following method, or one shown to give equally reliable results, may be used :

A sample of at least 25 ml from the monovalent pool should be neutralized by a high-titred antiserum against the specific type of poliovirus.

The immunizing antigen used for the preparation of the antiserum should be grown in tissue culture cells free from extraneous microbial agents which might elicit antibodies that could inhibit the growth of any adventitious agents present in the monovalent poliovirus pool.

This sample should be tested in primary *Cercopithecus* kidney tissue cultures or a cell line of demonstrated equal susceptibility to SV40 virus. The tissue cultures should be observed for 14 days. At the end of this observation period at least one subculture of fluid should be made in the same tissue culture system and both primary cultures and subcultures observed for an additional 14 days. If necessary, serum may be added to the primary cultures at this stage, provided that the serum does not contain SV40 antibody or other inhibitors.

The virus pool passes the test if there is no evidence of the presence of SV40 virus or other adventitious agent attributable to the virus pool.

3.3.3.3 *Test for B virus*

A sample of at least 100 ml of each monovalent pool shall be tested as soon as possible after harvesting by inoculation into ten healthy rabbits, each weighing between 1.5 and 2.5 kg, or proportionately larger volumes if more

animals are used. The inoculations shall be made at multiple sites, each rabbit being given a total of 1.0 ml of the monovalent pool by intradermal injection and 9.0 ml by subcutaneous injection. The animals shall be observed for at least three weeks for death or signs of illness. All rabbits that die after the first 24 hours of the test or that show signs of illness shall be examined by autopsy, with removal of the brain and organs for detailed inspection.

The monovalent pool passes the test if at least 80% of the rabbits remain healthy and if none of the rabbits shows lesions of any kind at the sites of inoculation or shows evidence of infection with B virus or with any adventitious transmissible agent attributable to the monovalent pool.

3.3.4 *Time of inactivation*

Inactivation shall be initiated as soon as possible and not later than 72 hours after filtration or clarification.

It is preferable to start inactivation within 24 hours. Since the purpose of the filtration or clarification step is to remove particulate matter and other interfering substances which may diminish the effectiveness of the inactivation process, and since aggregates tend to increase on standing after filtration or clarification, efforts should be made to keep within this time limit.

3.3.5 *Inactivation procedure*

The virus in the monovalent pools shall be inactivated through the use of an agent or a method that has been demonstrated to be consistently effective in the hands of the manufacturer.

The progress of inactivation should be followed by suitably spaced determinations of virus titres.

Formaldehyde is used as inactivating agent in the production of some vaccines. Most manufacturers have encountered some irregularities in the inactivation process that have not been fully explained. On the basis of the observation that a decrease in the concentration of free formaldehyde occurs in the course of the process, it has been recommended that tests for free formaldehyde be performed at intervals and its concentration maintained at the desired level by intermittent readjustments.

Introduction of a second filtration during the inactivation is another, more widely used, procedure which can effectively reduce the frequency of such irregularities and which is a requirement in some countries. In order to avoid a loss of antigen due to filtration, some producers use a combination of initial formaldehyde treatment with some other method of inactivation.

It should be emphasized that the requirements for consistency in production given in Part A, section 9.1 of the revised Requirements for Biological Substances No. 1 (General Requirements

for Manufacturing Establishments and Control Laboratories)[1] are applicable at this stage of production.

3.3.6 *Test for effective inactivation*

Two samples of at least 500 ml from each monovalent pool shall be tested, after removal or neutralization of the formaldehyde, by inoculation into tissue cultures for the absence of infective poliovirus. One sample shall be taken at the end of the inactivation period and the other before the end of this period after an interval equivalent at least to the time required to reduce the initial virus activity by a factor of 10^7. Each sample shall be inoculated into bottles of tissue cultures derived from at least two different batches of cells. Not more than 100 ml shall be inoculated into each bottle. The dilution of the vaccine in the nutrient fluid shall not exceed 1 : 4 and the area of the cell sheet shall be at least 3 cm^2 per ml of vaccine. One or more bottles of each batch of cultures shall be set aside to serve as uninoculated control bottles with the same medium.

The formaldehyde in samples of vaccine for tissue culture tests is usually neutralized at the time of sampling by addition of bisulfite. Usually, the samples are subsequently dialyzed. Dialysis is recommended because, in safety tests, it is desirable to use the highest possible concentration of vaccine, preferably undiluted.

It is possible to conduct tissue-culture tests on non-dialyzed material; however, this is often found to be toxic to cells even when diluted 1 : 4. If in such tests non-specific degeneration of cells occurs, or if the sensitivity of the tissue-culture system is reduced, the test should be repeated on dialyzed material.

In some countries this test is made using tissue cultures which are also sensitive to SV40 virus, as an additional measure to detect this extraneous agent.

The primary tissue-culture bottles shall be observed for at least two weeks. Not less than two subcultures shall be made from each original bottle, one at the end of the observation period and the other one week earlier. The subcultures shall be observed for at least two weeks.

If cytopathogenic effects occur in any of the cultures, the decision regarding the further use of the pool shall be deferred until the matter is resolved.

If active poliovirus is isolated, the monovalent pool shall not be used. The isolation of active poliovirus from a monovalent pool must be regarded as a break in the consistency record.

If viruses other than poliovirus are present, the pool shall not be used unless it can be demonstrated that such viruses have originated from a source other than the monovalent pool being tested.

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[1] See Annex 1 of this report.

The problem of detecting residual active poliovirus in a vaccine is not the same as that of measuring infective virus in untreated suspensions. Poliovirus that has been exposed to the action of formaldehyde without becoming inactivated has been shown to require a much longer time to produce cytopathogenic changes than does untreated virus. For this reason it is desirable that tissue cultures in tests for the presence of residual active virus be observed for as long a time as is technically possible. A satisfactory tissue-culture system for this purpose depends, therefore, not only on the sensitivity of the cells used for the preparation of the cultures but also on the nutrient fluid.

Kidney cells from some monkey species, for instance from the genera *Macaca*, *Cercopithecus* and *Papio*, appear to be more sensitive than others. If other tissue culture systems are used, they should have been shown to possess at least the same sensitivity as those specified above.

Cultures can be maintained in good condition for long periods of time by the addition to the medium of serum, albumin preparations, amniotic fluid, etc. Any such additional components of the medium should have first been shown to be free from virus inhibitors and antibodies.

Maintenance of the cultures in good condition may require frequent changes of culture medium. However, it should be borne in mind that by early changes of fluid unadsorbed virus might be removed and the validity of the test thus impaired.

3.4 *Control of trivalent bulk product*

3.4.1 *Test in tissue cultures for infective poliovirus*

A sample of at least 1500 ml shall be effectively tested for the absence of infective virus by the procedure given in Part A, section 3.3.6, of these requirements. If active poliovirus is isolated, this trivalent bulk product shall not be used.

3.4.2 *Monkey safety test*

A test for the absence of infective poliovirus shall be made in the trivalent bulk product according to the provisions of Part A, section 5.3.1 of these requirements, unless this test is to be done on vaccine from the final containers.

3.4.3 *Preservatives and other substances added*

Preservatives or other substances that might be added to or combined with the vaccine shall have been shown to have no deleterious effect on the product.

4. Filling and containers

The requirements concerning filling and containers given in Part A, section 4 of the revised Requirements for Biological Substances No. 1

(General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply with the addition of the following :

The containers shall be of colourless and fully transparent glass so as to permit the detection of visible changes in the vaccine.

Single- and multiple-dose containers may be used.

5. Control tests on final product

5.1 *Identity test*

An identity test shall be done on samples of vaccine from the final containers.

The potency test described in Part A, section 5.4 of these requirements may serve as the identity test.

5.2 *Sterility tests*

Each filling lot shall be tested for sterility according to the requirements given in Part A, section 5 of Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[2]

5.3 *Safety tests*

5.3.1 *Monkey safety test*

A test shall be made in *Macaca* or equally susceptible monkeys for the absence of infective poliovirus in vaccine from the final containers, if this test has not been performed on the trivalent bulk product.

It is highly desirable that the monkey safety test be performed on vaccine from final containers. Circumstances may exist, however, in which the performance of this test on vaccine from final containers is not desirable, e.g., in the case of vaccine to which adjuvants have been added, as this may reduce the infectivity of any active virus present and thus diminish the sensitivity of the test. In such circumstances, a monkey safety test on the bulk trivalent pool would be acceptable, providing that special care is taken to exclude contamination with poliovirus of the vaccine in the final container during the late stages of manufacture.

A total of not less than 20 monkeys in overt good health shall be used.

It is recommended that monkeys be conditioned for at least six weeks before they are subjected to the safety test procedure.

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[1] See Annex 1 of this report.

[2] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **200**, 13.

A pre-injection serum sample from each animal must contain no neutralizing antibody against any of the three poliovirus types in a dilution of 1 : 4 when tested against not more than 500 $TCID_{50}$ of virus.

Vaccine shall be injected, under deep anaesthesia, by combined intracerebral, intraspinal and intramuscular routes into monkeys. The intracerebral injection shall consist of 0.5 ml into the thalamic region of each hemisphere. The intraspinal injection shall consist of 0.5 ml of vaccine, or of vaccine suitably concentrated, into the lumbar spinal cord enlargement and may be divided between more than one site. The intramuscular injection shall consist of 1.0 ml into the right leg.

> In some countries the vaccine is concentrated 25-fold to 100-fold by a suitable method, e.g., by centrifugation in gelatin. It has been shown that concentration of vaccine in this way increases the sensitivity of the test.

