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**REQUIREMENTS FOR  
BIOLOGICAL SUBSTANCES**

**3. Requirements for Yellow Fever Vaccine**

**4. Requirements for Cholera Vaccine**

**Report of a Study Group**

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**STUDY GROUP ON  
REQUIREMENTS FOR YELLOW FEVER VACCINE AND  
REQUIREMENTS FOR CHOLERA VACCINE**

*Geneva, 1-6 September 1958*

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## REQUIREMENTS FOR BIOLOGICAL SUBSTANCES

### 3. Requirements for Yellow Fever Vaccine

### 4. Requirements for Cholera Vaccine

#### Report of a Study Group

The Study Group on Requirements for Yellow Fever Vaccine and Requirements for Cholera Vaccine met in Geneva from 1-6 September 1958.

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

The Deputy Director-General outlined the task of the Study Group which was to draw up requirements which should be fulfilled by preparations of yellow fever vaccine and cholera vaccine in order to ensure that these products are safe, reliable and potent prophylactic agents. The successful accomplishment of this task would be an essential part of the WHO project of issuing requirements that can be internationally recommended and would promote uniformity in the field of production, assay methods and requirements for important biological substances.

#### 1. GENERAL CONSIDERATIONS

The Study Group noted the report of the Study Group on Recommended Requirements for Biological Substances (1957)<sup>1</sup> and agreed that requirements for yellow fever vaccine and for cholera vaccine could be fitted into the framework suggested in that report.

In its discussion of requirements that could be internationally recommended, the Study Group considered the Standards for the Manufacture and Control of Yellow Fever Vaccine adopted by the Standing Committee on Health of UNRRA in 1945<sup>2</sup>—these contain the requirements for yellow fever vaccine that have up to the present formed the basis for WHO approval of laboratories producing yellow fever vaccine—and the first report of the

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<sup>1</sup> WHO Secretariat, unpublished working document WHO/BS/IR/27

<sup>2</sup> *Epidem. Inform. Bull.*, 1945, 1, 365

Expert Committee on Yellow Fever Vaccine,<sup>1</sup> which contains proposed revisions of the UNRRA Standards. The Study Group also considered the General Requirements for Manufacturing Establishments and Control Laboratories contained in Requirements for Biological Substances No. 1,<sup>2</sup> certain aspects of the International Sanitary Regulations relating to yellow fever vaccine,<sup>3</sup> the Yellow Fever Vaccine Requirements formulated by the Health Authorities of the USA,<sup>4</sup> as well as a number of working documents.<sup>5</sup>

As a further basis for its considerations the Study Group noted the work on standardization of cholera vaccines, cholera antigens, and cholera sera by Expert Committees on Biological Standardization,<sup>6</sup> the requirements on cholera vaccine adopted in some individual countries, as well as the draft requirements for yellow fever vaccine and for cholera vaccine prepared by the WHO Secretariat.<sup>7</sup>

The Study Group was of the opinion that the General Requirements for Manufacturing Establishments and Control Laboratories contained in Requirements for Biological Substances No. 1<sup>2</sup> were applicable to the manufacture and control of yellow fever vaccine and of cholera vaccine, and that the requirements for poliomyelitis vaccine contained in Requirements for Biological Substances No. 2<sup>8</sup> represented a suitable model for its work.

It noted that in this model the various sections embodying the requirements had been divided into two parts, the first part, printed in large type, being definitive requirements; the second part, printed in small type, being advice and additional information concerning the definitive requirements. The Study Group agreed that this arrangement was in accordance with the aim of the recommended requirements, namely that the definitive

<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1957, 136

<sup>2</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178, Annex 1

<sup>3</sup> Bonnel, P. H. (1956) *International regulation of yellow fever vaccination*. In: *Yellow fever vaccination*, Geneva, p. 193 (*World Health Organization: Monograph Series*, No. 30); World Health Organization (1957) *International Sanitary Regulations (Annotated ed.)*, Geneva, p. 35

<sup>4</sup> United States, Department of Health, Education and Welfare, National Institutes of Health, *Minimum requirements: yellow fever vaccine* (1st revision, May 18, 1949)

<sup>5</sup> Gallut, J., unpublished working document WHO/BS/IR/47; Gallut, J., unpublished working document WHO/BS/IR/48; Lahiri, D. C., unpublished working document WHO/BS/IR/49; Macnamara, F. N., unpublished working document WHO/BS/IR/50; WHO Secretariat, unpublished working document WHO/BS/IR/51; Panthier, R., unpublished working document WHO/BS/IR/52; Lahiri, D. C., unpublished working document WHO/BS/IR/53; WHO Secretariat, unpublished working document WHO/BS/IR/54; WHO Secretariat, unpublished working document WHO/BS/IR/55; USSR, unpublished working document WHO/BS/IR/56; Kerr, J. A., unpublished working document WHO/BS/IR/57

<sup>6</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1954, 86, 7; 1958, 147, 12

<sup>7</sup> WHO Secretariat, unpublished working documents, WHO/BS/IR/51, WHO/BS/IR/54 & WHO/BS/IR/55

<sup>8</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178, Annex 2

requirements could be incorporated as they stand into the national legislation of individual countries if the national health authorities of these countries wish to enforce these requirements in this way.

On the basis of the above-mentioned documents and with the above consideration in mind, the Study Group proceeded to prepare the drafts of the requirements for yellow fever vaccine and for cholera vaccine which are given in Annexes 1 and 2 to this report.

## 2. YELLOW FEVER VACCINE

### 2.1 Requirements for Yellow Fever Vaccine

The Study Group gave careful consideration to the recommendations made in the first report of the Expert Committee on Yellow Fever Vaccine.<sup>1</sup> It noted that in this report the opinion was expressed that "the choice of the virus strain to be employed should be left to the local authorities, having due regard to the local prevalence of yellow fever and the resulting morbidity", and that the Committee had agreed that "mass vaccination with vaccines produced from either of the two strains was preferable to no vaccination..." The Study Group accepted the validity of these views but was of the opinion that, in formulating requirements, recognition could only be given to preparations which were, as far as it was at present possible to ensure, both safe and potent. The Study Group wished to emphasize that, while the requirements drafted by the Group refer only to one type of vaccine, this was not to be construed as an indication that other vaccines had not, under special circumstances, a proper place in programmes of mass vaccination.

The Study Group came to the conclusion that no vaccine at present available for use by cutaneous scarification should be covered by the requirements. This conclusion was based on the fact that the so-called Dakar vaccine, although a potent antigen, carried a risk of occasional severe post-vaccination reactions, especially in children, whereas the vaccine for use by cutaneous scarification made from the 17D strain, although it was not known to have produced severe reactions, had not yet been prepared in a form which was antigenically adequate. It was therefore agreed that requirements (see Annex 1) should be provided only for yellow fever vaccine intended for use by subcutaneous injection.

The Study Group was of the opinion that the development of vaccines that would be fully satisfactory when used by cutaneous scarification was a matter of the first importance because of the reduction in the cost of mass vaccination and the greater flexibility of vaccine administration

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.* 1957, 136

that would result from their use. The Study Group therefore recommended that WHO continue actively to support research programmes, several of which were already in progress, designed to further the development of vaccines satisfactory for use by cutaneous scarification. The Study Group considered this matter of such potential importance that it prepared preliminary suggestions (Annex 1, Appendix 2) about the modifications that would probably be required in the future in order that the Requirements for Yellow Fever Vaccine could be made to cover a vaccine satisfactory for use by cutaneous scarification. It was considered that these suggestions might be a useful guide to those engaged upon developmental research on this problem.

## **2.2 Need for central advisory services**

During the discussions of the Study Group it became clear that laboratories manufacturing yellow fever vaccine, national health authorities, as well as WHO, would profit considerably if WHO instituted a central advisory service and a consultant group, with a view towards the creation of an international reference laboratory. Such a service backed by laboratory facilities could help to improve and co-ordinate the existing methods of production and testing which the Study Group recognizes are still far from perfect. Further work on these methods is required (see section 2.4) to enable amendments to be made from time to time to the present Requirements for Yellow Fever Vaccine.

Liaison between manufacturing laboratories in the complicated technical aspects of these problems is difficult and a central advisory service would be widely welcomed as a means of improving the free exchange of information. This service might also act as a focus for and stimulus to further research on the development of better vaccines, especially of a vaccine satisfactory for use by cutaneous scarification.

## **2.3 Responsibility of WHO in respect of International Certificates of Vaccination against Yellow Fever**

For an International Certificate of Vaccination against Yellow Fever to be valid, the vaccine used must have been approved by WHO. Approval of these vaccines has, in the past, been given by WHO after a single exhaustive investigation, both of the manufacturing establishment and of its product, by laboratories selected by WHO and by members of the WHO Expert Advisory Panel on Yellow Fever ; but, once given, this approval has been for an indefinite period of time. In considering the Requirements for Yellow Fever Vaccine, the Study Group became conscious of the need to devise some means whereby WHO could assure itself of the continued suitability of vaccines prepared by the approved laboratories.

