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REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

5. Requirements for Smallpox Vaccine

Report of a Study Group

WORLD HEALTH ORGANIZATION

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GENEVA

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STUDY GROUP ON REQUIREMENTS FOR SMALLPOX VACCINE

Geneva, 3-8 November 1958

Members :

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, Director, Institut d'Hygiène et d'Epidémiologie, Brussels, Belgium

Dr R. Muckenfuss, Technical Director, Naval Medical Research Institute, Bethesda, Md., USA

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

Dr G. Renoux, Director, Institut Pasteur, Tunis, Tunisia

Dr R. Sanjiva Rao, Assistant Director, Virus Research Centre, Poona, India (*Vice-Chairman*)

Secretariat :

Dr B. K. Bhattacharya, Medical Officer, Biological Standardization, WHO

Dr N. K. Jerne, Chief Medical Officer, Biological Standardization, WHO (*Secretary*)

Dr M. Kaplan, Chief, Veterinary Public Health, WHO [also representing the Food and Agriculture Organization]

Dr D. McClean, The Lister Institute of Preventive Medicine, Elstree, Herts., England (*Consultant*)

Dr S. S. Marennikova, The Metchnikov Institute, Moscow, USSR (*Consultant*)

Dr A. M.-M. Payne, Chief Medical Officer, Endemo-Epidemic Diseases, WHO

Dr A. C. Saenz, Medical Officer, Endemo-Epidemic Diseases, WHO

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REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

5. Requirements for Smallpox Vaccine

Report of a Study Group

The Study Group on Requirements for Smallpox Vaccine met in Geneva from 3-8 November 1958.

Dr M. G. Candau, Director-General of the World Health Organization opened the meeting and welcomed the members of the Group.

The Director-General outlined the task of the Study Group, which was to draw up an international recommendation on the requirements which should be fulfilled by a preparation of smallpox vaccine in order to ensure that the product is a safe, reliable and potent prophylactic agent. An international recommendation on requirements would tend to facilitate the exchange of vaccine between different countries, and would provide guidance to those concerned with the preparation of smallpox vaccine who may have difficulties in deciding upon appropriate methods of assay and control.

The Director-General reviewed the newer techniques that are being adopted as a result of modern developments in biology. Methods of producing smallpox vaccine in chick embryos and in tissue cultures, and the provision of vaccines in the dried form are of outstanding practical importance. These newer methods, as well as those which have been used for many generations, would demand the attention of the Study Group.

1. GENERAL CONSIDERATIONS

The Study Group noted the report of the Study Group on General Requirements for Manufacturing Establishments and Control Laboratories and Requirements for Poliomyelitis Vaccine [1] and agreed that the requirements for smallpox vaccine could be fitted into the framework adopted in that report.

In its discussions of requirements that could be internationally recommended, the Study Group considered the preliminary draft of requirements for smallpox vaccine [2] prepared by the Secretariat, and those sections in

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

[2] WHO Secretariat, unpublished working document WHO/BS/IR/65

the tenth,[1] eleventh [2] and twelfth [3] reports of the Expert Committees on Biological Standardization pertaining to the standardization of smallpox vaccine. The Study Group surveyed the regulations and requirements for the manufacture and control of smallpox vaccine that had been adopted in some countries [4] and it considered several working documents as well as unpublished data submitted by members of the Group.[5]

After a general discussion of smallpox vaccination and of the methods for the production and control of smallpox vaccine currently in use in various countries, the Study Group expressed the opinion that vaccines prepared according to the traditional methods, viz., in the skin of living animals, had proved of great value in eliminating smallpox from large areas of the world and should, therefore, not be replaced by newer types of vaccines without a satisfactory demonstration that the newer methods of production yield vaccines of at least equal prophylactic value and safety.

On the basis of the above-mentioned documents and with the above considerations in mind, the Study Group proceeded to prepare the draft of the Requirements for Smallpox Vaccine which is given in the annex to this report.