At the time of inoculation of vaccine, an intramuscular injection of 200 mg of cortisone acetate shall be given, as well as an intramuscular injection of 300 000 IU of procaine penicillin. The cortisone acetate may be given in divided doses over a period of several days, starting two days before the inoculation of vaccine. The monkeys shall be observed for 17-19 days and symptoms suggestive of poliomyelitis be recorded. Provided that at least 60% of the animals survive the first 48 hours after injection, those animals that do not survive this 48-hour period may be replaced by an equal number of test animals. If less than 60% of the original test animals survive the first 48 hours, or if the number of animals that survive the entire test period without significant weight loss is less than 80% of the initial number, the test must be repeated.

At the end of the observation period, samples of nervous tissue shall be taken for virus recovery and identification. Histological sections from both spinal cord enlargements shall be examined.

Doubtful histopathological findings necessitate (*a*) examination of samples of sections from several regions of the brain and spinal cord, and (*b*) attempts at virus recovery from the nervous tissues previously removed from the animal.

Evidence of intraspinal and intrathalamic trauma due to injection must be observed in at least 80% of the animals.

The final lot passes the test if the histological and other studies show no evidence of poliomyelitis infection.

5.3.2 *Innocuity test*

Each final lot shall be tested for abnormal toxicity by appropriate tests involving injection into mice and guinea-pigs. The tests shall be approved by the national control authority.

5.4 *Potency test*

Each final vaccine shall be tested for immunizing potency by a test approved by the national control authority. Such a test shall be done in suitable animals (monkeys, guinea-pigs or chicks) by a method that measures either the titre of antibody response or the limit of dilution at which the vaccine fails to give a response in 50% of the animals. The preparation under test shall meet the potency requirements of the national control authority.

The tests should be done in parallel with a reference vaccine. It would be valuable for comparison of results if antibody responses were expressed in international units.

Manufacturers should be encouraged to use the micro-precipitation (D-line) test as an in-process control of their production methods.

When an adjuvant has been added to the vaccine, the plain vaccine from which the final bulk has been made should have passed the same potency tests as the final vaccine.

At present, acceptance criteria cannot be based on the results of the various tests because they cannot be validly compared. This can only be achieved on the basis of comparisons of the relative potencies obtained in animals, using different methods and assay designs which include a common stable reference antigen, with the performance of the vaccines in the field.

5.5 *Protein nitrogen content*

Poliomyelitis vaccine (inactivated) shall not contain more than 0.02 mg of protein nitrogen per human dose.

5.6 *Penicillin content*

Poliomyelitis vaccine (inactivated) shall not contain more than the equivalent of 0.05 IU of penicillin per human dose.

6. Records

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

7. Samples

The requirements given in Part A, section 7, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply with the addition of the following :

[1] See Annex 1 of this report.

One sample shall be taken from the bulk vaccine at the latest possible stage of production, but before final preservatives, adjuvants, or other substances are added. The formaldehyde in such samples shall be neutralized at the time of sampling by the addition of bisulfite. Another sample shall be taken of the finished product in the final containers, and shall be representative of each filling lot. All samples shall be stored at a temperature between 0° and 10°C.

The samples required are relatively large in size, as much as 2500 ml of the trivalent bulk vaccine and 200 ml in final containers from each filling lot being needed to perform the tests for potency and safety.

Accidents, contaminations, technical difficulties, etc., may necessitate repetition of any of the required tests. Manufacturers should therefore follow the practice of retaining additional samples.

8. Labelling

The requirements given in Part A, section 8 of Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply with the addition of the following:

The leaflet accompanying the package shall include the following information:

the nature of the tissue and the virus strain used for the production of the vaccine and the method used for killing the virus.

9. Distribution and shipping

The requirements given in Part A, Section 9, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply.

10. Storage and expiry date

10.1 *Storage conditions*

Poliomyelitis vaccine (inactivated) shall be stored at all times at a temperature between 0° and 10°C.

10.2 *Expiry date*

The expiry date shall be fixed with the approval of the national control authority and shall be not more than 18 months after the date of the last

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[1] See Annex 1 of this report.

satisfactory potency test, the date of a potency test being that date on which the test animals were inoculated with the vaccine. The expiry date shall not, however, be more than 12 months from the date at which the vaccine was issued by the manufacturer.

In some cases the national control authority may decide, on the basis of experimental evidence, to leave the expiry date at 18 months after the date of the last satisfactory potency test, irrespective of the date of issue by the manufacturer.

Part B. National Control Requirements

1. General

The general requirements for control laboratories contained in Part B of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply with the addition of the following:

The national control authority shall specify potency requirements.

The national control authority shall be satisfied that the results of all tests, including those done on monovalent pools during the process of manufacture, are satisfactory and that consistency has been established.

2. Release and certification

Poliomyelitis vaccine (inactivated) shall be released only if it fulfils Part A of these requirements.

A statement signed by the appropriate official in charge of the national control laboratory shall be provided at the request of the producing laboratory and shall certify whether or not the lot of vaccine in question meets all national requirements as well as the requirements set forth in this document. The certificate shall also state the date of the last satisfactory potency test, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of biological substances between countries.

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[1] See Annex 1 of this report.

Annex 3

REQUIREMENTS FOR POLIOMYELITIS VACCINE (ORAL) (REQUIREMENTS FOR BIOLOGICAL SUBSTANCES No. 7)

Revised 1965 *

General Considerations

Since the Requirements for Poliomyelitis Vaccine (Oral) [1] were first formulated, more experience both in production of this vaccine and in its use in man has been gained. In the main, the Sabin strains have been used but in one country some new Koprowski strains have been tested. There have been a number of attempts to replace the type 3 Sabin strain.

Since the finding that simian virus SV40 may occur in the virus harvest from the rhesus monkey kidney tissue commonly used for vaccine production, a number of methods have been recommended for the removal of this

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* Requirements for Poliomyelitis Vaccine (Oral) (Requirements for Biological Substances No. 7) were first published in *Wld Hlth Org. techn. Rep. Ser.*, 1962, **237**. The original draft of these requirements was prepared by a Study Group on Requirements for Biological Substances which met in Geneva from 7-12 November 1960. The members of this Study Group were : Dr M. P. Chumakov, Institute for Poliomyelitis Research, Moscow, USSR ; Dr D. G. Evans, National Institute for Medical Research, London, England (Rapporteur) ; Dr S. Gard, Karolinska Institutet, Stockholm, Sweden ; Dr J. H. S. Gear, Poliomyelitis Research Foundation, Johannesburg, South Africa (Chairman) ; Dr R. Murray, Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA ; Dr F. P. Nagler, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada ; Dr F. Przesmycki, State Institute of Hygiene, Warsaw, Poland (Vice-Chairman) ; Dr D. Slonim, Institute of Sera and Vaccines, Prague, Czechoslovakia ; Dr M. K. Voroshilova, Institute for Poliomyelitis Research, Moscow, USSR ; Dr N. K. Jerne, Chief Medical Officer, Biological Standardization, WHO, acted as Secretary. This Annex comprises a revised version of these requirements, incorporating additions and amendments made by the WHO Expert Group on Requirements for Biological Substances which met in March 1965.

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1959, **237**.

virus from a contaminated harvest. None of these methods can be relied upon for the complete removal of SV40 virus and it is advisable to adopt rigorous steps to prevent contamination of the harvest with the virus. The use of alternative monkey species and possibly screening the animals for SV40 antibodies are good precautions, but even these measures do not eliminate the necessity for extensive tests for the detection of all extraneous agents.

Vaccine has been produced in a human diploid cell strain WI-38 with the Koprowski virus strains and has been administered orally to many thousands of persons in whom satisfactory immunity developed without, so far, producing untoward effects. Since this cell strain is free from all detectable adventitious agents, this development should be considered as a step towards a more reliable method for the production of oral poliomyelitis vaccine. The available information does not yet allow a decision on the use of such cell strains for production of this vaccine.

Each of the following sections constitutes a recommendation. The parts of each section that 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 included in definitive national requirements. The parts of each section that are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning oral poliomyelitis vaccine, it is recommended that a clause be included which would permit modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of safety and a potency of the vaccine at least equal to those provided by the requirements formulated below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms " national control authority " and " national control laboratory ", as used in these requirements, always refer to the country in which the vaccine is manufactured.

Part A. Manufacturing Requirements

1. Definitions

1.1 *International name and proper name*

The international name shall be " Vaccinum poliomyelitidis perorale Typus I, II, III " (whichever type or types apply). The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 *Descriptive definition*

Vaccinum poliomyelitidis perorale is a preparation of live attenuated poliovirus types 1, 2 or 3 containing any one type or any combination of the three types, and satisfying all the requirements formulated in this document.

1.3 *International standards and international reference preparations*

The International Standards for Antipoliovirus Sera Type 1, Type 2 and Type 3 (established in 1962) are dispensed in ampoules containing dried hyperimmune monovalent serum. Each ampoule of each type contains 10 IU.

These standards are in the custody of the International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen. Samples are distributed free of charge on request to national control laboratories. The international standards are intended for the calibration of national standards for use in the manufacture and laboratory control of poliomyelitis vaccines.

Since no international standards or international reference preparations of live attenuated polioviruses are yet available, no requirements based on comparisons with such preparations can at present be formulated. National control authorities should provide reference preparations of live polioviruses for use in tests of virus concentration (see Part A, section 3.5.4). Such standards or reference preparations would be useful as a check on sensitivity of cell cultures used in virus titration.

1.4 *Terminology*

Original vaccine : A monovalent vaccine, prepared according to the author's specification from the original seed virus, and shown on oral administration to man in extensive field trials to be immunogenic and free from harmful effects.

Seed lot : A quantity of virus processed together and of uniform composition. Primary and secondary seed lots are not more passages removed from an original vaccine than a number approved by the national control authority.

In some countries it is required that the seed lots are not more than four passages removed from an original vaccine.

Single harvest : A virus suspension of one virus type harvested from cell cultures prepared from the kidneys of one monkey.