The Group advised WHO to approve—for the issue of valid International Certificates of Vaccination against Yellow Fever—vaccines for subcutaneous use only if they satisfy all the Requirements for Yellow Fever Vaccine (Annex 1). Since no recommended requirements can as yet be drafted for vaccines intended for cutaneous scarification, no such basis for approval of these vaccines could be provided.

It was considered that the central advisory service envisaged above (section 2.2) could also act as an efficient agency for the collection of such information as would assure WHO of the continued high quality of the vaccines produced by the approved laboratories.

The Study Group wished to emphasize that the Requirements for Yellow Fever Vaccine (Annex 1) have been prepared according to the same pattern as that used by other Study Groups on Requirements for Biological Substances so that they can be used by the health authorities of different countries as a basis for control of yellow fever vaccine production. It hoped that requirements will eventually be available which can be used to cover all yellow fever vaccines made throughout the world. The responsibilities of WHO are limited to the approval of vaccines used in connexion with the issue of valid International Certificates of Vaccination against Yellow Fever. The creation of a central advisory service would enable WHO, if it so wished, by the provision of uniform strains and reagents, to promote the consistent production of more uniform vaccines than can be achieved solely by implementing the present Requirements for Yellow Fever Vaccine.

#### 2.4 Recommendations for research

The Study Group noted the recommendations for research made in the first report of the Expert Committee on Yellow Fever Vaccine,<sup>1</sup> and the proposed studies in South America on cutaneous vaccination with vaccines prepared from 17D strains.<sup>2</sup>

It agreed that the problems connected with vaccination by cutaneous scarification warranted a high priority in research programmes, and recommended that WHO should continue to assist and encourage research work on these questions.

It further recommended that research be undertaken upon :

- (a) the comparative effects of various diluents on the results of potency tests ; and
- (b) the comparative sensitivities to injection with vaccine virus of different strains of mice, and the comparative sensitivities of mice of the same strain according to age, weight, nutritional status and tem-

<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1957, 136

<sup>2</sup> WHO Secretariat, unpublished working document WHO/BS/IR/57

perature of rearing, as well as the effect of site and depth of intracerebral inoculations, and of seasonal variations in the mouse response ;

and that further studies be conducted on :

(c) the detection and quantitative estimation of specific yellow fever immune antibodies in man, and in other warm-blooded vertebrates.

### 3. CHOLERA VACCINE

#### 3.1 Requirements for Cholera Vaccine

In preparing the Requirements for Cholera Vaccine, the Study Group agreed that these should be formulated so as to cover only those vaccines in current use, namely vaccines consisting of approximately equal proportions of suspensions of killed *Vibrio cholerae* of the Ogawa and Inaba types. Draft requirements for this type of preparation are given in Annex 2 to this report.

Even when consideration is restricted to vaccines of this type, the diversity of the methods of preparation in current use is so wide that the formulation of definitive requirements is extremely difficult. Thus there are great variations in the strains of *Vibrio cholerae* used, in the methods of growing the cultures of the vibrios, in the method employed for killing them, and in the methods of preservation of the resulting vaccines. Added to this are the differences in methods of testing for the virulence of the strain, for the total bacterial count, for toxicity and, above all, for potency (see section 3.2).

These differences in techniques and in the nature of individual vaccines have made it necessary for the Study Group, in drafting the requirements, to leave many detailed specifications to be decided by national control laboratories, depending upon the methods in use in the manufacturing establishments in their own countries. Nevertheless, it has been possible to define certain properties which should apply to all satisfactory vaccines.

In connexion with the potency test, however, this has only been possible by devising an entirely new method, which is included in an Appendix to the requirements. It is still not known whether it will be a practicable method, and the Study Group therefore recommended that an immediate collaborative study of its suitability be organized by WHO. The new test involves comparison with the international reference preparations and the Study Group made certain recommendations in this connexion for consideration by the next Expert Committee on Biological Standardization (see section 3.3).

The final choice between the many techniques at present available and permitted under the draft requirements will depend upon the demon-

stration by clinical trial of the relative efficacy of different vaccines that pass these requirements (see section 3.2). At present, it is considered that the recommended requirements, as drafted, may help to bridge the gap until such time as this information becomes available.

### **3.2 Recommendation for an international study of the correlation between the results of laboratory potency tests and the prophylactic efficacy of cholera vaccines in the field**

The Study Group emphasized that the collaborative study of the new potency test (see section 3.1) was only a preliminary and that a field trial was absolutely necessary in order to demonstrate that the protective efficacy of cholera vaccine is correlated with its performance in a laboratory potency test.

The Study Group noted that efforts were being made in various countries to improve the basis of potency control, and it expressed the opinion that international co-operation would at this stage be possible and desirable. The Group recommended the organization of a study in which several laboratories of different countries, at present using different methods, would participate. This international study should be so designed as to obtain comparable data from different laboratories. It would be desirable to test several batches of cholera vaccine, one of which should be a reference vaccine (see section 3.3), by the methods laid down in the Requirements for Cholera Vaccine drafted by the Study Group—assuming that the immediate collaborative study proposed in section 3.1 indicated their practicability—as well as by the routine method or methods of each laboratory. Whatever method for potency determination is used, it should include the International Reference Vaccines in order to permit estimates of relative potency to be made. The results obtained in different participating laboratories should be evaluated and compared, so that the batches of cholera vaccine submitted to this international study can be graded in terms of their biological potency.

The above laboratory studies must be carried out in association with a field trial with a view to comparing the biological potency of cholera vaccine as determined in the laboratory with its prophylactic efficacy in man. Based on preliminary estimates of potency in the laboratories, three of the tested batches of vaccine, showing different potencies, should be selected for the field trial. It would be desirable to conduct the field trial in an area where epidemics of cholera have appeared every year. Such a field trial could indicate which of the potency tests were correlated with the prophylactic efficacy of cholera vaccine in man.

The Study Group therefore recommended that the World Health Organization make arrangements for initiating an international study as outlined above, and for collecting and evaluating the results. A successful

outcome of the field trial would imply that, for the first time, it would be possible to grade cholera vaccines validly by a laboratory potency test. This test could then be used to evaluate the importance of various factors involved in the production, testing, and storage of cholera vaccines.

### 3.3 International reference preparations

The Study Group noted that the proposed trial of laboratory potency tests (section 3.1) would make serious demands upon the stock of the International Reference Preparations. It therefore recommended that the next Expert Committee on Biological Standardization should arrange for sufficient quantities of monospecific cholera vaccines to be acquired as soon as possible to replace the existing International Reference Preparations. These new preparations should be included in the collaborative study. The Study Group also suggested that, at the same time, sufficient material from the same source, treated and stored in as nearly the same manner as possible, should be put aside, for use in the proposed field trial at a later date. In this way, a direct comparison of the behaviour in the laboratory and in the field of one and the same vaccine could be obtained. The Study Group wished to emphasize, however, that if an attempt to provide material on this scale seemed likely to delay the start of the proposed collaborative study of the assay methods, it would be preferable not to wait. In such circumstances, the reference vaccines used in the field trial should preferably be made by the same laboratory as the new international reference preparations and under as nearly similar conditions as possible.

The Study Group also requested that the next Expert Committee on Biological Standardization should consider whether the stocks of the existing International Reference Preparations of monospecific agglutinating sera would be adequate should the Recommended Requirements be brought into use, and to consider replacing them if necessary. It also suggested that the Expert Committee on Biological Standardization should investigate the possibility of providing an international reference preparation of *Vibrio cholerae* O rough serum.

## Annex 1

### REQUIREMENTS FOR YELLOW FEVER VACCINE (REQUIREMENTS FOR BIOLOGICAL SUBSTANCES No. 3)

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#### General Considerations

The present recommendation of requirements for yellow fever vaccine concerns only yellow fever vaccines to be used for subcutaneous injection. At present there are no vaccines available for use by cutaneous scarification which will satisfy the requirements of both safety and potency set forth in this document. The recommendations are based on methods and tests currently used to ensure a safe and potent vaccine; future revisions of these requirements 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 for their national regulations concerning yellow fever vaccine, it is

recommended that a clause be included which would permit modifications of manufacturing details on the condition that such modifications had been demonstrated, to the satisfaction of the national control authority and the World Health Organization, 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 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 febris flavae". 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 febris flavae shall consist of a preparation of viable, modified yellow fever virus. 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 yellow fever vaccine or sera have been established, no requirements of potency based on such standards can be formulated.