2. PROBLEMS NEEDING FURTHER INVESTIGATION

2.1 International and national reference preparations of smallpox vaccine

The Study Group noted that the Expert Committee on Biological Standardization has arranged an international collaborative study of different preparations of dried smallpox vaccine with the object of establishing an international reference preparation.[1, 2, 3] The Group was of the

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1957, **127**, 7

[2] *Wld Hlth Org. techn. Rep. Ser.*, 1958, **147**, 13

[3] *Wld Hlth Org. techn. Rep. Ser.*, 1959, **172**, 12

[4] Desbordes, J., unpublished working document WHO/BS/IR/61 (*Survey of requirements now in practice in 19 countries*); England and Wales, Therapeutic Substances Regulations, 1952, Third Schedule, Part IV, and Therapeutic Substances Amendment Regulations, 1957, Regulation 8; Gispen, R., unpublished working document WHO/BS/IR/64 (Netherlands requirements); Japan, Ministry of Health and Welfare (1957) *Minimum requirements of biologic products, smallpox vaccine*, p. 177; Union of Soviet Socialist Republics, unpublished working document WHO/BS/IR/60; United States, Department of Health, Education and Welfare (1951) *Minimum requirements : smallpox vaccine*, third revision (also additional proposals, unpublished working document WHO/BS/IR/69)

[5] Fisek, N. H., unpublished working documents WHO/BS/381 and WHO/BS/IR/67; Krag Andersen, E., unpublished working document WHO/BS/IR/66; Lafontaine, A., unpublished working document WHO/BS/IR/19; Marennikova, S. S., Ponomaryova, N. A., Ogorodnikova, Z. I. & Durasova, M. N., unpublished working document WHO/BS/IR/63 Rev. 1; Soloviev, M. D. & Mastyukov, U. N., unpublished working document WHO/BS/IR/68; Wang, S. P. & Grayston, J. T., unpublished working document WHO/BS/IR/62; WHO Secretariat, unpublished working document WHO/BS/IR/27

opinion that the international reference preparation finally to be adopted should be one which has been proven to protect man against smallpox without giving rise to vaccination complications. Samples of this preparation cannot be provided in sufficient quantities to serve as national reference preparations in the control of individual vaccine lots. The national control authorities should proceed with the preparation of their own national reference vaccines for use in comparative tests in the control of vaccine production. Such national reference vaccines could be made by using the international reference preparation as seed virus. If national reference preparations are made from other strains of virus they should be of proven value in man. All national reference preparations should be calibrated and compared in assays against the international reference preparation.

2.2 International standard for anti-vaccinia gamma globulin

The Study Group was of the opinion that there is an urgent need for the provision of an international standard preparation of anti-vaccinia gamma globulin and it considered that it would be of great advantage if the World Health Organization would arrange the investigations that must precede the establishment of such a standard.

These investigations should include field trials of anti-vaccinia gamma globulin (prepared from the serum of man or animals) in the prophylaxis and treatment of smallpox and of post-vaccination complications. The former trials should be conducted in areas where smallpox is still endemic. The investigations should also include collaborative laboratory studies of the validity and reproducibility of different methods of assaying antibodies against vaccinia virus and smallpox virus. Such laboratory studies are essential for the correct interpretation of the results of the field trials.

2.3 Virus strains

The Study Group noted that many different strains of vaccinia virus are at present being used by different laboratories in the preparation of smallpox vaccine and that very little information is available on the relative value of these strains.

In formulating the requirements that should be fulfilled by the seed virus to be used for the preparation of vaccine, the Study Group was unable to recommend the use of particular strains or to give an adequate description of the criteria for such strains. The Group was of the opinion, therefore, that a study should be made of the properties of different strains of vaccinia virus with the object of selecting satisfactory strains. In order to obtain the most valuable information in a study of limited scope, only a few strains

should be selected for comparison but these should show clearly different stable characteristics. The strains should be investigated for their protective properties in man as well as in laboratory animals by different routes of administration with the object of obtaining a correlation between the results of a laboratory test and protection in the field. The strains should also be investigated for their virulence and properties of producing vaccination reactions by studies in laboratory animals in comparison with field observations. The laboratory investigations should include studies of the relative immunogenicity of different strains of vaccinia virus in active and passive protection tests by various methods, including intracerebral challenge of immunized rabbits or mice with neuro-vaccinia or variola virus.

These investigations might also include a study of the antigenic pattern of various pock viruses.

2.4 Tissues suitable for preparing smallpox vaccine

The Study Group was of the opinion that although further data from long-term studies must be obtained on the efficacy of smallpox vaccine produced in chick embryos and in *in vitro* cultures of tissues, the requirements should not exclude the use of such vaccines. It recommended that field trials be undertaken in areas where smallpox is endemic in order to obtain further data on the protective value of vaccines prepared by these methods.