Bulk suspension : A pool of a number of single harvests of the same virus type.

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

Filling lot (final lot) : A collection of sealed, final containers or dragees that are homogeneous with respect to the risk of contamination during

filling or preparation with the finished vaccine. A filling lot must, therefore, have been filled or prepared in one working session.

Tissue culture infective dose 50% ($TCID_{50}$) : The smallest quantity of a virus suspension that will infect 50% of cell cultures.

Plaque-forming unit (PFU) : The smallest quantity of a virus suspension that will produce a plaque in monolayer cell cultures.

2. General manufacturing requirements

The general manufacturing requirements contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply to establishments manufacturing oral poliomyelitis vaccine, with the addition of the following :

In order to ensure that there will be no risk of contaminating the vaccine at any stage during manufacture by pathogenic micro-organisms that may have previously been present in the production areas, these areas shall be decontaminated before they are used for the manufacture of oral poliomyelitis vaccine.

The production of oral poliomyelitis vaccine shall be conducted by a separate staff, which shall consist of healthy persons, who shall submit to regular medical examination. Steps shall be taken to ensure that all persons in the production areas and monkey quarters are immune against poliomyelitis and do not excrete poliovirus or other micro-organisms of significance to the safety of the vaccine.

Visitors not directly concerned with the production processes shall not be permitted to enter the production areas.

3. Production control

3.1 *Control of source materials*

3.1.1. *Virus strains*

Strains of poliovirus used in the production of oral poliomyelitis vaccine shall be identified by historical records, which should include information on the origin of the strains, on the methods used in their attenuation, as well as on all other points listed in Part B, section 1 of these requirements, in which recommendations to national control authorities concerning strain selection are set forth. Only strains approved by the national control authority shall be used.

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[1] See Annex 1 of this report.

3.1.2 *Monkeys used for the production of seed virus*

Monkeys of a suitable species, in good health, and which have not previously been used for experimental purposes of significance to the safety of the vaccine, shall be used as the source of kidney tissue for the production of seed virus and vaccine. They shall conform to all the requirements given in Part A, section 3.2.1 of these requirements.

It is advisable that the monkeys used for vaccine production should be shown to be free from SV40 antibodies.

3.1.3 *Seed lot system*

The production of vaccine shall be based on the seed lot system. The seed virus for the production of vaccine shall be original vaccine, or a seed lot used in preparing original vaccine, or a seed lot prepared therefrom. Seed lots shall be prepared in monkey kidney-cell cultures under conditions satisfying the requirements of Part A, section 3.2 and shall be stored at a temperature below −20°C. All seed lots shall meet the criteria of section 3.1.4.

It is recommended that a large primary seed lot be set aside as the basic material to which the manufacturer can return for the preparation of secondary seed lots. It is desirable to store seed lots at a temperature below −60°C.

3.1.4 *Tests on seed lots*

The seed lot used for the production of vaccine shall be free from detectable extraneous viruses and shall satisfy the requirements of Part A, sections 3.3, 3.4, 3.5 and 3.6. Each seed lot shall have been tested in parallel with an original vaccine, according to the requirements of Part A, section 3.5.5.

The manufacturer should obtain a sufficient amount of original vaccine to enable him to perform all tests that are designed to ensure close similarity in laboratory performance between new seed lots and original vaccine.

3.2 *Production precautions*

The general production precautions as formulated in the requirements of Part A, section 3, of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply to the manufacture of oral poliomyelitis vaccine with the addition of the following:

[1] See Annex 1 of this report.

3.2.1 *Monkeys used for the production of vaccine*

Monkeys used for the production of vaccine shall conform to the requirements of Part A, section 3.1.2. They shall be kept in well-constructed animal rooms. The cages shall be covered on all sides except the front.

> It is recommended that not more than two monkeys be housed per cage and that the cages be spaced as far as possible, with adequate ventilation.

Monkeys shall be kept in quarantine groups and cage-mates shall not be interchanged. A quarantine group is a colony of selected, healthy monkeys kept in one room, with separate feeding and cleaning facilities, and having no contact with other monkeys during the whole of the quarantine period. The quarantine period shall be at least 6 weeks.

The monkeys shall be under veterinary supervision. If disease is observed in any monkey, none of the monkeys from the quarantine group concerned shall be used for vaccine production until the cause of the disease has been resolved and shown not to impair the safety of the vaccine.

> It is recommended that strict measures be enforced in order to reduce the risk of dissemination of infections within and between quarantine groups.
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> A further hazard exists because of the occurrence of B virus infection in some monkeys and it is recommended that the handling of these animals be reduced to a minimum. Workers should use protective clothing and other protective devices where possible. It is suggested that no monkey should be handled unless anaesthetized. It is further recommended that research be conducted towards developing prophylactic agents and protective sera against this infection.
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> Past experience has shown that monkeys may be infected with *Mycobacterium tuberculosis*. Adequate measures should therefore be taken to protect the personnel.

When monkeys are killed for removal of kidneys, they shall be thoroughly examined by a person experienced in the diagnosis of monkey diseases, particularly for evidence of tuberculosis and B virus infection. If a monkey shows any pathological lesion relevant to the use of its kidneys in the preparation of the vaccine, this monkey shall not be used, and none of the remaining monkeys of the quarantine group concerned shall be used for vaccine production unless it is evident that their use will not impair the safety of the vaccine.

All operations described in this section shall be conducted outside the actual production areas.

3.2.2. *Monkey kidney cell cultures for vaccine production*

Virus for the preparation of vaccine shall be grown by aseptic methods in cultures of monkey kidney cells that have not been propagated in series. The maintenance medium shall contain no added serum.

> Suitable antibiotics in small concentrations may be used. If penicillin is used its concentration should not exceed 200 IU per ml. Non-toxic pH indicators may be added, such as phenol-red in a concentration of 0.002%.

Each group of cell cultures derived from a single monkey is used to produce a single harvest and shall, therefore, be prepared and tested as an individual group.

3.2.3 *Tests of cell cultures used for vaccine production*

On the day of inoculation with seed virus, each cell culture shall be examined for degeneration. If this examination shows evidence that a culture is infected with cytopathic virus, other than foamy virus,[1] or with other microbial agents, the whole group of cultures concerned shall not be used for vaccine production.

A sample of 10 ml of the pooled fluid removed from the cell cultures of the kidneys of each single monkey on the day of inoculation with the seed virus shall be divided into two equal portions, one to be used for tests in monkey kidney-cell cultures prepared from the same species, but not the same animal, as that used for vaccine production and the other to be used for tests in monkey kidney-cell cultures from a species known to be sensitive to SV40 virus, as described in Part A, section 3.3.4 of these requirements. Another sample of 10 ml shall be inoculated into rabbit kidney-cell cultures as described in Part A, section 3.3.5. If by these tests evidence is found of the presence of an adventitious agent in a cell culture, the single harvest from the group of cell cultures concerned shall not be used for vaccine production. If these tests are not done immediately, samples shall be stored as required in Part A, section 3.3.2.

> It is suggested that, in addition to these tests, the fluid removed from the cell cultures on the day of inoculation with the seed virus be tested for the presence of adventitious agents by inoculation of 10-ml samples into human amnion-cell cultures. If evidence is found of the presence of an adventitious agent, the single harvest from the group of cell cultures concerned should not be used for vaccine production.

3.2.4 *Tests of control cell cultures*

On the day of inoculation with the seed virus, cultures prepared from 25% of the cell suspension obtained from the kidneys of each single monkey shall remain uninoculated, and shall serve as controls. These control

[1] In some countries, if foamy virus is detected the material is not acceptable. — ED.

cultures shall be incubated under the same conditions as the inoculated cultures for at least two additional weeks, and shall be examined during this period for evidence of cytopathic changes. For the tests to be valid, not more than one fifth of the control cultures shall be discarded for non-specific, accidental reasons.

At the end of the observation period, the control cell cultures shall be examined for degeneration. If this examination shows evidence that a culture is infected with cytopathic virus, other than foamy virus,[1] the whole group of cultures concerned shall not be used for vaccine production.

At the end of the observation period, the control cultures shall also be shown, by the addition of guinea-pig red blood cells, to be free from haem-adsorption viruses.

At the time of harvest, or not more than 7 days after the day of inoculation with the seed virus, a sample of 10 ml of the pooled fluid from each group of control cultures shall be divided into two equal portions, one to be used for tests in monkey kidney-cell cultures prepared from the same species, but not the same animal, as that used for vaccine production and the other to be used for tests in monkey kidney-cell cultures known to be sensitive to SV40 virus, as described in Part A, section 3.3.4 of these requirements. Another sample of 10 ml shall be inoculated into rabbit kidney cell cultures as described in Part A, section 3.3.5. Uninoculated cell cultures shall be used as controls. The control cultures pass the tests if the criteria of acceptability as described in Part A, sections 3.3.4 and 3.3.5 are met.

If by the tests required in this section evidence is found of the presence in a control culture of any adventitious agent, other than foamy virus,[1] the single harvest from the group of cell cultures concerned shall not be used for vaccine production.

> In several countries it is considered obligatory to make additional tests for adventitious agents in the fluid from the control cultures by inoculation of 10-ml samples into human amnion-cell cultures.

3.2.5 *Temperature of incubation*

During the period between inoculation and harvest, the cell cultures shall at no time be at a temperature outside the range of 34°-35°C.

3.3 *Control of single harvests*

3.3.1 *Single harvest*

Virus suspensions shall be harvested not later than 6 days after virus inoculation.

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[1] See footnote on previous page.

3.3.2 *Sampling*

Samples for testing single harvests shall be taken immediately on harvesting. If the tests described in Part A, sections 3.3.4 and 3.3.6 are not performed immediately, the samples for these tests shall be kept at a temperature below −60°C. Samples for detection of B virus may be held at 4°C provided that the test described in Part A, section 3.3.5 is done within 7 days of storage.

