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METHODOLOGY OF FREEZE-DRIED SMALLPOX VACCINE PRODUCTION

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1. Introduction

Freeze-dried vaccine is presently produced in 70 different laboratories in 45 countries. Less than one-third consistently produce vaccine which meets the standards of potency, stability and purity recommended by the WHO Expert Committee on Biological Standards, (Wld Hlth Org. techn. Rep. Ser., 1966, No. 323), standards which are referred to in the relevant provisions of the International Sanitary Regulations with respect to international certificates for vaccination against smallpox.

The various vaccine laboratories employ a wide variety of production methods evolved over many years through trial and error, experimentation, adaptation and arbitrary decision. Although the basic principles of smallpox vaccine production and testing have been elaborated,[1,2] a detailed methodology has never been published.

Accordingly, WHO convened a working group, composed of those with expertise in vaccine production and a broad knowledge of production problems in the developing countries, to consider alternative methods for the production and testing of freeze-dried smallpox vaccine and to recommend the simplest, most practicable methods. Since most laboratories prepare vaccine harvested from animal skin and intend this vaccine only for use by conventional scarification methods, the working group considered procedures for preparation of this type of vaccine only.

Because the majority of laboratories who experience difficulties in preparing vaccine of suitable quality are limited in terms of personnel, equipment, supplies of eggs for testing purposes, etc., the group sought particularly to simplify procedures at all stages of production and testing consistent with the preparation of a potent, stable and safe vaccine. It was recognized that special circumstances in certain laboratories may call for adopting alternative procedures to those described. However, the necessary tests should always demonstrate that the resulting vaccine is of sufficient potency, stability and purity and that production volume remains at a satisfactory level.

2. Production

2.1 Choice of vaccinifer

The choice of animal for the production of smallpox vaccine is largely controlled by availability to a given producer. The most commonly used animals are calves, sheep and water-buffaloes. There is no known difference in the efficiency of these animals as sources of vaccinia virus. Because soiling of scarified areas is less in female animals they are to be preferred to males. Young calves and sheep are normally used because of greater ease of handling. Calves are sometimes preferred to sheep because of the greater quantity of virus which may be harvested from each animal but they have the disadvantage of being more difficult to handle.

2.1.1 Every animal used for vaccine production should obviously be healthy and should have a negative tuberculin test. When possible, the animals should be obtained from a single reliable source. The animals should regularly be examined by a veterinarian. Any skin infection or infestation should lead automatically to the rejection of the animal if it cannot be controlled by appropriate therapy. Only those passed as healthy and free of ectoparasites should be accepted for quarantine. Before being introduced into the vaccine production building, the animals should spend at least two weeks in quarantine, during which time they must be kept separate from all animals not in quarantine. Rectal temperatures should be recorded daily. Only those animals which pass 14 consecutive days without fever or signs of disease should be inoculated. Those who show persistent fever, or signs of disease, should be removed from quarantine and the cause of illness sought. Competent veterinary advice should be sought about the type of prophylaxis or treatment needed.

At the start of the quarantine period the diet of the animals should be changed gradually to the type of food which they will be fed during the incubation period. The use of bedding should be avoided. Certain active and passive immunizations will be necessary depending on the local prevalence of disease. The whole animal should be clipped and washed before entry into quarantine and washed at least once, preferably more frequently, during quarantine. Soap containing hexochlorophene which exerts a residual antibacterial activity can be used if available.

2.2 Seed lots

The seed lot system should be used. A reasonably large freeze-dried or frozen primary seed lot (several hundred ampoules of 2-5 ml each) should be prepared from which secondary seed lots can be derived as required. Secondary seed lots shall be not more than five passages removed from a primary seed lot. Secondary seed virus should be prepared in the same type of animal as will be used for production. Details regarding the development and application of the seed lot system are presented in Appendix 1.