The Study Group also recommended investigations concerning the relative merits and dangers of using tissues of ectodermal and non-ectodermal origin, in primary cultures or in cultures propagated in series.

2.5 Potency tests

The Study Group was of the opinion that the only tests the results of which had so far been sufficiently compared with success rates obtained in the vaccination of humans were the rabbit scarification test and the potency tests in chick embryos. It therefore decided not to include other methods in the requirements at this stage. The Group recognized that the intracutaneous technique in the rabbit skin had provided excellent results in several laboratories, but noted that there was doubt regarding the validity of this method for certain strains of vaccinia virus used elsewhere. The Group therefore recommended that this method be used as an optional method, in addition to one of the methods required, so that further information on its general validity would become available.

Further studies are also needed on the use of tissue cultures in the determination of the potency of smallpox vaccine.

2.6 Heat deterioration tests on smallpox vaccine

The Study Group noted the extensive studies already undertaken by WHO of the stability of dried and fluid smallpox vaccine preparations.[1] It also considered other existing data on the stability of fluid vaccines. The Group emphasized, however, the importance of collecting further data, and recommended that systematic laboratory studies should be made of the rates of deterioration of liquid and dried vaccine when kept at various temperatures. The results of such studies would make it possible to formulate a better heat stability test than that now recommended by the Study Group. The data would also provide a more realistic basis for advice on storage and transport conditions, and requirements concerning the expiry date.

2.7 Post-vaccination complications

The Study Group recommended that all information on post-vaccination complications occurring in different countries be collected by a system of international notification in order to provide more basic data for further experimental and epidemiological studies directed towards the prevention of such complications.

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[1] *Bull. Wld Hlth Org.*, 1957, **16**, 63 & 1958, **19**, 123 ; WHO Secretariat, unpublished working documents WHO/Smallpox/3, WHO/Smallpox/4, WHO/Smallpox/6 & WHO/Smallpox/7

Annex

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

General considerations

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 use of different vaccinia virus strains, the use of preservatives, the issue of the vaccine in liquid or in dried form, the methods for testing the potency, and the use of different tissues for growing the virus.

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. 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 obvious 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 in the

preparation of smallpox 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.

In spite of these considerations, it is felt that certain essential requirements concerning manufacture and control can be formulated. The present recommendations are based on methods currently in use, and future revisions will be necessary.

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

Should individual countries wish to adopt these requirements as the basis of their national regulations concerning smallpox vaccine, it is recommended that a clause be included which would permit modifications of manufacturing requirements on the condition that such modifications had been demonstrated, to the satisfaction of the national control authority, to ensure that the degree of safety and the potency of the vaccine are at least equal to those provided by the requirements formulated below. 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. Definition

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 standards or reference preparations and international units*

Since no international standards or reference preparations of vaccinia virus have yet been established, no requirements of potency based on such standards can be formulated.

The provision of an international reference preparation of dried vaccinia virus is at present the subject of an international collaborative study arranged by the Expert Committee on Biological Standardization. In this study, different freeze-dried smallpox vaccines, including the proposed international reference preparation, are being compared by various assay methods.[1]

When an international reference preparation of smallpox vaccine has become established, the preparation will be in the custody of the International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, Denmark, and samples will be distributed free of charge on request from national laboratories for biological standards in all countries. The international reference preparation will be intended for the calibration of national reference preparations which could be made by using the international reference preparation as seed virus.

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 lots are one passage removed from a seed lot.

Bulk stage : Any stage of the product after harvesting and before filling into final containers.

Vaccine lot : All finished vaccine in final containers which has at some stage been processed together and which has, therefore, a uniform composition.

Final lot : A vaccine lot, or part thereof, which is homogeneous with respect to the risk of contamination during filling or drying. A final lot must, therefore, have been filled in one working session, and (if applicable) have been dried together.

LD_{50} : One LD_{50} is the quantity of vaccine estimated to kill 50% of developing chick embryos by the specific action of vaccinia virus.

2. General manufacturing requirements

The general requirements for manufacturing establishments contained in Requirements for Biological Substances No. 1 [2] shall apply to establishments manufacturing smallpox vaccine.

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[1] *Wld Hlth Org. techn. Rep. Ser.*, 1958, **147**, 13 ; 1959, **172**, 12

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

Manufacture of smallpox vaccine shall take place in a completely separate area, using separate equipment. Only strains of vaccinia virus used for the production of smallpox vaccines shall be permitted in the manufacturing area.