The volumes of single harvests required to be tested in Part A, sections 3.3.3, 3.3.4 and 3.3.5 are based on the assumption that the virus concentrations in these samples are within the range between 10^7 $TCID_{50}$ and 5×10^7 $TCID_{50}$ per ml. If the titre is significantly higher, the volumes to be tested may be reduced accordingly. If the titre is below 10^7 $TCID_{50}$ per ml, the volumes to be tested shall be increased accordingly.

3.3.3 *Sterility tests*

A volume of 10 ml, or at least 0.5% of each single harvest shall be tested for bacterial and mycotic sterility as described in Part A, sections 5.2.1.2 and 5.3.1.2 of requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[1]

Each single harvest shall also be tested for the presence of *Mycobacterium tuberculosis* by appropriate culture methods and for mycoplasma [2] by a method approved by the national control authority.

3.3.4 *Tests of neutralized single harvests*

A sample of at least 10 ml of each single harvest shall be neutralized by type-specific poliomyelitis antiserum prepared in animals other than monkeys. In preparing antisera for this purpose the immunizing antigens used shall be cultured in non-simian cells.

Half (5 ml) of the neutralized suspension shall be tested in monkey kidney-cell cultures prepared from the same species, but not the same animal, as that used for vaccine production. The other half of the neutralized suspension shall be inoculated into monkey kidney-cell cultures known to be sensitive to SV40 virus.

The neutralized suspensions shall be inoculated into bottles of these cell cultures, in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 : 4. The area of the cell sheet shall be at least 3 cm^2 per ml of neutralized suspension. At least one bottle of uninoculated cell culture shall serve as a control and shall be maintained by nutrient medium containing the same concentration of the specific serum used for neutralization. The cultures shall be incubated at a temperature of 37°C

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **200**.

[2] Tests for mycoplasma should be done using both solid and liquid media which have been shown to be capable of supporting the growth of mycoplasma. — ED.

and shall be observed for a period of at least 2 weeks. At the end of this observation period at least one subculture of fluid from each of these cultures shall be made in the same tissue culture system and both primary cultures and subcultures observed for an additional 2 weeks. If necessary, serum may be added to the primary cultures at this stage provided that the serum does not contain SV40 antibody or other inhibitors. If any cytopathic changes occur in any of the cultures the causes of these changes shall be investigated. If the cytopathic changes are shown to be due to unneutralized poliovirus the test shall be repeated. The single harvest passes the test if there is no evidence of the presence of SV40 virus or other adventitious agent attributable to the single harvest.

In some countries this test is carried out on the final bulk as described in Part A, section 3.6.2.

It is also suggested that fluorescent antibody techniques may be useful for detecting SV40 virus in the cells.

3.3.5 *Test in rabbit kidney-cell cultures*

A sample of at least 20 ml of each single harvest shall be tested in rabbit kidney-cell cultures. The sample shall be inoculated into bottles of these cell cultures, in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 : 4. The area of the cell sheet shall be at least 3 cm^2 per ml of suspension. At least one bottle of uninoculated cell cultures shall serve as a control. The cultures shall be incubated at 37°C and maintained for a period of at least 2 weeks. Serum used in the nutrient medium of rabbit kidney-cell cultures shall have been shown to be free from B virus inhibitors. The single harvest passes the test if there is no evidence of the presence of B virus.

If the presence of B virus is demonstrated in this test, or in the test described in Part A, section 3.4.3, the manufacture of oral poliomyelitis vaccine shall be discontinued. It shall not be resumed until a thorough investigation has been completed and the necessary precautions have been taken, and then only with the approval of the national control authority.

3.3.6 *Tests in animals*

The single harvests shall be tested in animals, as described in Part A, section 3.4, unless these tests are performed on the bulk suspension.

3.3.7 *Preservatives and stabilizers*

Preservatives or stabilizers that may be added to the single harvests or to the bulk suspension shall have been shown to the satisfaction of the national control authority not to impair the safety and effectiveness of the vaccine.

All tests described in Part A, sections 3.3 and 3.4 shall be done on samples taken before any preservatives or stabilizers are added.

3.4 *Control of the bulk suspension before filtration*

3.4.1 *Bulk suspension*

Each bulk suspension shall be tested in animals as described in the following sections unless these tests have already been made on single harvests (see Part A, section 3.3.6).

3.4.2 *Sampling*

Samples for testing the bulk suspension before filtration shall be taken immediately after the bulk suspension has been prepared and, if not tested immediately, shall be kept at a temperature below −60°C until the tests for the presence of adventitious micro-organisms described in Part A, sections 3.4.4, 3.4.5 and 3.4.6 are performed. Samples for detection of B virus may be held at +4°C provided that the test described in Part A, section 3.4.3 is done within 7 days of storage.

The volumes of bulk suspensions required to be tested in these sub-sections are based on the assumption that the virus concentration in the samples is within the range between 10^7 and 5×10^7 $TCID_{50}$ per ml. If the titre is significantly higher, the total volumes of undiluted bulk suspension to be tested may be reduced accordingly, without reducing the total number of animals required in these tests. If the titre is below 10^7 $TCID_{50}$ per ml, the total volumes to be tested shall be increased accordingly.

3.4.3 *Test in rabbits*

A sample of the bulk suspension shall be tested for the presence of B virus by injection into at least 10 healthy rabbits each weighing between 1.5 kg and 2.5 kg. The sample shall consist of 100 ml if 10 animals are used or a proportionately larger volume if more animals are used. Each rabbit shall receive 10 ml of the bulk suspension, at multiple sites, 1 ml being given intradermally and the remainder subcutaneously. The rabbits shall be observed for at least 3 weeks for death or signs of illness. All rabbits that die after the first 24 hours of the test or that show signs of illness shall be examined by autopsy with removal of the brains and organs for detailed inspection.

The bulk suspension passes the test if at least 80% of the rabbits remain healthy and if none of the rabbits shows lesions of any kind at the sites of inoculation or shows evidence of infection with B virus or with any adventitious transmissible agent attributable to the bulk suspension.

If the presence of B virus is demonstrated in this test, the measures described in Part A, section 3.3.5 shall be taken.

3.4.4. *Test in adult mice*

Each bulk suspension shall be tested for the presence of adventitious agents pathogenic to mice by the intracerebral inoculation of 0.03 ml and intraperitoneal inoculation of at least 0.5 ml amounts of the suspension into each of at least twenty adult mice, each weighing 15-20 grams. The mice shall be observed for at least 3 weeks. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined for evidence of viral infection ; this shall be done macroscopically by direct observation as well as by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least 5 additional mice, which shall be observed for 3 weeks.

The bulk suspension passes the test if at least 80% of the inoculated animals survive the observation period and none of the mice shows evidence of infection with adventitious transmissible agents, attributable to the bulk suspension.

3.4.5 *Test in suckling mice*

Each bulk suspension shall be tested for the presence of adventitious agents pathogenic to mice by the intracerebral inoculation of 0.01 ml and intraperitoneal inoculation of at least 0.1 ml of the suspension into each of at least 10 mice less than 24 hours old originating from more than one litter. The mice shall be observed daily for at least 14 days. All mice that die after the first 24 hours of the test or that show signs of illness shall be autopsied and examined for evidence of viral infection ; this shall be done macroscopically by direct observation and the tissues shall be examined microscopically and by subinoculation of appropriate tissue suspensions by the intracerebral and intraperitoneal routes into at least 5 additional suckling mice, which shall be observed daily for 14 days.

> In some countries, in addition, a blind passage is made of a suspension of the pooled emulsified tissue (minus skin and viscera) of all mice surviving the original 14-day test.

The bulk suspension passes the test if at least 80% of the mice originally inoculated remain healthy and if none of the mice shows evidence of infection with adventitious transmissible agents attributable to the bulk suspension.

3.4.6 *Test in guinea-pigs*

Each bulk suspension shall be tested for the presence of *Mycobacterium tuberculosis* or other adventitious agents by the intraperitoneal inoculation of 5.0 ml of the virus pool into each of at least five guinea-pigs of 350-450 g weight. The animals shall be observed for at least 42 days for death or signs of disease. All guinea-pigs that die after the first 24 hours of the test

or that show signs of illness shall be examined macroscopically and the tissues shall be examined both microscopically and culturally for evidence of infection with *Mycobacterium tuberculosis*. Animals that survive the observation period shall be examined in a similar manner.

The bulk suspension passes the test if at least 80% of the original group of guinea-pigs remain healthy and survive the observation period, and if none of the animals shows evidence of infection with *Mycobacterium tuberculosis* or any adventitious transmissible agent attributable to the bulk suspension.

The test may be made using the deposit centrifuged from a large volume of bulk suspension and resuspended in a small volume of saline or supernatant.

It is desirable to record the rectal temperature of the animals daily during the first three weeks.

3.5 *Control of bulk suspension after filtration*

3.5.1 *Filtration of bulk suspension*

The bulk suspension shall be filtered through a filter having a porosity that will retain bacteria and other large micro-organisms.

3.5.2 *Sampling of filtered bulk suspension*

Samples of the filtered bulk suspension shall be taken immediately after filtering and shall be kept at a temperature below —20°C until the tests described in the following sections are made.

3.5.3 *Identity test*

The poliovirus type in the filtered bulk suspension shall be serologically identified.

3.5.4 *Virus concentration*

The determination of the amount of infective poliovirus per ml of filtered bulk suspension shall be made in cell cultures. This determination shall be made in terms of PFU per ml and/or in terms of $TCID_{50}$ per ml, in parallel with the determination of the virus concentration of a known reference preparation of the same poliovirus type. The determination of the number of PFU per ml shall be based on a total count of at least 100 clearly defined plaques on at least 5 different cell sheets. The determination of the number of $TCID_{50}$ per ml shall be based on the use of 10-fold dilution steps with 10 tubes per dilution, or on any other arrangement of dilutions and tubes yielding equal precision.