A WHO Expert Committee on Biological Standardization has taken steps to establish an international reference preparation of anti-yellow-fever serum.<sup>1, 2</sup>

A quantity of freeze-dried human serum, non-immune to yellow fever, is being held and distributed on behalf of the World Health Organization by the Statens Seruminstitut, Copenhagen, Denmark.<sup>1</sup>

#### 1.4 *Terminology*

*Seed lot* : A quantity of virus processed together and of uniform composition. In each manufacturing establishment the *primary seed lot* is

<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1958, **147**, 17

<sup>2</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, **172**, 16

that from which material is drawn for inoculating tissues for the preparation of all *secondary seed lots*. *Secondary seed lots* are one passage removed from a *primary seed lot* and material is drawn from them for inoculating tissues for the preparation of all *vaccine lots*. Such use of seed lots is known as the *seed lot system*.

*Vaccine lot (vaccine batch)* : All finished material in final containers which has at some stage been processed together and which therefore has 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.

*Constituted vaccine* : Vaccine ready for administration.

*Date of distribution* : The date on which a particular portion of a lot is distributed from the manufacturing establishment.

*Expiry date* : The date after which an issued vaccine may not be used.

*LD<sub>50</sub>* : One mouse LD<sub>50</sub> is the quantity of virus estimated to produce fatal specific encephalitis in 50% of mice of a highly susceptible strain, four to six weeks of age, after intracerebral inoculation.

*French neurotropic yellow fever virus* : A strain of neuro-adapted yellow fever virus commonly referred to as the French neurotropic strain. The passage level must be high and at least the last 200 consecutive passages must have been by the intracerebral route in mice. The stock virus is prepared by taking a 10% suspension of adult mouse brain in undiluted, heat-inactivated, non-immune human or simian serum, clearing by centrifugation, and desiccating under vacuum from the frozen state. The desiccated virus is subsequently stored at a temperature below  $-20^{\circ}\text{C}$ . The rehydrated freeze-dried stock virus must contain not less than 100 000 mouse LD<sub>50</sub> in 0.03 ml.

*Diluent for yellow fever virus* : A 0.75% solution of bovine albumin, fraction V, in phosphate-buffered isotonic sodium chloride solution pH 7.4, prepared from water purified by ion-exchange resins or by glass distillation ; or any other diluent which has been demonstrated to the satisfaction of the control authorities to be equivalent.

*Reference immune serum* : A freeze-dried serum or pool of sera taken not less than six months after inoculation from one or more monkeys immunized by a single subcutaneous injection of French neurotropic or unmodified pantropic yellow fever virus. The neutralizing power of this serum must have been determined by titration in a yellow fever virus neutralization test, as described in section 3.1.4.1 (p. 16), in comparison with an established reference immune serum or with the international reference preparation of yellow fever immune serum when this becomes available.

*Immune* : Adjective applied to a person or an animal whose serum will pass the yellow fever virus neutralization test, as described in section 3.1.4.1 (p. 16).

*Reference non-immune serum* : A freeze-dried serum or pool of sera of human or simian origin shown to be devoid of yellow fever antibodies and tested in a quantitative yellow fever virus neutralization test, as described in section 3.1.4.1 (p. 16), in comparison with an established reference non-immune serum, such as referred to in section 1.3 (p. 12).

*Non-immune* : Adjective applied to a person or an animal whose serum fails to pass the yellow fever virus neutralization test, as described in section 3.1.4.1 (p. 16).

## 2. General manufacturing requirements

The general requirements for manufacturing establishments contained in Requirements for Biological Substances No. 1<sup>1</sup> shall apply to establishments manufacturing yellow fever vaccine, with the addition of the following :

All work involving the use of any virus or micro-organism other than that used for the manufacture of the vaccine shall be conducted in a completely separate area, using separate equipment and separate washing and sterilizing facilities.

Written procedures for the preparation of yellow fever vaccine adopted by the manufacturer shall be submitted for approval to the national control authorities. Proposals for modifications shall be submitted for approval to the national control authorities and the World Health Organization before their implementation.

## 3. Production control

### 3.1 Control on source materials

#### 3.1.1 Virus strains

The strain of virus used shall be modified yellow fever virus of a type not transmissible from man to man by the bite of an insect.

The strains and substrains used in the production of vaccine shall be identified by historical records. They shall have been shown to the satisfaction of the national control authorities to yield immunogenic vaccines.

The virus should be of a type which induces protection against yellow fever for a period lasting from not more than nine days after vaccination until at least six years after vaccination.

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178, Annex 1

The strains in general use which meet the requirements set forth in the present document are known as the 17D strain and substrains of yellow fever virus. They are of the 200-300th subculture cultivated in chick-embryo tissue or in developing chick embryo. The 17D strain has in well-conducted trials evoked the production of protective antibodies against yellow fever in the blood of more than 95% of persons that were devoid of such antibodies before vaccination.

Samples of tested primary or secondary seed lots of a 17D strain of virus may be obtained for the purpose of establishing a primary seed lot to be used in vaccine production by application directly or through the World Health Organization to specialized laboratories.\*

### 3.1.2 *Tissues for virus production*

Virus for the preparation of all primary and secondary seed lots and of all vaccine lots shall be grown in the tissues of chick embryos. The seed lots and vaccine lots shall not contain any human protein or added serum.

The production method employed should be essentially as described in the literature.<sup>1</sup>

The colonies or stocks from which the eggs are derived should be free from pathogens capable of being transmitted to man by the route to be used for administering the vaccine.

### 3.1.3 *Seed lot system*

The production of vaccine shall be based on the seed lot system.

Primary and secondary seed lots shall be prepared.

As soon as possible after harvesting and processing, all seed lots shall be dried in the final container from the frozen state under high vacuum and shall be stored under conditions optimum for the stability of the strain of virus. Storage for desiccated seed lots shall be at a temperature lower than  $-50^{\circ}\text{C}$ . If for any reason a seed lot is not desiccated it shall be kept continuously at a temperature lower than  $-70^{\circ}\text{C}$ .

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\* The following laboratories have expressed their willingness to supply samples of strains for this purpose: Instituto Oswaldo Cruz, Rio de Janeiro, Brazil; Instituto de Estudios Especiales "Carlos Finlay", Bogota, Colombia; The Wellcome Research Laboratories, Beckenham, Kent, England; Instituut voor Tropische Hygiene en Geographische Pathologie, Koninklijk Instituut voor de Tropen, Amsterdam, Netherlands; Federal Laboratory Services, Lagos, Nigeria; Division of Biologics Standards, National Institutes of Health, Bethesda, Md., USA

<sup>1</sup> Fox, J. P., Kossobudzki, S. L. & Fonseca da Cunha, J. (1943) *Amer. J. Hyg.* **38**, 113; Hargett, M. V. & Burruss, H. W. (1945) *Amer. J. trop. Med.*, **25**, 19; Hargett, M. V., Burruss, H. W. & Donovan, A. (1943) *Publ. Hlth Rep. (Wash.)*, **58**, 505; Panthier, R. (1956) *Bull. Soc. Path. exot.*, **49**, 616; Penna, H. A. (1956) In: *Yellow fever vaccination*, Geneva, p. 67 (*World Health Organization: Monograph Series*, No. 30); Smith, H. H. (1951) In: Strode, G. K., ed. *Yellow fever*, McGraw-Hill, New York, p. 612; Theiler, M. & Smith, H. H. (1937) *J. exp. Med.*, **65**, 767

Primary seed lots shall be desiccated so that the preparations contain less than 1.0% of moisture as determined by the phosphorus pentoxide method.

The moisture content of all desiccated seed lots should preferably be less than 0.5%.

The inoculum for infecting the tissues used in the production of any vaccine lot shall be a preparation of a secondary seed lot. The diluent used for the purpose of rehydrating or diluting the seed lot virus shall not contain any human protein or added serum.

The seed lot system ensures that no vaccine shall be made that is more than one passage removed from a seed lot that has passed all the safety tests.

#### 3.1.4 *Monkey safety test on primary and secondary seed lots*

Primary and secondary seed lots from their final containers shall pass a test in monkeys for viscerotropism and neurotropism.

The monkeys shall be of the species *Macaca mulatta* (*Macacus rhesus*) or of an equally sensitive and responsive species and shall have been demonstrated to be non-immune just prior to injecting the seed virus. They shall not be the progeny of a mother known to be immune to yellow fever. They shall be healthy and shall not have been previously subjected to intracerebral or intraspinal inoculation. Also, they shall not have been inoculated by other routes with neurotropic viruses or with antigens related to yellow fever. Not less than ten monkeys shall be used for each test. The test dose shall consist of 0.25 ml containing not less than 5000 mouse LD<sub>50</sub>, as shown by a titration conducted by the method described in section 5.4 (p. 21). The test dose shall be injected into the frontal lobe of each monkey. The monkeys shall be observed for a minimum period of 30 days.