The seed lot should be of high potency since the higher the potency of the virus, the higher the probability of infection of the maximum number of cells in the dermis of the vaccinated animal. Seed virus with a potency of not less than 5×10^8 p.f.u./ml should be used. If necessary, highly potent seed may be prepared by concentration of less potent material, e.g. by pervaporation (Appendix 2). Seed virus should pass the tests for safety, bacterial content, etc. (Appendix 3). If possible, it should be sterile.

Secondary seed virus should be stored in containers of such size that one animal can be inoculated from a single container. Any material taken from cold storage and not used to vaccinate production animals in one session should be discarded. Glycerolated seed virus stored at -20°C retains potency for one year. In laboratories with unreliable electricity supplies, it may be advisable to freeze dry the secondary seed, despite a possible loss of potency which may ensue, and store it at 4°C or less.

The choice of a virus strain for smallpox vaccine has, in the past, been arbitrary and many strains of vaccine virus have only recently been subjected to proper investigation. It is important to choose a strain of vaccinia virus which will induce adequate immunity in man but as little vaccination illness as possible. It is an advantage if the chosen strain produces compact clearly-visible white pocks on the chorioallantoic membranes of chick embryos, thus making assay easier. Strains of virus unsuitable for vaccine production should be discarded and replaced by a suitable existing vaccine strain which may be obtained through WHO. Approximately one-third of the producing laboratories employ either the Elstroe (Lister) strain or the EM-63 strain.

2.3 Preparation of vaccinifer

The proper preparation of the skin of the vaccinifer before vaccination is one of the most important steps in obtaining a vaccine which meets requirements. The vaccinifers should be anaesthetized or tranquillized by general anaesthesia or a local (intraspinal) anaesthetic

before they are washed, shaved, again washed and scarified. It is reported that 95% ethyl alcohol (5 ml/kg, diluted 50% with water) given by stomach tube is an effective tranquillizer which makes the animal easy to handle and still able to walk to its pen at the end of the operation.

The animal should be shaved extensively enough to permit a reasonably large area to be vaccinated. No hair or wool should encroach on the area to be vaccinated. The areas used vary from about 2500 cm^2 up to 1 m^2.

A rigorous schedule of cleansing should be instituted and strictly supervised. Thorough washing of the skin with soap and water is the most important single factor in ensuring a final product complying with the regulations. Additionally, there is some evidence that heavily contaminated skins produce less vaccinia virus than skins relatively free of bacteria. The shaved area should be repeatedly and thoroughly washed with warm water and soap applied either by gloved hands or a soft cellulose sponge for three periods of at least 10 minutes each followed each time by copious rinsing with warm water. Finally, the animal's skin should be treated with 0.1% solution of quarternary ammonium salt or 70% alcohol. The quarternary ammonium salt or alcohol is rinsed off with sterile distilled water and the skin mopped dry and covered with sterile cloths until the animal is vaccinated. It is preferable that the shaving and washing procedures be performed in a different room from the one in which the animal is vaccinated. If the water supply is of doubtful purity, it may be advisable to insert a sterile Berkefeld V or similar type of filter in the water supply pipe. When in use, the filters should be replaced daily. The efficiency of the washing procedure may be monitored by taking swabs from the skin at different times.

A suitable table for handling animals is pictured in Appendix 4. Working drawings to permit fabrication can be obtained from WHO on request.

2.3.1 Scarification

The scarification should be thorough. It is best done with a sterile special scarifying device having a row of sharp teeth not more than 5 mm apart. A suitable instrument, available through WHO, is shown in Appendix 5. The scarification should be made horizontally and vertically and then diagonally in each direction so as to stimulate an evident erythema. The seed virus is applied thoroughly to the scarified area. It has been found that yields from calves are improved if successive small areas (30 x 30 cm) are scarified and inoculated immediately until the whole of the available area of the skin has been dealt with. This will require about 50 ml of seed virus per square metre of skin. It is extremely important during preparation of the animal, scarification and vaccination to keep movement in the operating room to a minimum and to keep the floor and other flat surfaces wet to reduce the amount of dust. Doors and windows should be kept closed. Dust inevitably increases bacterial contamination of the animal skin and of the material harvested from it, and thus makes production of an acceptable smallpox vaccine much more difficult.