It is desirable for the national control laboratory to issue reference virus preparations to manufacturers (see Part A, section 1.3).

3.5.5 *Tests for constancy of vaccine quality*

The poliovirus in the filtered bulk suspension shall be tested in comparison with the seed virus with regard to certain characteristics, as described in the following sub-sections.

The object of these tests is to ensure that the virus has not undergone changes during its multiplication in vaccine preparation. Since the seed virus may be either original vaccine, or a seed lot that has been tested in comparison with original vaccine, the tests also ensure that the virus in the vaccine prepared has the same characteristics as the virus in original vaccine that has been shown to be safe and effective in man.

The tests in monkeys will also serve to detect contamination with wild polioviruses.

3.5.5.1 *Tests in monkeys*

The pathogenicity of the bulk suspension to *Macaca* or *Cercopithecus* monkeys shall be tested in comparison with that of the seed virus by inoculation by the intrathalamic and the intraspinal routes of injection. The number of monkeys inoculated by each route shall be approved by the national control authority. The monkeys shall have been shown to be free from antibodies to all 3 types of poliovirus. For the test to be valid, the virus suspension shall contain at least 10^7 $TCID_{50}$ per ml or 10^7 PFU/ml. The volume of virus suspension inoculated into each thalamus shall be 0.5 ml and that injected into the lumbar spinal cord not less than 0.1 ml. All monkeys shall be observed for at least 18 days for symptoms suggestive of poliomyelitis or other virus infection. Those monkeys that die after the first 24 hours of the test as well as those that survive the observation period shall be examined by autopsy and histological examinations made on the lumbar cord, the cervical cord, the lower medulla, the upper medulla, the mesencephalon and the motor cortex of each monkey. Negative histological findings in monkeys showing evidence of faulty injection technique shall not be considered.

In some countries, reliance is placed mainly on the intrathalamic test. In this case, 30 monkeys are inoculated intrathalamically with undiluted vaccine and 3 groups, each of 5 monkeys, are inoculated intraspinally with undiluted vaccine, vaccine diluted to 10^{-3} and vaccine diluted to 10^{-4} respectively. In other countries, reliance is placed mainly on the intraspinal test. In this case, intraspinal inoculations of undiluted vaccine and vaccine dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} are made, using

5 monkeys for each dilution and intrathalamic inoculation is made with undiluted vaccine only, using 10 monkeys.[1]

The filtered virus suspension passes the test if at least 80% of the animals in each group remain healthy, survive the observation period and show evidence of valid inoculation trauma, and if none of the clinical and histopathological findings indicates a significant difference in pathogenicity between the vaccine virus and the seed virus.

3.5.5.2 *Tests in vitro*

The poliovirus in the filtered bulk suspension shall be tested for its property of reproducing at the temperatures of 36°C and 40.3°C (rct/40 marker) in comparison with the seed virus and with appropriate reference strains of poliovirus of the same type.

It is desirable for national control laboratories to issue reference viruses for this test.

The incubation temperatures used in this test shall be strictly controlled.

The bulk suspension shall have passed the test if, for both the virus in the bulk suspension and the seed virus, the titre determined at 36°C is

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[1] At a Symposium on Testing for Neurovirulence of Virus Vaccines held by the Permanent Section of Microbiological Standardization of the International Association of Microbiological Societies at Munich, 25-28 August 1965, recommendations were made regarding the minimum number of sections to be taken and examined at each level of the central nervous system. These are summarized in the table below.

It may be desirable to reserve part of the material from which sections are taken for virus isolation, if necessary.

Route of injection	Level of CNS	Number of sections to be taken per monkey [a]	
		A	B
Intrathalamic route	Lumbar cord	10	14
	Cervical cord	10	10
	Medulla	2	3
	Mid-brain	2	1
	Thalamus	1	2
	Motor cortex	1	2
Intraspinal route	Lumbar cord	12	14
	Cervical cord	10	10
	Medulla	2	1
	Mid-brain	1	1
	Thalamus	0	0
	Motor cortex	1	2

[a] A = tests in which reliance is placed mainly on the intrathalamic tests; B = tests in which reliance is placed mainly on the intraspinal tests.

The inclusion of the information given in this footnote was suggested by the eighteenth WHO Expert Committee on Biological Standardization. — ED.

at least 100 000 times that determined at 40.3°C. Unless the titres obtained for the reference viruses show expected values, the test shall be repeated.

It is strongly recommended that the manufacturer should perform at least one other test for a genetic marker, since a genetic change could occur which may not be detected by the rct/40 marker test only. Other marker tests currently used are (*a*) tests based on the study of antigenic character of the strain, (*b*) tests for the sensitivity of reproduction to different concentrations of sodium bicarbonate (d-marker) and (*c*) tests for the capacity to reproduce in tissues of different origin, such as the so-called monkey-stable line (MS-marker).

3.6 *The final bulk*

The final bulk shall be prepared from either one filtered bulk suspension or a blend of filtered bulk suspensions, or a dilution thereof. The operations necessary for preparing a final bulk shall be conducted in such a manner as to avoid contamination of the product.

A stabilizer may be added to the product in preparing the final bulk. Such stabilizers should have been shown to the satisfaction of the national control authority not to impair the safety and effectiveness of the vaccine.

3.6.1 *Sterility test*

The final bulk shall pass the tests for bacterial and mycotic sterility in accordance with the requirements given in Part A, section 5 of Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[1]

3.6.2 *Test for SV40 virus*

A sample of at least 25 ml of the final bulk should be neutralized by specific poliomyelitis antisera and tested for SV40 virus as described in Part A, section 3.3.4, unless all components of the final bulk have already passed the test.

The sample should not contain substances that may interfere with the growth of SV40 virus in tissue culture.

4. Filling and containers

The requirements concerning filling and containers given in Part A, section 4 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [2] shall apply to vaccine filled out in the liquid form. Vaccine incorporated into a solid medium (as in the case of dragees) shall be processed in accordance with the regulations governing the production of tablets and capsules in pharmaceutical manufacture.

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **200**, 13.

[2] See Annex 1 of this report.

5. Control tests on final product

Samples shall be taken from each filling lot for the following tests.

5.1 *Identity test*

The poliovirus type or types shall be serologically identified.

5.2 *Sterility test*

Samples of each filling lot of liquid vaccine shall be tested for bacterial and mycotic sterility according to the requirements given in Part A, section 5 of Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances). [1]

5.3 *Virus titration*

The determination of the poliovirus titre shall be made as described in Part A, section 3.5.4 of these requirements.

5.4 *Innocuity tests*

The innocuity of each filling lot shall be tested by appropriate tests in mice, guinea-pigs, and rabbits, using parenteral injections. The tests shall be approved by the national control authority.

6. Records

The requirements given in Part A, section 6 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [2] shall apply.

7. Samples

The requirements given in Part A, section 7 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [2] shall apply.

8. Labelling

The requirements given in Part A, section 8 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **200**, 13.

[2] See Annex 1 of this report.

Establishments and Control Laboratories) [1] shall apply with the addition of the following:

The label on the container shall include the following information:

the fact that the vaccine is for oral administration only.

The leaflet accompanying the package shall include the following information:

the designation(s) of the strain(s) of poliovirus contained in the vaccine;

the fact that the vaccine was prepared in monkey kidney tissue;

the nature and amount of stabilizers present in the vaccine;

the nature and amount of antibiotics used in the preparation of the vaccine;

the amount of virus of each type contained in one recommended human dose.

9. Distribution and shipping

The requirements given in Part A, section 9 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [2] shall apply.

10. Storage and expiry date

The statements concerning storage temperatures and expiry dates appearing on the label and the leaflet, as required in part A, section 8 of these requirements, shall be based on experimental evidence and shall be submitted for approval to the national control authority.

10.1 *Storage conditions*

Before being distributed by the manufacturing establishment, or before being issued from a depot for the maintenance of reserves of vaccines, all vaccines in their final containers shall be kept continuously at a temperature below −20°C. After distribution or issue the vaccine in the final containers shall be stored at a temperature below 0°C.

Liquid vaccine, after thawing for intended use, may be kept at a temperature between 2°C and 10°C for 30 days.

Vaccine may be kept for longer periods of time if a suitable stabilizer has been added.

10.2 *Expiry date*

The date after which the vaccine may not be used shall be not more than two years after it has passed the last virus titration as described in Part A,

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **200**, 13.

[2] See Annex 1 of this report.

section 5.3 of these requirements, provided that the vaccine has been stored continuously at a temperature below −20°C. The expiry date shall not, however, be more than one year from the date of issue by the manufacturer, provided that the vaccine has been stored below 0°C.

Part B. National Control Requirements

1. General

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

The national control authority shall give directions to manufacturers concerning the poliovirus strains to be used in vaccine production and concerning the recommended human dose.

The strains used in the production of oral poliomyelitis vaccine must have been shown to yield vaccines which are immunogenic and free from harmful effects upon oral administration to susceptible children and adults.

A number of attenuated strains have been developed for this purpose during recent years.

The national control authority should take into consideration all available information on strains before deciding on the strain permitted for vaccine production.

2. Release and certification

A vaccine lot shall be released only if it fulfils Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify whether the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall, furthermore, state the date of the last satisfactory determination of virus concentration, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of biological substances between countries.

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[1] See Annex 1 of this report.