##### 3.1.4.1 *Viscerotropism*

The criterion of viscerotropism (as indicated by the amount of circulating virus) which must be fulfilled shall be as follows :

Sera obtained from each of the test monkeys on the second, fourth and sixth days after inoculation shall be injected undiluted and in at least two tenfold dilutions in aliquots of 0.03 ml intracerebrally into groups of at least six mice of the same quality as used in the potency test described in section 5.4 (p. 21). Virus shall be demonstrated in at least one 0.03 ml sample of serum from each of at least nine of the monkeys, but in no case shall 0.03 ml of serum contain more than 500 mouse LD<sub>50</sub> and in not more than one case shall 0.03 ml of serum contain more than 100 mouse LD<sub>50</sub>.

One or more brains of mice that have been injected with one of these positive serum samples, and that show symptoms of specific encephalitis shall be harvested and ground in nine times their weight of diluent for yellow fever virus. After centrifugation at about 2000 revolutions per minute for 10 minutes, the supernatant shall pass the identity test as described in section 5.1 (p. 19).

The criterion of sufficient virus-neutralizing antibody in the sera shall be as follows :

Not more than 10 % of the test monkeys shall fail to become immune within 30 days subsequent to injection of the test dose, as shall be determined by examining their sera in the yellow fever virus neutralization test described below.

Two or more containers of French neurotropic yellow fever virus shall be rehydrated and pooled in an amount of diluent for yellow fever virus equal to ten times the original volume of the virus suspension in the containers. The pooled virus shall be allowed to stand at room temperature for 15 minutes. The virus dilution to be used in the test shall then be prepared in the same diluent. This test-dilution shall be such that after addition of an equal volume of non-immune serum and after incubation at 37°C for one hour, this serum-virus mixture will contain approximately 100 mouse LD<sub>50</sub> in 0.03 ml. Samples of each of the monkey sera to be tested shall be mixed with an equal volume of the test-dilution of virus. No serum-virus mixture shall be allowed to remain at room temperature for more than 15 minutes before incubation. The serum-virus mixtures shall be incubated in a water bath at 37°C for one hour and then chilled in an ice-water bath.

Healthy four- to six-week old mice of a strain highly susceptible to intracerebral inoculation of yellow fever virus shall be distributed at random into groups of not less than six for the test, and into groups of not less than twelve for the controls.

From each serum-virus mixture 0.03 ml volumes shall then be injected intracerebrally under ether anaesthesia into each of at least six mice. The inoculations shall begin at once after incubation of the serum-virus mixtures and be completed as expeditiously as possible. The mice shall be observed for 10 days. Only deaths occurring after the third day and considered to be specific shall be taken into account. A serum shall have passed the yellow fever virus neutralization test if more than two-thirds of the mice survive and if the LD<sub>50</sub> determination shows that the test dose of virus contains more than 50 LD<sub>50</sub>. A serum shall have failed the yellow fever virus neutralization test if less than one-third of the mice survive, and if the LD<sub>50</sub> determination shows that the test dose contains less than 200 LD<sub>50</sub>. All sera that neither pass nor fail the test shall be retested until they either pass or fail by these criteria.

The following two controls shall be carried out simultaneously with the above test :

An LD<sub>50</sub> determination shall be done by preparing progressive tenfold dilutions of virus and by adding to each of a series of at least five of these dilutions an equal volume of reference non-immune serum. The mixtures shall be incubated and injected into mice as above, but groups of at least 12 mice shall be used for each serum-virus mixture, and the range from zero to 100% mortality of the mice should be covered.

A control of the specificity of the virus should be done by determining the potency of a reference immune serum. Progressive fourfold dilutions should be prepared of the reference immune serum in undiluted reference non-immune serum and to each of a series of at least six of these dilutions should be added an equal volume of the test-dilution of virus. The serum-virus mixtures should be incubated and injected into mice as described above, but groups of at least 12 mice should be used for each serum-virus mixture, and the range from zero to 100% mortality should be covered. The result of this potency determination should be compared with the known neutralizing potency of the reference immune serum previously established.

#### 3.1.4.2 *Neurotropism*

The criterion of neurotropism (as indicated by the incidence of clinical manifestations of encephalitis and death) shall be as follows :

Not more than 20% of the test monkeys shall develop encephalitis manifested by paralysis or inability to stand, with or without subsequent death of the animal.

Neither the onset and duration of the febrile reaction nor the nature of the symptoms and pathological findings should be such as to indicate a change in the properties of the virus.<sup>1</sup>

#### 3.1.5 *Sterility test on primary and secondary seed lots*

Each final lot of seed virus in the final containers shall pass the test described in section 5.2 (p. 20).

#### 3.1.6 *Guinea-pig safety test on primary and secondary seed lots*

Each seed lot shall pass the test described in section 5.3 (p. 21).

### 3.2 *Production precautions*

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

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<sup>1</sup> Fox, J. P. & Penna, H. A. (1943) *Amer. J. Hyg.*, **38**, 152

Section 3, of Requirements for Biological Substances No. 1<sup>1</sup> shall apply to the manufacture of yellow fever vaccine.

### 3.3 *Harvesting and treatment of the bulk product before desiccation*

Chick embryos that have been injected with virus shall be living and typical when harvested. The age of a harvested embryo shall be reckoned from the initial introduction of the egg into the incubator, and shall be not more than 12 days.

The inclusion of the heads of the embryos in preparing the embryo-pulp is optional. No requirements are made concerning the quantity of diluent which may be added to the embryo pulp or virus suspension provided that the requirements both concerning the presence of human protein and added serum and concerning the quantity of protein nitrogen in the final constituted vaccine are observed.

## 4. **Filling and containers**

The requirements concerning filling and containers given in Part A, Section 4 of the General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply with the addition of the following :

The containers of the final vaccine shall be of neutral glass of high quality, especially with regard to resistance against temperature fluctuations and breakage.

The containers shall be tested for leaks before filling and again during the process of desiccation ; all defective containers shall be discarded.

The containers of the final vaccine shall be flame-sealed.

As soon as possible after harvesting, vaccine should be dried in a manner similar to that applied to primary seed lots. Failure to achieve adequate desiccation will result in a product that is liable to rapid deterioration even at 0°C.

Containers should be sealed under vacuum or filled with dry nitrogen.

Single- and multiple-dose containers may be used.

## 5. **Control tests on final product**

### 5.1 *Identity test*

The following identity test shall be performed on at least one final container from each vaccine lot after constituting the vaccine according to the indications of the manufacturer for preparing constituted vaccine for human administration.

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, **178**, Annex 1

Progressive dilutions in steps not larger than fivefold shall be made of the constituted vaccine in diluent for yellow fever virus. Aliquots of each virus dilution shall be mixed with equal volumes of reference immune serum. Other aliquots of each virus dilution shall be mixed with equal volumes of reference non-immune serum. The serum-virus mixtures shall each be incubated at 37°C and injected into groups of not less than six mice as required in the yellow fever virus neutralization test described in section 3.1.4.1 (p. 16). All mice shall be observed daily for 21 days, and all deaths shall be recorded. Only deaths considered to be caused specifically by yellow fever virus infection shall be taken into account in the computations. Mice paralysed on the twenty-first day shall be counted as alive. The virus dilution calculated to give 50% mortality in mixture with non-immune serum shall be more than tenfold higher than the virus dilution calculated to give 50% mortality in mixture with immune serum.

### 5.2 *Sterility test*

Each final lot of vaccine in the final containers shall pass the following bacteriological sterility test.

Samples from final containers selected at random from each final lot shall be tested for bacteriological sterility. The random sampling shall be made in such a manner that all stages of the filling from the bulk container will have an equal chance of being represented. Not less than 10 containers from each final lot shall be tested. Not less than 1.0 ml from each container, or the entire content if less than 1.0 ml is present, shall be tested. The vaccine samples 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 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 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 with the same number of containers. A final lot shall be discarded if the same type of organism appears in more than one test, but no lot shall be passed unless the final test shows no growth throughout.

The sterility of the product, except for the presence of live yellow fever vaccine, should be maintained at all times while processing.

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

### 5.3 *Guinea-pig safety test*

The equivalent of 8-10 human doses from a vaccine lot shall be injected intraperitoneally into each of two normal guinea-pigs weighing 300-500 g each. The animals shall remain healthy for 21 days. If both of the animals show reactions the entire vaccine lot shall be regarded as unsatisfactory. If one animal shows reactions the test shall be repeated using three test animals. If in the repeated test one of the three animals shows reactions the product is unsatisfactory.

### 5.4 *Potency-test* (test for the active virus content of the vaccine)

Three final containers shall be selected at random from each vaccine lot and shall be tested individually as follows :

Constituted vaccine shall be prepared with the volume and kind of diluent recommended by the manufacturer for constituting the vaccine for human administration (see section 8).

After preparation, the constituted vaccine should stand at a temperature between 20°C and 30°C for 20 minutes before further dilution.