2.3.2 The incubation period

After vaccination, the scarified area may be covered loosely with a sterile cotton cloth. If used, it should be changed at least once each day. The pen in which the animal is housed should have a hard floor and walls capable of withstanding frequent washing. A slatted wooden platform the length of the animal, sloping gently from front to back, should be provided for the animal to stand on (Appendix 6). This platform prevents its being grossly soiled by excreta and the animal can lie on it with reduced risk of contamination. A simple leather harness, in which the animal can stand in its pen but which prevents the animal from lying down is even more effective in reducing contamination. Excreta should be removed promptly and floors washed frequently with running water. Some authorities also recommend spraying the vaccinated area with 0.1% Roccal three times daily. Others use a mixture of brilliant green + neomycin + streptomycin (0.01%: 0.01%: 0.02%).

During the incubation period, many animals do well on a pelleted diet to which they have been introduced during the quarantine period (see section 2.1). This reduces the amount of dust in the immediate environment. Dry hay or similar natural foods should never be supplied to animals during the incubation period since they are a very likely source of contamination by many different microbes, including Actinomycetes which are very difficult to get rid of. If pelleted diets are not available, cereal mashes and similar mixtures may be given.

During the incubation period, the animal should be protected from extremes of temperature. The floor might be kept wet to reduce dust. The animal's temperature should be taken twice each day. Normally, temperature rises on the third day. If it rises prematurely, another infection may be present and the animal should not be used.

2.3.3 Harvest

The pulp should be collected about 96 hours after inoculation. The skin should again be prepared by careful and thorough washing with warm water and soap in three 10-minute cycles. Before the pulp is collected, the animal must be killed humanely and exsanguinated. The deep curettage necessary to obtain maximum yield and high titre pulp is not possible with living animals, nor is the pulp of living or unbled animals capable of yielding a clean-looking vaccine preparation because of its high concentration of haemoglobin. The pulp is collected with a curette which may be easily made by sharpening the edge of a hemispherical domestic spoon made of stainless steel. About 300 g of pulp may be collected from one square metre of scarified skin. After collection of the material, the animal shall be necropsied and if any serious pathological condition is found, the pulp must not be used for vaccine production. If all the details of production have been carefully followed the pulp should have a virus content of about 10^{10} p.f.u./g. Harvests from individual animals should be kept separately in sterile containers closed tightly enough to prevent drying by evaporation. The longer the pulp is to be stored before being worked into a vaccine, the lower the storage temperature should be. Pulp stored continuously at -20°C may be expected to retain potency for 12 months without deterioration.

2.4 Preparation of vaccine

2.4.1 Extraction and treatment of virus

A simple method of extracting pulps with a good virus content is to homogenize the material in 10 volumes[a] of 0.004 M McIlvaine's buffer (Appendix 7) + one volume trifluorotrichloroethane (Arcton, Freon 113, or Genetron 113) (for example, pulp 100 g: McIlvaine's buffer 1000 ml: trifluorotrichloroethane 100 ml). Homogenization should be done with provision made for constant cooling of the pulp. Machines of the Silverson Laboratory Mixer type are immersed in a melting-ice bath; machines of the Eppenbach Colloid Mill type have a water-cooled jacket. In the latter machine, the pulp is milled down to a scale reading of eight in about 30 minutes. The mixture is then centrifuged at about 750 g for 10 minutes to separate the fluorocarbon and aqueous phases. If no centrifuge is available the material may be allowed to sediment at 4°C overnight. The fluorocarbon phase will separate rapidly with a sharp boundary because of its specific gravity of 1.5. The aqueous phase may then be syphoned off. The bulk of the virus remains in the aqueous phase. To reduce microbial contamination to acceptable limits, phenol (analytic grade) should be introduced into the aqueous phase which should then be incubated at 25°C for 24 hours with constant mixing either with a magnetic stirrer or by mechanical shaking (Appendix 8). If the ambient temperature is higher than 25°C the incubation should be reduced to a period determined experimentally to be suitable. The amount of phenol added must be carefully measured. Too much will destroy the infectivity of the virus. The amount of phenol should be 0,5% by weight.