Annex 4

REQUIREMENTS FOR SMALLPOX VACCINE (REQUIREMENTS FOR BIOLOGICAL SUBSTANCES No. 5)

Revised 1965 *

General Considerations

Since the Requirements for Smallpox Vaccine (Requirements for Biological Substances No. 5) [1] were published in 1959, there have been advances in production and control which necessitate modification of the require-

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* Requirements for Smallpox Vaccine (Requirements for Biological Substances No. 5) were first published in *Wld Hlth Org. techn. Rep. Ser.*, 1959, **180**. The original draft of these requirements was prepared by a Study Group on Requirements for Biological Substances which met in Geneva from 3-8 November 1958. The members of this Study Group were: Dr J. Desbordes, Directeur du Contrôle bactériologique, Laboratoire national de la Santé publique, Ministère de la Santé publique et de la Population, Paris, France; Dr D. G. Evans, Director, Department of Biological Standards, National Institute for Medical Research, London, England (Chairman); Dr R. Gispen, Director, National Institute of Public Health, Utrecht, Netherlands; Dr L. Greenberg, Chief, Biologics Control Laboratories, Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Canada; Dr E. Krag Andersen, Statens Seruminstitut, Copenhagen, Denmark; Dr U. Krech, Chief, Virus Department, Serum and Vaccine Institute, Berne, Switzerland (Rapporteur); Dr A. Lafontaine, Directeur, Institut d'Hygiène et d'Epidémiologie, Brussels, Belgium; Dr R. Muckenfuss, Technical Director, Naval Medical Research Institute, Bethesda, Md., USA; Dr C. Puranananda, Director, Queen Saovabha Memorial Institute, Bangkok, Thailand; Dr G. Renoux, Directeur, Institut Pasteur, Tunis, Tunisia; Dr R. Sanjiva Rao, Assistant Director, Virus Research Centre, Poona, India (Vice-Chairman); Dr N. K. Jerne, Chief Medical Officer, Biological Standardization, WHO, acted as Secretary. This Annex comprises a revised version of these requirements, incorporating additions and amendments made by the WHO Expert Group on Requirements for Biological Substances which met in March 1965.

[1] *Wld Hlth Org. techn. Rep. Ser.*, 1959, **180**.

ments. Furthermore, there has been a meeting of a WHO Expert Committee on Smallpox [1] and some of the recommendations of that Committee suggest the need for modification of these requirements.

The recommendation of international requirements for smallpox vaccine is complicated by the fact that a number of different manufacturing and testing procedures are in use in various countries. The procedures differ in the vaccinia virus strain used, the preservatives added, the form in which the vaccine is issued, the methods for testing the potency, and the animal or tissues used for growing the virus.

The origin of the different strains is not known. Some vaccine strains are more pathogenic for man than others and since there is no evidence that a strain producing severe local lesions and marked systemic disturbance confers better protection than strains producing milder clinical reactions, the less pathogenic strains giving satisfactory immunity should be preferred for vaccine production. Further studies should be made of the various strains used and the vaccine obtained by different procedures. The local systemic reactions after vaccination and revaccination in the field should be compared in order to test the validity of the present requirements for virus strains. Studies on the antigenic and immunogenic properties of strains in relation to their reactivity would be useful and the genetic characters of these strains should be investigated.

Smallpox vaccines prepared in the skin of living animals have been in world-wide use for generations and considerable evidence has been accumulated on the protective value of vaccination with this type of vaccine. Adequate precautions must be taken, however, to ensure that no infectious agent transmissible to man exists in the animal. More recently, vaccine prepared by growing the virus in the developing chick embryo has come into use, but although this type of vaccine has certain advantages, there is, as yet, only limited information on its efficacy and safety in the field and on the duration of the immunity which it induces. As a result of the advances in tissue-culture techniques, an increasing interest has now developed in the use of such cultures for production of vaccine. Further investigations of smallpox vaccine prepared in this way are highly desirable in order that information may be collected over a sufficient period to permit a final evaluation of its efficacy and safety.

It is recognized that prolonged repeated passage of a virus strain in tissue culture or on the chorio-allantoic membrane may reduce its immunogenic property. The seed lot used for inoculating the tissue cultures or eggs for vaccine production should not be more than five passages removed from the animal host to which the virus was adapted. When eggs or tissue derived from chick embryos are used, the eggs must be obtained from a flock free from avian leucosis viruses. Similarly, tissue cultures should be

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1964, **283**.

free from known adventitious agents. It has been reported that smallpox vaccine prepared in human diploid cell strains has been used on an experimental scale.

Some observations suggest that vaccines of relatively low potency, though adequate for primary vaccination, may be inadequate for revaccination. Evidence of consistent successful revaccination in comparison with a suitable reference vaccine should be obtained before a vaccine produced on a new tissue is accepted. In the present requirements for virus concentration, full stress has been laid on the enumeration of pock-forming units in the chorio-allantoic membranes of chick embryos, as it is only with this test that the results have been correlated with the results of vaccination and revaccination in man.

Liquid vaccines deteriorate rapidly at moderately high atmospheric temperatures and on exposure to sunlight. Since a more stable product can be obtained by freeze-drying, the production of vaccine in a dried form should be preferred for hot countries and where transport or refrigeration is difficult. The use of freeze-dried smallpox vaccine is particularly important in extensive vaccination programmes involving several countries and every effort should be made to develop methods for producing large quantities of freeze-dried vaccine that conform to the requirements recommended for international use.

The present requirements have been formulated principally to cover vaccines intended for administration by the multiple pressure or single scratch method of vaccination.

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 included in definitive national requirements. The parts of each section which are printed in small type are comments and recommendations for guidance.

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning smallpox vaccine, it is recommended that a clause be included which would permit modifications of manufacturing requirements on the condition that it be demonstrated, to the satisfaction of the national control authority, that such modified requirements ensure a degree of safety and a potency of the vaccine at least equal to those provided by the requirements formulated below. It is desirable that the World Health Organization should then be informed of the action taken.

The terms " national control authority " and " national control laboratory ", as used in these requirements, always refer to the country in which the vaccine is manufactured.

Part A. Manufacturing Requirements

1. Definitions

1.1 *International name and proper name*

The international name shall be " Vaccinum variolae ". The proper name shall be the equivalent of the international name in the language of the country of origin.

The use of the international name should be limited to vaccines that satisfy the requirements formulated below.

1.2 *Descriptive definition*

Vaccinum variolae is a fluid or dried preparation of vaccinia virus grown in the skin of living animals or in the membranes of the chick embryo or in *in vitro* cultures of suitable tissues. The preparation shall satisfy all the requirements formulated below.

1.3 *International standard and reference preparations*

The International Reference Preparation of Smallpox Vaccine (established in 1962) is dispensed in ampoules containing 14 mg of freeze-dried smallpox vaccine.

This reference preparation is in the custody of the International Laboratory for Biological Standards, Statens Serum-institut, Copenhagen. Samples are distributed free of charge on request to national control laboratories. The international reference preparation is intended for the calibration of national reference preparations for use in the manufacture and laboratory control of smallpox vaccine.

The provision of an international standard for anti-smallpox serum is at present the subject of an international collaborative study sponsored by the World Health Organization. Such a standard can be used for the assay of variola and vaccinia antibodies.[1]

1.4 *Terminology*

Primary seed lot : A quantity of virus adapted to, and grown on the skin of a living animal, which has been processed together and has a uniform composition.

Secondary seed lot : A quantity of virus grown in the skin of living animals or in the chorio-allantoic membranes of chick embryos or in tissue cultures, which is uniform with respect to composition and is not more than 5 passages removed from a primary seed lot.

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[1] The International Standard for Anti-Smallpox Serum was established by the eighteenth WHO Expert Committee on Biological Standardization (*Wld Hlth Org. techn. Rep. Ser.*, to be published) — ED.

Single harvest : A quantity of material harvested from one animal or a quantity of material harvested from a group of chick embryos or tissue cultures inoculated, incubated and harvested together.

Bulk material : The material at any stage after harvesting and before filling into final containers. Bulk material may be prepared from one or a number of single harvests.

Final bulk : A quantity of vaccine after completion of preparations for filling and present in the container from which the final containers are filled.

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

Pock-forming unit : The smallest quantity of virus suspension that will produce a single pock on the chick chorio-allantoic membrane.

Plaque-forming unit (PFU) : The smallest quantity of virus suspension that will produce a single primary plaque in monolayer cell cultures.

2. General manufacturing requirements

The general requirements for manufacturing establishments contained in the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply to establishments manufacturing smallpox vaccine.

3. Production control

3.1 *Control of source materials*

3.1.1. *Virus strains*

The strains of virus used in the production of all seed lots shall be identified by historical records. They shall have been shown to the satisfaction of the national control authority to yield immunogenic vaccines which produce typical vaccinal lesions in the skin of man followed by insusceptibility to subsequent challenge by revaccination with a strain of virus known to protect man against variola. The strains shall produce a characteristic vesicular eruption in the skin of rabbits and reproducible characteristic pock lesions in the membranes of chick embryos. In addition, the vaccine strains shall be characterized by serological tests and animal inoculation.

Records shall be maintained of all tests made periodically for verification of strain characters.

The strain used for vaccine production should be one that has never shown a greater tendency to produce generalized

[1] See Annex 1 of this report.

lesions or lesions of the nervous system in either man or animals than other strains of vaccinia virus which have been in general use for many years and have been found to be satisfactory without producing severe local lesions and marked systemic disturbance. Strains of so-called " neurovaccine " should be excluded.

3.1.2 *Animals or tissues for the production of seed virus and vaccine*

Only healthy animals or tissues from healthy animals, susceptible to ectodermal inoculations with vaccinia virus, or chick embryos obtained from healthy flocks shall be used for vaccine production. They shall conform to all the requirements given in Part A, section 3.2 of these requirements. If cell cultures are used for vaccine production they shall be shown to be free from detectable adventitious agents.

Different species of animals may be used for vaccine production or for preparing seed virus. Calves, sheep, buffaloes, donkeys and rabbits are used successfully in different countries.