Serial tenfold dilutions of the constituted vaccine shall then be made in diluent for yellow fever virus.

Mice of a highly susceptible strain, 4-6 weeks of age, shall be injected intracerebrally under ether anaesthesia with 0.03 ml of the vaccine dilutions. Groups of not less than six mice shall be used for each dilution, and the series of dilutions shall embrace the range from 0%-100% mortality of the mice.

Injection of the mice should be commenced immediately after the dilutions have been made.

During a period of observation of 21 days, all deaths shall be recorded. Only survivors and deaths caused by typical yellow fever infections shall be counted in the computations. Mice paralysed on the twenty-first day of observation shall be counted as survivors. The computations shall demonstrate that the constituted vaccine contains not less than 1000 mouse LD<sub>50</sub> in the volume of the individual dose for use in man recommended by the manufacturer (see section 8).

If any of the three containers tested fails the test, the vaccine lot shall not be used. If among the results computed for the three containers the highest titre is more than 300 times the lowest titre, seven more containers shall be tested as described in this section, and if any of these fails the test, the vaccine lot shall not be used.

The potency of the vaccine of a vaccine lot that has passed this test should be expressed as the geometrical mean of the computed numbers of mouse LD<sub>50</sub> per human dose.

### 5.5 *Protein nitrogen content*

A human dose of constituted vaccine shall contain not more than 0.25 mg of protein nitrogen.

## 6. Records

The requirements given in Part A, Section 6, of the General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply, with the addition of the following :

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

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

The records should be kept in three groups : (a) primary seed lots ; (b) secondary seed lots ; and (c) vaccine lots. It is advisable to number the lots in each group serially. This facilitates checking the consistency of all lots processed. (See Appendix 1.)

## 7. Samples

The requirements given in Part A, Section 7, of General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply, with the addition of the following :

In addition to the samples of vaccine lots, samples of all seed lots shall be retained by the manufacturing laboratory and stored under the same conditions as those pertaining to the remainder of the lot until the expiry date of all vaccine lots prepared from these seed lots.

## 8. Labelling

The label printed on or affixed to each container shall show at least :

- The name and address of the manufacturer, suitably abbreviated if necessary ;
- the words " *Vaccinum febris flavae* " and/or the proper name of the product ;
- the vaccine lot number ;
- the expiry date.

<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178, Annex 1

Moreover, this label, or the label of the carton enclosing one or more 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 words “ living yellow fever vaccine prepared from... ”, specifying the tissue in which the virus was grown ;  
the conditions recommended during storage and shipping (if shipment at a temperature higher than that recommended is considered detrimental to the potency, it shall be so stated) ;  
the volume and kind of diluent to be added to constitute the vaccine ;  
the volume of the recommended individual dose, and the mode of administration (subcutaneous injection) ;  
the words “ the dose shall be the same for persons of all ages ” ;  
instructions for the administration of the vaccine including the statement that the constituted vaccine must be used or discarded within one hour after the container is opened.

A statement that constituted vaccine should be held at a temperature close to 0°C may be included. In addition, the leaflet should contain information concerning the contraindications, and the reactions which may follow vaccination.

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

## 9. Distribution and shipping

The requirements given in Part A, Section 9 of General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply.

## 10. Storage and expiry date

### 10.1 *Storage conditions*

Before being distributed by the manufacturing establishment all vaccines shall be kept constantly at a temperature below —5°C.

If possible storage should be at a temperature of less than —25°C. Since storage below —25°C may prevent deterioration of insufficiently dried products that would deteriorate at higher temperatures, some ampoules should always be stored at higher temperatures in order to test the stability of the vaccine at such temperatures as may prevail after distribution.

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, **178**, Annex 1

The manufacturer shall recommend such conditions of storage and shipping as will ensure that the constituted vaccine conforms to the requirements of potency until the expiry date as stated on the label.

Distributed vaccine should normally be stored at a temperature lower than 4°C although higher temperatures may be permitted for a short interval. Nevertheless, vaccine stored at temperatures between 0°C and 4°C may lose a considerable degree of potency during the course of one year.<sup>1</sup> Therefore, if the vaccine is to be stored at this temperature for periods of more than three months the vaccine should initially have a sufficiently high titre to comply with the requirement in this section.

#### 10.2 *Expiry date*

The expiry date shall be not more than 18 months after the date of the last satisfactory potency test, the date of a potency test being that on which the test animals were inoculated with the vaccine. The expiry date shall not, however, be more than 12 months from the date on which the vaccine was issued by the manufacturer.

### **Part B. National Control Requirements**

#### **1. General**

The general requirements for control laboratories given in Part B of General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>2</sup> shall apply.

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<sup>1</sup> Burruss, H. W. & Hargett, M. V. (1947) *Publ. Hlth Rep. (Wash.)*, **62**, 940; Fox, J. P. & Gard, S. (1940) *Amer. J. trop. Med.*, **20**, 447; Hahn, R. G. & Bugher, J. C. (1953) *J. Immunol.*, **70**, 352

<sup>2</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, **178**, Annex 1

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**Appendix 1**

**SUMMARY PROTOCOLS OF YELLOW FEVER VACCINE PRODUCTION**

**A. PRIMARY/SECONDARY SEED LOT**

Name and address of manufacturing laboratory .....

Laboratory reference number of lot . . . . .

Date when the processing was completed . . . . .

Has this seed lot passed the requirements . . . . .

**Information on manufacture**

1. Virus used to inoculate tissues for the manufacture of the lot :
  - (a) strain and substrain .....
  - (b) passage level .....
  - (c) source of origin and reference number .....
  - (d) remarks .....
2. Date of harvesting .....
3. Number of containers prepared .....
4. Conditions of storage .....

**Information on safety tests**

1. *Monkey safety test*
  - (a) Date of inoculation of monkeys .....
  - (b) Dilution of the rehydrated seed lot used for the inoculum .....
  - (c) Titration in mice of inoculum .....

<i>Dilution</i>	<i>Mortality ratio (MR)</i> (no. of dead mice/total number)	<i>Average survival time (AST)</i> <i>of the mice that died *</i>
inoculum undiluted		
inoculum diluted 1 : 10		
inoculum diluted 1 : 10 <sup>2</sup>		
inoculum diluted 1 : 10 <sup>3</sup>		
inoculum diluted 1 : 10 <sup>4</sup>		
inoculum diluted 1 : 10 <sup>5</sup>		
Calculated end-point		Mouse LD <sub>50</sub> administered to each monkey

\* For all mice that have died of specific encephalitis between the fourth and twenty-first day after inoculation, add up all days from the first day until the days of death and divide by the number of mice that died.

## (d) Record of monkeys

Monkey	Weight kg	Circulating virus demonstrated on days	Maximum titre of circulating virus	Para- lysis *	Death *	Development of immunity *
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						

\* State "yes" or "no"

(e) Identity test Date of test .....

(i) Titre of immune-serum plus virus .....

(ii) Titre of non-immune serum plus virus .....

2. Sterility test Date of test .....

(a) Number of containers examined .....

(b) Number showing contamination .....

(c) Remarks .....

3. Guinea-pig safety test Date of test .....

(a) Number of animals injected .....

(b) Number of animals showing reaction .....

(c) Remarks .....

4. Other safety tests .....

5. Moisture content of ampoules Date of test .....

(a) Test method used .....

(b) Number of ampoules examined .....

(c) Percentage of moisture determined .....

(d) Test not performed .....

6. Remarks .....

Signature of director of manufacturing laboratory .....

Date .....

Remarks of control authority .....

Signature of control authority .....

Date .....

**B. VACCINE LOT**

Name and address of manufacturing laboratory .....

Laboratory reference number of lot . . . . .

Date when the desiccation was completed . . . . .

Has this vaccine lot passed the requirements . . . . .

**Information on manufacture**

1. Virus used to inoculate tissues for the manufacture of the lot :
  - (a) strain and substrain .....
  - (b) passage level .....
  - (c) secondary seed lot reference number and place of origin .....
2. Date of harvesting .....
3. Containers : sealed under vacuum/filled with dry nitrogen
4. Number of containers prepared .....
5. Recommended human dose .....
6. Dilution of the tissues or juices in which the virus was grown present in constituted vaccine .....

**Information on potency tests**

Date of test .....

Mortality ratios (MR) and average survival times (AST) of mice inoculated with rehydrated vaccine diluted as follows :

Ampoule number	Undiluted		1:10		1:10 <sup>2</sup>		1:10 <sup>3</sup>		1:10 <sup>4</sup>		1:10 <sup>5</sup>	
	MR	AST	MR	AST	MR	AST	MR	AST	MR	AST	MR	AST
1												
2												
3												

Ampoule number	Titre	Mouse LD <sub>50</sub> per human dose	Geometrical mean of mouse LD <sub>50</sub> per human dose
1		.....	} .....
2		.....	
3		.....	