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[a] A smaller volume of buffer may be required if the titre of pulps is less than 10^{10} p.f.u./g.

2.4.2 After incubation the bacterial contamination is estimated by an aerobic plate count and a potency test is made (Appendix 3). While the results of these tests are awaited, the virus suspension should be stored at 4°C. It should not be allowed to freeze. The period of storage should be as short as possible and not more than two weeks. If the bacterial count is less than 500 per ml and the potency is such that the final product may be expected to meet the requirements, the suspension is accepted as suitable for the preparation of dried smallpox vaccine. A suspension with a potency of 10^9 p.f.u./ml would certainly be satisfactory. If the bacterial count exceeds 500 per ml, the suspension should again be incubated at 25°C and retested. This may be repeated, if necessary, up to a total of five days. It should be recognized, however, that continued exposure to phenol destroys virus as well as bacteria.

2.4.3 Pooling of single extracts

The volume of suspension obtained from a single extract (harvest from a single animal) may not be sufficient for a full drying-machine batch. Suitable volumes may be produced by pooling single extracts. Pooling of extracts may also be useful in permitting the use of a slightly understrength suspension after it has been mixed with a higher strength suspension. Pooling of this sort should not be resorted to if the stronger suspension would be diluted to less than a suitable potency for drying. Proper records must be kept of the origin of the pool. Suspensions or pools of suspensions should not be admixed with peptone until shortly before they are to be filled into containers for drying.

Enough peptone solution of appropriate concentration should be added to give a virus titre not less than 5 x 10^8 p.f.u./ml in a final concentration of 5% peptone (Appendix 9). Normally, a 10% peptone solution would be added to achieve this final concentration. Since some types of peptone have been found unsatisfactory, each lot or type of peptone should be tested before use to assure that vaccine dried with this peptone is satisfactorily stable. Bactopeptone (Difco) has been found to be uniformly satisfactory.

2.5 Filling

The filling room should be a small separate room rather than part of a larger one and, if possible, should be air-conditioned. Movement in it should be kept to a minimum. The room should be kept clean. To reduce the amount of dust the floor may be kept wet. Strict dust control measures and aseptic techniques shall be enforced to ensure that the product is not contaminated during the filling process. Suspensions of vaccine should be mixed continuously during the filling process.

2.5.1 Final containers (Appendix 10)

Whatever type of container is used, it must be clean and sterile. Hot water or steam may be employed for cleansing followed by sterilization in a suitable oven. Detergents should not be used.

Manufacturers prefer, as a rule, to dry as large a number of doses per container as possible. However, large numbers of doses in a single container are usually not suitable for field use and waste is considerable. The volume filled into each container should be between 0.15 and 0.25 ml which provides 15 to 25 doses by conventional scarification techniques and 40 to 100 doses by vaccination with the bifurcated needle.

2.6 Freeze-drying

2.6.1 Types of machines and closure of containers

Two types of freeze-drying machines are used for large-scale vaccine production: the centrifugal type and the shelf type.

In the centrifugal dryer, initial freezing takes place by evaporative freezing ("self" or "snap" freezing) under reduced pressure. No artificial cooling or mechanical refrigeration is employed. To avoid foaming under vacuum and to speed up the initial freezing (and later the drying process) the chamber is equipped with a centrifuge which causes the liquid and hence the frozen vaccine to assume the form of a wedge, thereby giving a large surface area in relation to volume. Centrifugal dryers are normally used for the so-called primary drying stage only, i.e. to reduce moisture to about 5%. For further drying, the containers are transferred to a secondary dryer which consists of a number of manifolds, connected to a vacuum system, and with a chemical trap for moisture instead of a cold trap (condenser) as used on the primary dryer. On this type of drying machine, ampoules are the standard form of containers used, and are sealed whilst still connected to the manifolds, either under vacuum or, more easily, after the vacuum has been broken with dry, oxygen-free nitrogen.