Written procedures for the preparation of smallpox vaccine shall be submitted for approval to the national control authorities. Proposals for modifications shall be submitted for approval to the national control authorities before their implementation.

3. Production control

3.1 *Control of source materials*

3.1.1 *Virus strains*

The strains and sub-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 authorities to yield immunogenic vaccines which produce typical vaccinal lesions in the skin of man followed by insusceptibility to subsequent vaccination with a strain of virus known to protect man against variola. The strain shall produce a characteristic vesicular eruption in the skin of rabbits, or characteristic pock lesions in the membranes of chick embryos.

> The strain used for vaccine production should be one that has never shown a greater tendency to produce generalized 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. Strains of so-called " neurovaccine " should be excluded.
>
> Samples of duly tested seed virus may be obtained for the purpose of preparing a primary seed lot to be used in vaccine production by application directly, or through the World Health Organization, to specialized laboratories.[1]

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

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[1] The following laboratories have expressed their willingness to supply samples of strains for this purpose: Institut d'Hygiène et d'Epidémiologie, Brussels, Belgium; Statens Seruminstitut, Copenhagen, Denmark; Rijks Instituut voor de Volksgezondheid, Utrecht, Netherlands; Institut Pasteur, Tunis, Tunisia; The Lister Institute of Preventive Medicine, Elstree, Herts., England; Institute for Control of Sera, Vaccines and Antibiotics (L. A. Tarasevich), Moscow, URSS; Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA

from healthy birds, shall be used for vaccine production. They shall conform to all the requirements given in section 3.2 below.

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 prepared by growing vaccinal virus in the skin of a living animal. If chick embryos or tissue cultures are used for the preparation of *further seed lots*, these seed lots shall be as few passages as possible, and not more than ten passages, removed from a primary seed lot. All vaccine lots shall be prepared from a seed lot without intervening passage.

Until further knowledge is available it would seem desirable to use only seed lots prepared in the skin of living animals in order to avoid passages in other tissues which may cause a change in the immunogenic properties of the virus.

All 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 all subsections of section 3.3.4 (p. 16).

The seed lot used for the production of vaccine in the membranes of chick embryos or in tissue cultures shall, after rehydration if applicable, satisfy the requirements of section 3.3.5 (p. 18).

3.2 *Production precautions*

The general precautions as formulated in the requirements of Part A, section 3 of 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

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

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 authorities.

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 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, and also during the process of harvesting if the animal is kept alive.

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

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

A post-mortem examination of all the animals killed in the production of vaccine shall be made. 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 highly communicable one, the harvest from the entire group of animals exposed shall be discarded.

Before harvesting, the animal should be killed. This should be done by a painless device and should be immediately followed by exsanguination, in order to avoid heavy admixture of the vaccinal material with blood.

If the animals are not killed at harvesting, they should be kept under veterinary supervision for at least two weeks and the measures to be taken in the event of disease or death should be determined by the national control authorities.

3.2.2 *Vaccines produced in the chick embryo*

Only eggs from flocks known to be free from disease shall be used.

Special attention should always be given to salmonellosis, tuberculosis, Newcastle disease, fowl pest, chronic respiratory disease, and the avian lymphomatosis complex, but in some areas it may be necessary to consider other diseases.

Living embryos after incubation for a suitable period shall be inoculated with seed virus which satisfies the requirements of sections 3.1.3 and 3.1.4 (p. 13). 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. Tissues originating from man shall not be used. The virus shall be grown and harvested with aseptic precautions.

Suitable antibiotics in minimum concentrations required for sterility may be used.

3.3 *Control at the bulk stage of the product*

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 section 3.3.4.

If the vaccine is intended for issue in the liquid form, this treatment may consist of the addition of glycerol 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.

The treated material should be stored at a temperature below 0°C if containing glycerol, and below 5°C if not containing glycerol. It may be stored in concentrated form.

3.3.2 *Final bulk*

After the initial treatment, a quantity of vaccinal material, which may be made up of a number of single harvests, may be diluted by the addition of glycerol and/or another suitable diluent. In the concentration ready for filling into the final containers, this material will be referred to as the *final bulk* in the subsequent sections.

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 *Potency tests on the final bulk*

The final bulk shall pass one of the potency tests described in the sub-sections of section 5.2 (p. 19).