State, if known, the drop in potency of the vaccine resulting from the desiccation procedure .....

**Information on safety tests**

1. *Sterility test on bulk lot* Date of test .....

.....

2. *Sterility test* Date of test .....

(a) Number of final lots examined .....

(b) Number of containers examined from each final lot .....

(c) Number showing contamination .....

(d) Remarks .....

3. *Guinea-pig safety test* Date of test .....

(a) Number of animals injected .....

(b) Number of animals showing reaction .....

(c) Remarks .....

4. *Identity test* Date of test .....

(a) Titre of immune-serum plus virus .....

(b) Titre of non-immune serum plus virus .....

(c) Remarks .....

5. *Protein nitrogen content* Date of test .....

.....

6. *Remarks*

Signature of director of manufacturing laboratory Date .....

.....

Remarks of control authority .....

Signature of control authority Date .....

.....

## Appendix 2

### **Modifications to the Requirements for Yellow Fever Vaccine given in Annex 1, which will probably be necessary in order to cover any vaccine intended for use by cutaneous scarification**

A yellow fever vaccine to be given by cutaneous scarification should in general meet the same requirements as a vaccine intended for subcutaneous injection.

Nevertheless, several modifications would be necessary, such as :

(a) Strains other than those derived from strain 17D may be found to be satisfactory.

(b) The seed lots and the vaccine lots might be grown in tissues from animals or in animals other than the chick embryo. The animals from which the tissues might be derived should be free from pathogens capable of transmitting disease to man by cutaneous scarification. Tests of a non-specific character for the presence of pathogens in the animal stocks would be required and tests for specified agents would also be recommended.

(c) Minor modifications in the preparation of the vaccine for the potency test might be necessary.

(d) The *constituted vaccine* for cutaneous scarification should contain more than 500 000 LD<sub>50</sub> per ml.

(e) No requirement would be necessary concerning the quantity of protein nitrogen in the constituted vaccine.

(f) There should be a statement on the leaflet accompanying the vaccine stating the animal species and the tissue on which the virus was grown.

(g) Minor modifications would be necessary in the suggested summary protocols of production.

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## Annex 2

### REQUIREMENTS FOR CHOLERA VACCINE (REQUIREMENTS FOR BIOLOGICAL SUBSTANCES No. 4)

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#### General Considerations

The recommendation of international requirements for cholera vaccine is complicated by the fact that a number of different manufacturing and testing procedures are in use in various countries. The procedures differ mainly in the choice of the strains—especially with respect to virulence—the method of killing the vibrios, the use of preservatives, the concentration of vibrios in the final vaccine, and the tests for evaluation of the biological potency of the vaccine. 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 for their national regulations concerning cholera vaccine, it is recommended that a clause be included which would permit modifications of manufacturing details 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 cholerae". 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 cholerae shall consist of an aqueous suspension of *Vibrio cholerae* containing approximately equal parts of the types Inaba and Ogawa, grown in a suitable culture medium and subsequently killed by a suitable method. The preparation shall satisfy all the requirements formulated below.

The preparation may contain suitable preservatives and adjuvants, and it may be dispensed in a freeze-dried form.

#### 1.3 *International standards or reference preparations and international units*

The following International Reference Preparations of cholera antigens, vaccines, and sera were established in 1953;<sup>1</sup> no international units have been assigned to these preparations.

International Reference Preparation of Cholera antigen (Inaba):

dispensed in ampoules containing approximately 100 mg of dried antigen

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1954, 85, 7

International Reference Preparation of Cholera antigen (Ogawa) :

dispensed in ampoules containing approximately 100 mg of dried antigen

International Reference Preparation of Cholera vaccine (Inaba) :

dispensed in ampoules containing 20 mg of dried vaccine ( $1.6 \times 10^{10}$  organisms per ampoule)

International Reference Preparation of Cholera vaccine (Ogawa) :

dispensed in ampoules containing 20 mg of dried vaccine ( $1.6 \times 10^{10}$  organisms per ampoule)

International Reference Preparation of Cholera agglutinating serum (Inaba) :

dispensed in ampoules containing 0.6 ml of monospecific serum

International Reference Preparation of Cholera agglutinating serum (Ogawa) :

dispensed in ampoules containing 0.6 ml of monospecific serum

International Opacity Reference Preparation (established in 1953) :<sup>1</sup>

dispensed in ampoules containing 20 ml of a suspension of Pyrex-glass particles, roughly of bacterial size, in water. The opacity of this suspension has been defined as 10 International Units of Opacity per ml. By dilution of the International Opacity Reference Preparation in water, suspensions containing correspondingly fewer International Units of Opacity can be made up.

All international reference preparations mentioned above are in the custody of the International Laboratory for Biological Standards, Statens Seruminstitut, Copenhagen, Denmark, and samples are distributed, free of charge, on request to national laboratories for biological standards in all countries. The international reference preparations are intended for the calibration of national reference preparations. Such national reference preparations may replace the international reference preparations wherever these are mentioned in the requirements formulated below.

An Expert Committee on Biological Standardization has requested the Statens Seruminstitut, Copenhagen, to acquire, as soon as possible, quantities of monospecific cholera vaccines large enough both to replace the present international reference preparations and to be used in a field trial at a later date.<sup>2</sup>

Arrangements are being made for replacing the present International Reference Preparations of Cholera Agglutinating Sera, and for establishing an International Reference Preparation of Cholera O Rough Serum.

<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1954, **86**, 14

<sup>2</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, **172**, 13

## 2. General manufacturing requirements

The general manufacturing requirements contained in Requirements for Biological Substances No. 1<sup>1</sup> shall apply to establishments manufacturing cholera vaccine, with the addition of the following:

Written procedures for the preparation of cholera vaccines adopted by the manufacturer 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 on source materials

#### 3.1.1 Strains of *Vibrio cholerae*

Strains of *Vibrio cholerae* of both type Inaba and type Ogawa shall be used. They shall conform to the following morphological, cultural, biochemical, haemolytic, serological, and pathogenic characters of *Vibrio cholerae* in the smooth phase of growth. Any strains that fulfil the requirements of this section and that will yield a vaccine meeting the requirements set forth in subsequent sections may be used.

(a) Morphological properties. Smears shall be made from cultures of the strains selected after growth at 37°C for 18-24 hours on a suitable nutrient medium adjusted approximately to pH 8.0. The smears shall be stained by Gram's method, and examined microscopically. The vibrios shall appear as Gram-negative slightly curved rods, of uniform size and shape.

(b) Cultural properties. The strains selected shall be grown on a nutrient agar medium adjusted approximately to pH 8.0. The medium may contain 0.5% bile salt. When examined after 18-24 hours growth, the colonies shall appear typically smooth and translucent.

(c) Biochemical properties. The strains selected shall be grown in a set of peptone water media each containing different sugars which are required for identification of *Vibrio cholerae*. After 18-24 hours growth the reactions shall be noted. A strain shall be considered suitable if it ferments mannose and saccharose with the production of acid and no gas, and if, at the same time, it fails to ferment arabinose.

(d) Haemolytic properties. The strains selected shall be grown at 37°C for 24 hours in a nutrient broth, adjusted to approximately pH 8.0. Equal volumes of this culture, and of a 5% suspension of sheep erythrocytes shall be carefully mixed. The mixtures shall be incubated at 37°C for

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, **178**, Annex 1

two hours, and subsequently placed in a refrigerator overnight. The result shall indicate that the strains are nonhaemolytic.

(e) Serological properties. The strains selected shall be agglutinated specifically by non-differential cholera O group 1 antiserum. When a suspension of living *Vibrio cholerae* is tested by the slide-agglutination method against the monospecific International Reference Preparations of Cholera agglutinating serum—Inaba and Ogawa—or against corresponding National Reference Preparations, the respective strains shall be agglutinated to a titre of at least 1 : 100. When the same suspension of living organisms is tested against cholera O rough serum, there shall be no agglutination.

(f) Properties of stability in suspension. A suspension in physiological saline of the strains grown for 18-24 hours at 37°C on nutrient agar medium adjusted approximately to pH 8.0 shall be stable during at least five hours at 37°C.

(g) Pathogenic properties. The virulence of the strains selected shall be determined in guinea-pigs weighing 200-500 g by intraperitoneal injection of aqueous suspensions of cultures grown for 3-5 hours at 37°C. The number of organisms injected shall be determined as described in section 3.3.7 (p. 37). The virulence shall be such that  $4 \times 10^9$  organisms, or less, will kill a guinea-pig within 72 hours after injection.

The virulence of the strains selected should also be tested in a highly susceptible strain of mice. For the purpose of this test the organisms should preferably be grown for 3-5 hours at 37°C, so as to ensure that the great majority of the vibrios are in the logarithmic phase of growth. They should not be suspended in mucin. Different strains of mice vary considerably in susceptibility. The number of organisms that should kill a mouse depends, therefore, on the colony of mice available in the laboratory.