The chorio-allantoic membrane of the developing chick embryo and tissues from the embryos or young animals of susceptible species have also been found suitable for virus propagation.

3.1.3 *Seed lot system*

A primary seed lot shall be used as original material for the preparation of a secondary seed lot. The secondary seed lot shall be not more than five passages removed from a primary seed lot. If vaccine is produced in the skin of a living animal the secondary seed lot shall be prepared from the primary seed lot without passage in chick embryos or tissue cultures. Vaccines shall be prepared from a seed lot without intervening passage.

Seed lots should be maintained either in dried, frozen, or glycerinated form. If a glycerinated seed lot is used it should be kept continuously at a temperature below 0°C.

3.1.4 *Tests on seed lots for the presence of extraneous micro-organisms*

The seed lot, in the dilution used as inoculum for the production of vaccine in the skin of animals, shall satisfy the requirements of Part A, section 3.3.4 of these requirements.

The seed lot used for the production of vaccine in chick embryos or in tissue cultures shall, after rehydration if applicable, satisfy the requirements of Part A, section 3.3.5.

3.2 *Production precautions*

The general precautions as formulated in the requirements of Part A, section 3 of the revised Requirements for Biological substances No. 1 (General Requirements for Manufacturing Establishments and Control

Laboratories) [1] shall apply to the manufacture of smallpox vaccine with the addition of the following:

3.2.1 *Vaccines produced in the skin of living animals*

The animals shall be freed of ectoparasites, and each animal shall be kept in quarantine under veterinary supervision for at least two weeks prior to the inoculation of the seed virus. Before inoculation the animals shall be cleaned, and thereafter kept in scrupulously clean stalls until the vaccinal material is harvested.

> The use of bedding, unless sterilized and changed frequently, should be avoided. The stalls, including feed boxes, should be designed so as to make cleaning easy, and dust-producing food should be avoided.

During a period of five days before inoculation and during incubation the animals shall remain under veterinary supervision, they shall remain free from any sign of disease, and daily rectal temperatures shall be recorded. If any abnormal rise in temperature occurs, or if any clinical sign of disease is observed, the production of vaccine from the group of animals concerned shall be suspended until the cause of these irregularities has been resolved. The prophylactic and diagnostic procedures adopted to exclude the presence of infectious disease shall be submitted for approval to the national control authority.

> According to the species of animal used and the diseases to which that animal is liable in the country where the vaccine is being produced, the prophylactic and diagnostic procedures to be used will vary. They must exclude the possibility of transmitting diseases within the country where the vaccine is prepared, but consideration should also be given to the danger of spreading diseases to other countries or continents to which the vaccine may be shipped.
>
> Special attention should always be given to foot-and-mouth disease, brucellosis, Q fever, tuberculosis, and dermatomycosis, but in some areas it will be necessary to consider diseases such as contagious pustular dermatitis (orf), pulpy kidney disease, sheep pox, anthrax, rinderpest, haemorrhagic septicaemia, Rift Valley fever, and many others.

The inoculation of seed virus shall be made on such parts of the animal as are not liable to be soiled by urine and faeces. The surface used for inoculation shall be so shaved and cleaned as to procure the nearest possible approach to surgical asepsis. If any antiseptic substance deleterious to the virus is used in the cleaning process it shall be removed by thorough rinsing with sterile water prior to inoculation. During inoculation, the exposed

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[1] See Annex 1 of this report.

surface of the animal not used for inoculation shall be covered with sterile covering.

Many workers prefer to inoculate the ventral surface of female animals. If male animals are used this area is more liable to soiling by urine and faeces than the flank, which may be equally susceptible to vaccinia virus and easier to keep clean, especially since the animal tends to rest on the uninoculated side.

It is recommended that narcotic or anaesthetic drugs be used to save the animal from unnecessary discomfort and pain during the process of shaving, cleaning and inoculation.

After inoculation the area may be covered with suitable antibiotics.

Before the collection of the vaccinal material, any antibiotic shall be removed and the inoculated area shall be subjected to a repetition of the cleaning process. The uninoculated surfaces shall be covered with sterile covering.

Before harvesting, the animal shall be killed painlessly. The animals shall be exsanguinated before harvesting to avoid heavy admixture of the vaccinal material with blood.

The vaccinal material from each animal shall be collected separately with aseptic precautions.

All animals used in the production of vaccine shall be examined by autopsy. If evidence of any generalized or systemic disease other than vaccinia is found, the vaccinal material from that animal shall be discarded. If the disease is considered to be a communicable one, the harvest from the entire group of animals exposed shall be discarded.

3.2.2 *Vaccines produced in the chick embryo*

Only eggs from flocks known to be free from disease, including avian leucosis, shall be used.

In particular, it is desirable that the eggs should be derived from flocks free from *Salmonella pullorum*, *Mycobacterium tuberculosis*, Rous virus, mycoplasma and other agents pathogenic for chickens.

Living embryos after incubation for a suitable period shall be inoculated with seed virus which satisfies the requirements of Part A, sections 3.1.3 and 3.1.4 of these requirements. After further incubation for a suitable period, the vaccinal material shall be harvested with aseptic precautions.

3.2.3 *Vaccines produced in tissue culture*

Only primary tissue cultures from animals known to be free from disease shall be used. The virus shall be grown and harvested with aseptic precautions. No material of human origin shall be added to the cultures at any stage.

Suitable antibiotics in minimum concentrations required for sterility may be used but the use of penicillin and streptomycin should be prohibited.

3.3 *Control of the bulk material*

3.3.1 *Initial treatment*

The vaccinal material harvested from the skin of each animal shall be subjected to a treatment designed to reduce its content of living extraneous micro-organisms, if this is necessary, to satisfy the requirements of Part A, section 3.3.4 of these requirements. No antibiotics shall be added to the bulk material.

If the vaccine is intended for issue in the liquid form, this treatment may consist of the addition of glycerol or other suitable diluent, with or without an antibacterial substance, and temporary storage at a suitable temperature.

If the vaccine is intended for issue in the dried form, the treatment may consist of the addition of a suitable antibacterial substance and/or of the removal of micro-organisms by centrifugation.

Vaccinal material collected from chick embryos or tissue cultures does not need such treatment, but glycerol and/or an antibacterial substance should be added as a precaution against later contamination.

It is recognized that antibiotics are used in the preparation of vaccines from tissue cultures, but in general the addition of antibiotics to smallpox vaccine should be discouraged.

3.3.2 *Final bulk*

After the initial treatment, vaccine intended for issue in the liquid form may be made up by dilution of bulk material with glycerol and/or another suitable diluent.

Vaccine intended for issue in the dried form may be subjected to additional processes before dilution of the bulk material.

Before making up a final bulk, it is advisable to do preliminary tests on the single harvests for potency and for the presence of living extraneous micro-organisms.

3.3.3 *Tests for virus concentration on the final bulk*

The final bulk shall pass the test for virus concentration described in Part A, section 5.2.1.

3.3.4 *Tests for the presence of living extraneous micro-organisms in the final bulk prepared in the skin of living animals*

The final bulk shall pass the following tests for the presence of living extraneous micro-organisms, unless these tests have already been passed by each of the single harvests represented in the final bulk.

3.3.4.1 *Tests for total bacterial content*

Suitable dilutions of the final bulk shall be made in a suitable diluent not deleterious to living bacteria. At least three 1-ml samples of each dilution shall be cultured on nutrient-broth-agar plates. The plates shall be incubated for 72 hours between 15°C and 22°C and for a further period of 48 hours between 35°C and 37°C. From the number of colonies appearing on the plates the number of living bacteria in 1 ml of the final bulk shall be calculated. If this number exceeds 500, the final bulk shall be subjected to further treatment or be discarded.

Suitable control plates containing higher dilutions of the final bulk shall be included in this test in order to make sure that the number of colonies appearing on the test plates has not been influenced by the inhibitory action of any preservative present in the final bulk.

3.3.4.2 *Test for the presence of* Escherichia coli

At least three 1-ml samples of a 1 : 100 dilution of the final bulk shall be cultured on plates of a medium suitable for differentiating *E. coli* from other bacteria. The plates shall be incubated for 48 hours at 35°C to 37°C. If *E. coli* is detected, the final bulk shall be subjected to further treatment or be discarded.

The presence of *E. coli* in this test might indicate a heavy faecal contamination.

3.3.4.3 *Test for the presence of haemolytic streptococci, coagulase-positive staphylococci, or any other pathogenic micro-organisms which are known to be harmful if introduced into the human body by the process of vaccination*

At least three 1-ml samples of a 1 : 100 dilution of the final bulk shall be cultured on plates of blood agar. The plates shall be incubated for 48 hours at 35°C to 37°C and the colonies appearing shall be examined.

If any of the organisms mentioned are detected, the final bulk shall be subjected to further treatment or be discarded.

In some countries culture of the final bulk in salt meat broth is made for the purpose of detecting staphylococci.

3.3.4.4 *Test for the presence of* Bacillus anthracis

Any colony seen on any of the plates used in the tests described in Part A, sections 3.3.4.1, 3.3.4.2 and 3.3.4.3 which morphologically resembles *B. anthracis* shall be examined. If the organisms contained in the colony are non-motile, further tests for the cultural character of *B. anthracis* shall be made, including pathogenicity tests in suitable animals. If *B. anthracis* is found to be present, the final bulk shall be discarded.

In countries where anthrax presents a serious risk, this test should be based on a larger number of colonies.

3.3.4.5 *Test for the presence of* Clostridium tetani *and other pathogenic spore-forming anaerobes*

A total volume of not less than 1 ml of the final bulk, taken preferably from the depth of the bulk and not from the upper surface, shall be distributed in equal amounts into ten tubes, each containing not less than 10 ml of a medium suitable for the growth of anaerobic micro-organisms. The tubes shall be held at 65°C for one hour in order to reduce the content of non-spore-forming organisms, after which they shall be incubated for at least one week between 35°C and 37°C.