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 tests for the presence of living extraneous micro-organisms, described in the following sub-sections, 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. One ml of each dilution shall be cultured in at least three nutrient-broth agar plates. The plates shall be incubated

for 72 hours between 18°C and 22°C and for a further period of 48 hours between 32°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 computed. If this number exceeds 1000, 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 preservatives present in the final bulk.

3.3.4.2 *Test for the presence of* Escherichia coli

One ml samples of a 1 : 100 dilution of the final bulk shall be cultured in at least three plates of a medium suitable for differentiating *E. coli* from other bacteria. The plates shall be incubated for 48 hours at 32°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 may prove harmful if introduced into the human body by the process of vaccination*

One ml samples of a 1 : 100 dilution of the final bulk shall be cultured in at least three plates of blood agar. The plates shall be incubated for 48 hours at 32°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.

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 subsections 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 forthwith.

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 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 32°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 appearing shall be examined morphologically and if any organisms resembling pathogenic *Clostridia* are found the tube culture from which the subculture was made shall be tested for pathogenicity by inoculation into animals as follows: Groups of not less than two guinea-pigs and five mice shall be used for each tube culture to be tested. 0.5 ml of the cultures shall be 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 shall be injected intramuscularly into each of the mice. The animals shall be 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 shall be discarded.

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

Any pathogenic *Clostridium* that may be found should be identified. This may be facilitated by the inoculation of control groups of animals protected by specific antitoxins.

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

The final bulk shall pass the following sterility test:

Not less than two samples of 0.1 ml of the final bulk shall be inoculated into fluid thioglycollate medium or any other medium equally effective for the growth of aerobes and anaerobes and into Sabouraud medium or any other medium equally effective for the growth of yeasts and fungi. The final dilution of the vaccine in the sterility media shall be such that the preservative no longer exerts bacteriostatic activity. The media inoculated for the detection of bacteria shall be incubated at 30°-32°C for not less than one week or they shall be divided into two portions, one being incubated at 35°-37°C and the other at 15°-22°C for not less than one week. The media inoculated for the detection of yeasts and fungi shall be incubated at 20°-25°C for not less than two weeks. If growth appears in any of the cultures, the test may be 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.

If other methods are used for this test they shall have been demonstrated, to the satisfaction of the national control authorities, to be at least equally effective for demonstrating sterility.

General requirements for sterility of biological preparations will be formulated as Requirements for Biological Substances No. 6.

4. Filling and containers

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

All containers of the final vaccine shall be sterilized before use. If glass containers are used, the glass shall be neutral and of high quality, especially with regard to resistance against temperature fluctuation and breakage. If containers of metal or other materials are used it shall have been demonstrated, to the satisfaction of the national control authorities, that these materials have no deleterious effect on the vaccine.

If containers of liquid vaccine are not hermetically closed, the form of closure shall be submitted to the national control authorities 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*

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

A potency test as described in section 5.2 may serve as identity test.

5.2 *Potency tests on vaccine in final containers*

Liquid vaccine from its final containers shall pass at least one of the potency tests described in the following sub-sections.

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

Dried vaccine from its final containers, after reconstitution to the form in which it is to be used for human inoculation, shall pass one of the potency tests described in the following sub-sections.

5.2.1 *Potency test in the scarified skin of rabbits*

Serial tenfold dilutions shall be made of the vaccine and of a reference vaccine approved for this purpose by the national control authorities. The 1/1000 and 1/10 000 dilutions of both the vaccine under test and the reference vaccine shall be applied to scarified areas of the skin on the back or flank of at least two rabbits. The volume of vaccine used may be either 0.1 ml applied to an area of approximately 5 cm^2 or 0.2 ml applied to an area of approximately 10 cm^2. After four to seven days, both the vaccine under test and the reference vaccine shall have produced confluent lesions characteristic of vaccinia in the areas to which the dilutions of 1/1000 were applied and more than one characteristic discrete vesicle in the areas to which the dilutions of 1/10 000 were applied.

In order to evaluate the potency of the vaccine more accurately, many laboratories also apply other dilutions to additional scarified areas.

It is advisable to use rabbits with white skin and to remove the fur by clipping. If the rabbits are shaved or depilated, this should be done at least a day before the test.

5.2.2 *Potency test by enumeration of pock-forming units after application of vaccine to the chorio-allantoic 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 computed. This number shall exceed 5×10^7.