The strains may be preserved by freeze-drying. In addition to such preserved strains, however, it is preferable to use one or more freshly isolated strains from a prevailing epidemic. Freshly isolated strains of *Vibrio cholerae* may be obtained by application directly or through the World Health Organization to specialized laboratories.<sup>1</sup>

### 3.1.2 Culture medium for production of vibrios

Either a solid or a liquid growth medium may be used.

If a liquid medium is used that will form part of the finished vaccine it is particularly important that it should be free from ingredients that may cause toxic or allergic reactions in man.

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<sup>1</sup> The following laboratories have expressed their willingness to supply samples of strains for this purpose: The Wellcome Research Laboratories, Beckenham, Kent, England; Institut Pasteur, Paris, France; Calcutta School of Tropical Medicine, Calcutta, India; Central Research Laboratory, Kasauli, Punjab, India; Haffkine Institute, Bombay, India; Department of Microbiology, University of Chicago, Ill., USA; Walter Reed Army Institute of Research, Washington, D.C., USA

### 3.2 *Production precautions*

The general production precautions, as formulated in the requirements of Part A, Section 3, of General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply to the manufacture of cholera vaccine.

### 3.3 *Control at the monovalent stages of the product*

#### 3.3.1 *The period of growth*

The organisms shall be grown at 37°C for a period chosen by the manufacturing laboratory and approved by the national control authority.

#### 3.3.2 *Purity control*

Immediately before the cultures are killed, their purity shall be tested by the examination of samples from the culture flasks by morphological and cultural methods, as described in section 3.1.1 (a) and (b) (p. 34).

#### 3.3.3 *Killing of harvested cholera vibrios*

After sampling for purity control, the cholera vibrios shall be killed by a method selected by the manufacturing laboratory and approved by the national control authorities.

#### 3.3.4 *Control of the killing procedure*

Whenever a manufacturing laboratory selects a new killing agent, or a new strain of *Vibrio cholerae*, or changes other relevant conditions, a comparative test shall be done of the agglutinability of the organisms before and after killing, by determining the titre of the International Reference Preparations of Cholera Agglutinating Sera—Inaba and Ogawa—or of equivalent National Reference Preparations. If the titres of the sera, when using the killed organisms, are one fourth or less of the titres when using the living organisms, this killing procedure shall not be used.

#### 3.3.5 *Control of smooth to rough variation and of suspension stability*

A random sample drawn from the harvested growth prior to killing, as well as another sample collected from the same suspension after killing, shall be tested both for the agglutinability of the vibrios with cholera O rough serum, and for the stability of their suspension in physiological saline, as described in section 3.1.1 (e) and (f) (p. 35). Vibrios that are

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178, Annex 1

found to be agglutinated in cholera O rough serum, or to form an unstable suspension, shall not be used for the preparation of vaccine.

This recommendation is based on the fact that preparations showing the presence of rough elements and preparations forming unstable saline suspensions have always been found to give poor protection in animal experiments.

#### 3.3.6 *Sterility test*

The killed suspension of cholera vibrios shall be sterile. A sample of each monovalent bulk product of not less than 10 ml and divided into not less than 10 portions, shall pass the sterility test described in section 5.2 (p. 38).

#### 3.3.7 *Control of the concentration of vibrios per ml of the monovalent bulk product*

The concentration of vibrios in the monovalent bulk product shall be determined within 24 hours after harvesting and before the addition of a preservative. The number of organisms shall be measured either by actual count in a counting chamber, or by comparison of the opacity of the product with that of the International Opacity Reference Preparation, or of an equivalent National Reference Preparation.

Recount of the vibrios, or determination of the opacity of their suspension, at a later date, may not reveal the initial concentration of the organisms.

If the manufacturing laboratory wishes to use opacity determinations only, it should have conducted previous experiments to establish the correlation between the actual count of organisms and the corresponding opacity which prevails under the existing local conditions of production.

#### 3.3.8 *Storage of monovalent bulk product*

If the crude suspensions have been killed with formalin, they shall not be stored for a period longer than one month without dilution, or without removing or neutralizing the formalin. The storage temperature shall be below 10°C.

### 3.4 *Control of the divalent bulk product*

#### 3.4.1 *Preparation*

The divalent bulk product shall be prepared by mixing monovalent bulk products of types Inaba and Ogawa, in suitable proportions. Dilution, if necessary, shall be done in buffered physiological saline of pH 6.8 to 7.4, so as to produce a divalent vaccine containing at least  $2 \times 10^9$  vibrios of type Inaba and  $2 \times 10^9$  vibrios of type Ogawa per ml.

The largest concentration compatible with the requirements for safety should be preferred.

### 3.4.2 *Chemicals added for killing and preservation*

A suitable preservative shall be added to the bulk product, in its monovalent or divalent stage, if all or part of this material is to be issued in multidose containers. The preservative used shall have been demonstrated, to the satisfaction of the national control laboratory, not to affect the antigenicity and the safety of the vaccine.

All chemicals used for killing and preservation shall fulfil the requirements of the International Pharmacopoeia or of the national pharmacopoeia.

## 4. **Filling and containers**

The requirements concerning filling and containers given in Part A, Section 4, of General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply.

Single- and multiple-dose containers may be used.

## 5. **Control tests on final product**

### 5.1 *Identity test*

A specific identity test shall be performed on at least one final container from each vaccine lot. Cholera-agglutinating reference sera—Inaba and Ogawa—shall be used for this purpose. Both of these monospecific sera shall agglutinate partially the organisms in the vaccine, as described in section 3.1.1 (e) (p. 35).

If non-differential cholera O group 1 serum is used there shall be complete agglutination of the organisms, and the supernatant shall be clear.

### 5.2 *Sterility tests*

Each final lot of vaccine in the final containers shall pass the following sterility tests.

Samples from final containers selected at random from each final lot shall be tested for sterility. The random sampling shall be made in such a manner that all stages of the filling from the bulk container will have an equal chance of being represented. Not less than 10 containers from each final lot shall be tested. If the volume of each final container is 1.0 ml or less, the entire content of the containers shall be tested. If the volume of the final containers is greater than 1.0 ml, the volume tested from each container shall be not less than 1.0 ml.

<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, **178**, Annex 1

The vaccine samples 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. Other methods may be used if they have been demonstrated to the satisfaction of the national control authorities to be at least equally effective.

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

### 5.3 *Safety tests*

#### 5.3.1 *Test for abnormal toxicity*

Absence of toxicity of the vaccine in the final containers shall be demonstrated by tests in guinea-pigs and mice.

These tests may be carried out by parenteral injections of quantities of vaccine equivalent to five human doses into each of two or more guinea-pigs weighing 250-300 g or of quantities equivalent to one half of the human dose into two or more mice weighing 15-20 g. The animals should be observed daily for not less than seven days, during which time they should not show any signs of illness attributable to the vaccine injected.

#### 5.3.2 *Test for abnormal toxicity of phenol*

If phenol has been used in the preparation of the vaccine, three or more white mice weighing 15-20 g shall be injected subcutaneously with 0.5 ml of vaccine. If after injection one or more of the mice are observed to react with tremor and spasms persisting for more than 30 minutes, the test shall be repeated in twice the number of mice. If the reactions occur again in the second test the vaccine shall be discarded.

Attempts should be made by post-mortem examinations to ascertain the cause of death or illness of the animals used for the tests described in sections 5.3.1 and 5.3.2.

### 5.4 *Potency tests*

The vaccine in the final containers shall pass two potency tests, one being an active animal-protection test and the other an agglutination test.

The details of these tests shall be decided by the national control authorities.

Proposed details for these two tests of antigenic potency are given in the Appendix (p. 43). These tests will be incorporated in the present section of this document, as they appear, or with amendments, as soon as an international collaborative study has shown their suitability.

#### 5.5 *Hydrogen-ion concentration*

The hydrogen-ion concentration of the finished vaccine shall be within the range 6.8 to 7.4.

#### 5.6 *Homogeneity of suspension*

Final containers that show any coarse deposit which does not disperse on shaking into a fine homogeneous suspension shall be discarded.

Vaccines containing clumped organisms, or particulate matter derived from culture media are unsuitable for human use.

### 6. Records

The requirements given in Part A, Section 6, of General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply, with the addition of the following:

Protocols showing the morphological, cultural, biochemical, serological and pathogenic characteristics of the strains used for the preparation of the vaccine, the detailed history of its manufacture giving all the stages of its production, and the results of control tests done at intermediate stages of production and on the final product shall be submitted for approval to the national control laboratory as required.

### 7. Samples

The requirements given in Part A, Section 7 of General Requirements for manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply.

Samples should be submitted as required by the national control laboratory in sufficient volumes to permit a repetition of all tests. All samples should be stored at a temperature between 2°C and 5°C.