From every tube showing growth, subcultures shall be made on to plates of a suitable medium which shall be incubated anaerobically at the same temperature. All anaerobic colonies shall be examined and identified and if *Cl. tetani* or other pathogenic spore-forming anaerobes are present the final bulk shall be discarded.

Organisms resembling pathogenic *Clostridia* found in the tube culture from which the subculture was made may be tested for pathogenicity by inoculation into animals as follows: Groups of not less than two guinea-pigs and five mice are used for each tube culture to be tested. 0.5 ml of the cultures is mixed with 0.1 ml of a freshly prepared 4% solution of calcium chloride and injected intramuscularly into each of the guinea-pigs; 0.2 ml of the cultures mixed with 0.1 ml of this calcium chloride solution are injected intramuscularly into each of the mice. The animals are observed for one week. If any animal develops symptoms of tetanus, or if any animal dies as a result of infection with spore-forming anaerobes, the final bulk should be discarded.

If other methods are used for this test, they should have been demonstrated, to the satisfaction of the national control authorities, to be at least equally effective for detecting the presence of *Cl. tetani* and other pathogenic spore-forming anaerobes.

3.3.5 *Test for bacteriological sterility of the final bulk prepared in chick embryos, or in tissue cultures*

Each final bulk shall be tested for bacterial sterility according to the requirements given in Part A, section 5 of Requirements for Biological Substances No. 6 (General Requirements for the Sterility of Biological Substances).[1]

If growth appears in any of the cultures the final bulk shall be discarded or the test repeated. The final bulk shall be discarded if the same type of organism appears in more than one test, but no final bulk shall be passed unless the final test shows no growth throughout.

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1960, **200**, 13.

4. Filling and containers

The requirements concerning filling and containers given in Part A, section 4 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply with the addition of the following:

All containers of the final vaccine shall be shown to be sterile before filling and shall be made of a material demonstrated, to the satisfaction of the national control authority, to have no deleterious effect on the vaccine.

In some countries, containers of liquid vaccine may not be hermetically closed; if this is permitted the form of closure shall be submitted to the national control authority for approval.

Containers of dried vaccine shall be hermetically sealed under vacuum or after filling with pure, dry, oxygen-free nitrogen or any other gas not deleterious to the vaccine.

All hermetically sealed containers shall be tested for leaks after sealing. All defective containers shall be discarded.

Single- and multiple-dose containers may be used. Each container of dry vaccine should be issued together with an ampoule of sterile reconstituting fluid. This fluid may contain glycerol and/or some suitable antiseptic substance. The containers should be issued in a form that renders the process of reconstitution as simple as possible.

5. Control tests on final product

5.1 *Identity test*

An identity test shall be performed on at least one labelled container from each filling lot by appropriate methods.

The test for virus concentration as described in Part A, section 5.2.1 of these requirements may serve as an identity test.

A test may also be made in the scarified skin of rabbits. Suitable dilutions of vaccine are applied on scarified areas of skin. After four to seven days the vaccine should produce lesions characteristic of vaccinia.

5.2 *Tests for virus concentration on vaccine in final containers*

A test for virus concentration shall be made on each filling lot in accordance with the requirements described in Part A, section 5.2.1 of these requirements. Dried vaccine shall be reconstituted to the form in which it is to be used for human inoculation before the test is made.

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[1] See Annex 1 of this report.

Tests should be done in parallel with a reference vaccine which has been calibrated against the international reference preparation of smallpox vaccine.[1]

5.2.1 *Test for virus concentration in membranes of chick embryos*

At least ten chick embryos, each of about 12 days' incubation, shall be divided into two equal groups. To the chorio-allantoic membrane of each embryo of the first group, 0.1 ml or 0.2 ml of a suitable dilution of the vaccine shall be applied. To the membrane of each of the second group of embryos 0.1 ml or 0.2 ml of another suitable dilution of the vaccine shall be applied. After the optimal time of incubation the total number of discrete specific lesions shall be counted on the membrane of each embryo. The dilutions shall be so chosen that the membranes of at least one of the groups yield countable numbers of lesions exceeding ten per membrane. From the number of lesions counted in this group and from the dilution and volumes used, the number of pock-forming units in one ml of the undiluted vaccine shall be calculated. This number shall exceed 1×10^8.

It has been shown that the severity of systemic reactions after successful vaccinations in young adults is related to the virus strain rather than to the number of pock-forming units present in the dose of vaccine applied.

5.2.2 *Other tests*

Tests for virus concentration in the scarified skin of rabbits may also be used provided it has been shown that the results correlate with those obtained using the membranes of chick embryos.

In some countries tests are made in tissue culture either by counting the number of plaque-forming units per ml of vaccine or by determining the dilution of vaccine which will produce cytopathic effects in 50% of tissue cultures. More experience should be gained in the use of these tests and in the relationship of the results to those obtained in the test performed in chick embryos before these tests alone can be used for determining virus concentration.

5.3 *Tests for the presence of extraneous living micro-organisms in the vaccine in final containers*

Not less than four final containers (or not less than 10 if single-dose containers) giving a total pooled volume of not less than 0.5 ml shall be taken at random from each filling lot in such a manner that all stages of the filling from the bulk container shall be represented. Dried vaccine shall be

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[1] The purpose of using a reference vaccine is to ensure that the system used has adequate sensitivity. The International Reference Preparation of Smallpox Vaccine when reconstituted with 1 ml of fluid per ampoule has been shown in a number of different laboratories to have approximately 1×10^8 pock-forming units per ml. — ED.

reconstituted to the form in which it is to be used in human inoculation. The vaccine thus collected shall pass the test described in Part A, section 3.3.4.1 or 3.3.5 of these requirements, whichever is applicable.

5.4 *Innocuity test*

Each filling lot shall be tested for abnormal toxicity by appropriate tests involving injection into rabbits. The tests shall be approved by the national control authority.

Mice and guinea-pigs may also be used for this test.

5.5 *Heat-resistance test on dried vaccine*

At least one container of dried vaccine from each filling lot shall be incubated at a temperature of not less than 37°C for not less than 4 weeks and tested for virus concentration. The vaccine passes the test if the requirements described in Part A, section 5.2.1 are fulfilled and at least one tenth of the virus concentration is retained.

In some countries a more rapid stability test is made by heating the vaccine to 100°C for 1 hour. The vaccine passes the test if at least one tenth of the virus concentration is retained.

5.6 *Preservatives and other substances added*

No antibiotics shall be added to smallpox vaccine.

If the liquid vaccine or reconstituted dried vaccine contains preservatives or other added substances such substances shall have been shown, to the satisfaction of the national control authority, to have no deleterious effect on the product in the amounts present and to cause no untoward reactions in vaccinated subjects. If phenol is present, its concentration shall not exceed 0.5%. Further, the substance used shall fulfil the requirements of the International Pharmacopoeia or a pharmacopoeia approved by the national control authority.

6. Records

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

7. Samples

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

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[1] See Annex 1 of this report.

8. Labelling

The Requirements given in Part A, section 8 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply with the addition of the following:

The leaflet accompanying the package shall include the following information:

the tissue or animal in which the vaccine was prepared;

any antibiotics used in the preparation of the vaccine (except such antibiotics as may have been applied to the skin of inoculated animals and removed before harvesting);

if the vaccine is in the dried form, a statement that, after rehydration of the dried vaccine, the vaccine shall be used within 24 hours or within 7 days if it can be stored under conditions in which potency and sterility can be maintained.

Instructions for the use of dried vaccine when issued in a container hermetically sealed under vacuum should specify the precautions to be taken when opening a container in order to avoid dispersion of the vaccine into the surroundings.

9. Distribution and shipping

The requirements given in Part A, section 9 of the revised Requirements for Biological Substances No. 1 (General Requirements for Manufacturing Establishments and Control Laboratories) [1] shall apply.

10. Storage and expiry date

The statements concerning storage temperatures and expiry dates appearing on the label and the leaflet, as required in Part A, section 8 of these requirements, shall be based on experimental evidence and shall be submitted for approval to the national control authority.

10.1 *Storage conditions*

Before being distributed by the manufacturing establishment, or before being issued from a depot for the maintenance of reserves of vaccine, all liquid vaccines in their final containers shall be kept constantly at a temperature below −10°C, and all dried vaccines in their final containers at a temperature below +10°C.

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[1] See Annex 1 of this report.

10.2 *Expiry date*

The date after which liquid vaccine may not be used shall be not more than 12 months after passing the last test for virus concentration. The date after which dried vaccine may not be used shall be not more than 36 months after passing the last test for virus concentration. The expiry date shall not, however, be more than three months for liquid vaccine or more than twelve months for dried vaccine from the date on which the vaccine was issued by the manufacturer or from a depot.

Part B. National Control Requirements

1. General

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

2. Release and certification

A vaccine lot shall be released only if it fulfils Part A of the present requirements.

A statement signed by the appropriate official of the national control laboratory shall be provided at the request of the manufacturing establishment and shall certify whether the lot of vaccine in question meets all national requirements as well as Part A of the present requirements. The certificate shall also state the date of the last satisfactory potency test, the lot number, the number under which the lot was released, and the number appearing on the labels of the containers. In addition, a copy of the official national release document shall be attached.

The purpose of the certificate is to facilitate the exchange of biological substances between countries.

3. Efficacy and safety of the vaccine in the field

The appropriate health authorities should satisfy themselves, on the basis of vaccination results, that the vaccine lots released give close to 100% " takes " in susceptible children and do not give rise to complications.

It is also important that similar studies should be made periodically to determine the success rate in revaccination.

[1] See Annex 1 of this report.

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