5.2.3 *Potency test by determination of LD_{50} after application of vaccine to the chorio-allantoic membranes of chick embryos*

Chick embryos of about 12 days incubation shall be divided into at least four groups of at least six each. Suitable progressive dilutions of the vaccine shall be made. 0.2 ml or 0.3 ml of each dilution shall be applied

to the chorio-allantoic membrane of each embryo of a group. After inoculation the eggs shall be incubated for a suitable period. All embryos that die within 24 hours shall be excluded from the test. From the number of embryos that die after 24 hours and that show the presence of specific lesions, and from the dilutions and volumes used, the LD_{50} shall be computed. One ml of the undiluted vaccine shall contain at least 7×10^7 LD_{50}.

The dilutions chosen should cover the range from 0%—100% mortality

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

Not less than four final containers from each final lot shall be selected at random in such a manner that all stages of the filling from the bulk container will be represented. Dried vaccine shall be reconstituted to the form in which it is to be used for human inoculation. The contents of the containers selected shall be pooled together and the total volume shall be not less than 0.25 ml.

A 0.1 ml volume of the vaccine thus collected shall pass the test described in sub-section 3.3.4.1 (p. 16) or in section 3.3.5 (p. 18), whichever is applicable.

5.4 *Innocuity tests*

It may be desirable to test for the absence of toxicity of the vaccine in the final containers. This may be done by tests in guinea-pigs and mice.

5.5 *Heat-resistance test on dried vaccine*

At least one container of dried vaccine from each final lot shall be incubated at a temperature of not less than 37°C for not less than 4 weeks, after which the vaccine shall pass one of the potency tests described in one of the sub-sections of section 5.2 (p. 19).

5.6 *Preservatives and other substances added*

The liquid vaccine in its final containers and the reconstituting fluid for the dried vaccine shall not contain preservatives or other added substances in concentrations that exceed the limits prescribed by the national control authorities. If phenol is present its concentration shall not exceed 0.5%.

The use of antibiotics as additives to smallpox vaccine should be discouraged.

5.7 *Protein content*

The vaccine in its final containers shall contain no human protein.

6. Records

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

Written records shall be kept of all seed lots, and of all vaccine lots produced by the manufacturing establishment, irrespective of the results of safety and potency tests.

The records shall be of a type approved by the national control authorities.

> Records should be kept in three groups: (*a*) seed lots; (*b*) vaccinal material collected from each animal, if applicable; (*c*) vaccine lots. In each group the lots should be numbered serially. The use of serial numbers is recommended because it will facilitate checking the consistency of all lots processed.

7. Samples

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

8. Labelling

The label printed on or affixed to each container, or affixed to the wrapper of the package in which the container of the vaccine is distributed, shall show at least:

the name and address of the manufacturer;
the words "Vaccinum variolae" and/or the proper name of the product;
the vaccine lot number;
the temperature of storage and the expiry date if kept below that temperature.

Moreover, this label or the label of the carton enclosing several final containers, or the leaflet accompanying the containers, shall contain the following additional information:

the fact that the vaccine fulfils the requirements of this document;
the tissue or animal in which the vaccine was prepared;
the preservatives present and their quantity;
the antibiotics used in the preparation of the vaccine;
the instructions for the use of the vaccine;

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

the conditions recommended during storage and shipping, with information on the reduced stability of the vaccine if exposed to higher temperatures than that stated on the label;
if the vaccine is in the dried form, an additional statement that, after rehydration of the dried vaccine, the potency cannot be assured for more than 24 hours.

Instructions for the use of dried vaccine when issued in a container 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.

The leaflet should also contain information concerning the contra-indications and the reactions which may follow vaccination.

The above requirements for labelling have been drafted pending the formulation of general requirements on labelling applicable to all biological products.

9. Distribution and shipping

The requirements given in Part A, section 9 of the 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 section 8 (p. 22) shall be based on experimental evidence and shall be submitted for approval to the national control authorities.

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.

Precautions shall be taken to maintain the vaccine below +10°C during transport.

10.2 *Expiry date*

The date after which the vaccine may not be used shall be not more than 12 months after passing the last potency test. After issue by the manu-

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

facturer or from a depot, the expiry date shall be not more than three months from the date of issue for liquid vaccine and not more than six months from the date of issue for dried vaccine.

Part B. National Control Requirements

1. General

The general requirements for control laboratories given in Part B of 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.

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