Samples required for control at intermediate stages of production would usually amount to about 50 ml each of

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178, Annex 1

living and killed suspensions of Inaba and Ogawa types respectively. Samples required for repetition of all the control tests on the final product would be larger in size. As much as 200 ml of the vaccine in final containers from each batch may be needed to perform these tests.

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 label printed on or affixed to each container shall show at least :

- the name and address of the manufacturer ;
- the words "Vaccinum cholerae" and/or the proper name of the product ;
- the number of the vaccine lot, and, if applicable, the number under which the lot was released by the national control authorities ;
- and the expiry date.

Moreover, this label, or the label of the carton enclosing one or more 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 nature of the preparation and the methods used for killing the vibrios ;
- the nature and quantity of the preservatives and the remaining amount of formaldehyde, if left unneutralized ;
- the conditions recommended during storage and shipping (if shipment at a temperature higher than that recommended is considered detrimental to the potency, it shall be so stated) ;
- the recommended individual dose, and the mode of administration.

In addition, the leaflet should 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 for labelling applicable to all biological products.

## 9. Distribution and shipping

The requirements given in Part A, Section 9, of General Requirements for Manufacturing Establishments and Control Laboratories (Requirements for Biological Substances No. 1)<sup>1</sup> shall apply.

<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178, Annex 1

## 10. Storage and expiry date

### 10.1 *Storage conditions*

The manufacturer shall recommend such conditions of storage and shipping as will ensure that the vaccine conforms to the requirements of potency until the expiry date as stated on the label.

Whenever it is possible to do so, the vaccine should be stored at a temperature between 2°C and 5°C. Otherwise it should be stored in a cool, dark place.

### 10.2 *Expiry date*

The expiry date shall not be more than two years after the date of the last satisfactory potency test, the date of a potency test being that on which the test animals were inoculated with the vaccine.

The vaccine should meet the requirements set forth in section 5 throughout the duration period. Fluid vaccine should not be used later than two years after its manufacture. A shorter period of duration may be prescribed if the manufacturer or the national control authorities so decide.

## Part B. National Control Requirements

### 1. General

The general requirements for control laboratories contained in Requirements for Biological Substances No. 1,<sup>1</sup> Part B shall apply.

### 2. Tests to be applied by the national control laboratory in the country in which the vaccine is produced

Section 3.2 of Requirements for Biological Substances No. 1,<sup>1</sup> Part B shall apply.

### 3. Release and certification

A vaccine lot shall be released only if it fulfils all requirements set forth in Part A.

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 the requirements set forth in this document.

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<sup>1</sup> *Wld Hlth Org. techn. Rep. Ser.*, 1959, 178, Annex 1

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.

#### 4. Pre-release vaccination

It is still not possible to devise laboratory tests which will ensure that a vaccine lot will not cause reactions in man. National health authorities are advised that, except in emergency situations, vaccine lots should be stored for six weeks and then be used for vaccination of a small group of at least five volunteers. Only after this has been done, and no untoward reactions have been observed, should the lot be released for mass immunization.

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### Appendix

#### Proposed Potency Tests \*

##### 5.4.1 *Active animal protection test*

In this test the protective power of the vaccine from the final containers shall be compared with that of two monovalent reference vaccines, Ogawa and Inaba respectively. The reference vaccines shall be either re-suspensions in 16 ml of physiological saline of the total contents ( $16 \times 10^9$  organisms) of the ampoules containing the International Reference Preparation of cholera vaccine, or they shall be equivalent suspensions of monovalent national reference vaccines.

At least 60 animals shall be divided at random into four equal groups. The animals of the first and second group shall be immunized subcutaneously with equal volumes of the vaccine from the final containers; the animals of the third group with the same volume of the reference vaccine Ogawa; and the animals of the fourth group with the same volume of the reference vaccine Inaba. The total immunizing volume for each

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\* Proposed text of tests for the antigenic potency of cholera vaccine, to be inserted into section 5.4 of part A of the Requirements for Cholera Vaccine, with amendments if necessary, after an international collaborative study of their suitability.

animal shall preferably be given in one injection, but may, if necessary for obtaining a sufficient degree of immunity, be given in two or three injections separated by a time interval. The animals of each group may be divided into two or three sub-groups, such that the total immunizing dose given to the animals of one sub-group differs by a factor five or ten from that given to the animals of the next sub-group, but whatever system of dosage is chosen it shall be applied equally to each of the four main groups.

After a time interval from the last vaccine injection, each animal of the first and the third group shall be challenged intraperitoneally with the same lethal dose of cholera vibrios type Ogawa with or without the addition of mucin or any other virulence-enhancing substance, whereas each animal of the second and the fourth group shall be challenged similarly with cholera vibrios type Inaba.

All animals shall be observed for 72 hours after the challenge.

In the first and third group, taken together, at least five animals shall survive and at least five animals shall die after the challenge with Ogawa vibrios. In the second and fourth group, taken together, at least five animals shall survive and at least five animals shall die after the challenge with Inaba vibrios.

The vaccine shall have passed the Ogawa part of the test if the number of survivors in the first group is equal to or larger than the number of survivors in the third group. The vaccine shall have passed the Inaba part of the test if the number of survivors in the second group is equal to or larger than the number of survivors in the fourth group.

If the vaccine fails both parts of this test, it shall be discarded. If the vaccine fails only one of the two parts of this test, this part may be repeated twice. If the vaccine fails either of the two repeated tests it shall be discarded.

The animals used for the active protection test shall be either mice, about four weeks old, or guinea-pigs weighing 200-250 g. All animals shall be in good health and of strains which are highly susceptible to the intraperitoneal inoculation of living cholera vibrios.

The immunizing doses, the time intervals, and the challenge conditions chosen for this test by the manufacturing establishment on the basis of its experience shall be submitted for approval to the national control authorities.

The cholera vibrios used for challenge should have adequate virulence and should not be of the same strains as those incorporated into the vaccines.

The virulence of the vibrios may be enhanced by suspending the living cholera vibrios in 5% mucin in distilled water, or by other methods. It would, in any case, be preferable to use young growing cultures of maximum natural virulence; a 3-5 hour growth at 37°C in a nutrient medium would in most

cases be suitable. A suitable challenging dose should be such that about 50% of the animals, immunized with the reference preparations, survive the challenge of Inaba and Ogawa vibrios respectively. Unimmunized control animals should be used to demonstrate the virulence of the organism in the dose used for challenge.

#### 5.4.2 *Agglutination test*

In this test the vaccine in the final containers shall be compared with a reference vaccine with respect to its agglutinability in monospecific reference sera. The reference vaccine shall be either a mixture in equal proportions of the International Reference Preparations of cholera vaccine, Ogawa and Inaba, obtained by suspending the total contents of the ampoules containing these International Reference Preparations in 4 ml of physiological saline to which the same preservative that is present in the vaccine in the final containers has been added, or it shall be an equivalent suspension of a national reference preparation of cholera vaccine. The monospecific reference sera shall be the International Reference Preparations of cholera agglutinating sera, Ogawa and Inaba, or equivalent monospecific national reference sera.

For *each* of the monospecific reference sera two series of twofold dilutions shall be prepared in two sets of nine test tubes. The first set of test tubes shall contain 0.5 ml of serum dilutions ranging from 1:10 to 1:2560, whereas the second set of test tubes shall contain 0.5 ml of serum dilutions ranging from 1:80 to 1:20480. To each test tube of the first set 0.5 ml of the vaccine from the final containers shall be added, and to each test tube of the second set 0.5 ml of the reference vaccine shall be added. In case a laboratory prefers to use a lower density of vibrios in the test, both the vaccine under test and the reference vaccine shall be diluted by the same factor.

The vaccine shall have passed the Ogawa part of this test if, on comparing the two sets of tubes containing the monospecific Ogawa reference serum, the degree of agglutination appearing in each of the nine tubes containing the vaccine from the final containers is found to be equal to or superior to the degree of agglutination appearing in the corresponding nine tubes containing the reference vaccine.

The vaccine shall have passed the Inaba part of the test if, on comparing the two sets of tubes containing the monospecific Inaba reference serum, the degree of agglutination appearing in each of the nine tubes containing the vaccine from the final containers is found to be equal to or superior to the degree of agglutination appearing in the corresponding nine tubes containing the reference vaccine.

If the vaccine fails both parts of this test, it shall be discarded. If the vaccine fails only one of the two parts of this test this part may be repeated

twice. If the vaccine fails either of the two repeated tests it shall be discarded.

There is no uniformity in the choice of optimum temperature of agglutination and the length of time for permitting this reaction to proceed. Each laboratory may use the conditions it prefers. Incubation for two hours at 37°C, 18 hours at 55°C, two hours at 56°C and subsequently 22 hours in a refrigerator, four hours at 52°C and subsequently overnight in the cold, are some illustrations of the diversity of practice.

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