Shelf-type dryers will usually have shelves that can be cooled as well as heated. In most cases the cooling capacity on the shelves is adequate to carry out the initial freezing directly in the chamber. Initial freezing, however, may be carried out in a separate pre-freezer. In the case of small containers with very small liquid fills care must be taken to avoid thawing of the vaccine during the transfer from pre-freezer to dryer and before sufficient vacuum has been created in the freeze-drying chamber to give an adequate evaporative heat loss. Shelf-type dryers normally perform the primary as well as the secondary drying. One make and model of shelf-dryer has a built-in chemical trap which is brought into action in the secondary phase, but in most types the secondary drying is an extension of the primary phase with the continuation of controlled heating up to a safe level.

If the shelf-dryer is equipped with an automatic stoppering device, vials or vampoules may be employed. At the end of the drying run, vials or vampoules may be closed in the machine under vacuum (50 microns or less) or may be filled with dry oxygen-free nitrogen (Appendix 11), at atmospheric pressure before closing. This avoids exposing the product to ambient atmospheric gases and water vapour. When vials are employed, the vials and specially slotted rubber stoppers for this purpose must be made to very close tolerances. The rubber stoppers must be made of butyl rubber as the rate of diffusion of atmospheric gases and water vapour through ordinary rubber is such that appreciable amounts of oxygen and water vapour may enter in a relatively short time and cause deterioration of the vaccine. The stoppers may be silicone-coated to permit easier insertion into the vials. Finally, an aluminium collar is affixed to hold the rubber stopper in place. Vampoules (Appendix 9) are also sealed in the machine before removal. This is accomplished with a slotted stopper of ordinary rubber; final sealing is achieved by fusing the glass at the constriction. The upper portion of the container containing the rubber stopper is discarded; the rubber stopper may be reused several times.

Ampoules, in contrast to vials and vampoules cannot be sealed in the machine. Although shelf-dryers may also be used for drying vaccine in ampoules, provided the shelf interspace is high enough, it is necessary to remove these from the dryer before they are sealed thereby exposing the vaccine to the ambient atmosphere. As the dried vaccine is very hygroscopic, exposure to ambient humidity for brief periods will result in a substantial uptake of moisture by the vaccine. Thus, provision should be made for sealing the ampoules under vacuum or nitrogen in a room with controlled low relative humidity. Additionally, vaccine vials before sealing should be held in vacuum dessicators or preservation cabinets until minutes before sealing.

The type of dryer to be used in any given situation can only be decided after a close consideration of local factors, such as the type of container best produced locally, or if imported, the type of container for which a constant and reliable supply can be expected in view of possible currency problems and import regulations. With respect to the freeze-dryer, the availability of maintenance, repair services and spare parts should be carefully considered. The makes and models of freeze-dryers and refrigeration compressors which are in use elsewhere in the area should be considered. Generally speaking it is better to use

several smaller machines than a single large one in order to reduce the risk of total stoppage of production in case of a machine breakdown. Capacities of several machines in common use for smallpox vaccine production are shown in Appendix 12.

Selection of the type of container must also be carefully considered. While vials and vampoules may be sealed before removal from the freeze-dryer thus avoiding exposure to ambient air, these containers must be made to very close tolerances and thus are often not produced locally. Ampoules on the other hand, if hand sealed, do not need to be made to such exacting standards and thus might be obtainable from local manufacturers. If ampoules are to be sealed by machine, however, close tolerances will be required. Further, in contrast to vials, ampoules have a glass as opposed to a rubber seal and are thus somewhat more permanent - a consideration when long-term storage is contemplated.

Containers closed under vacuum should be tested to ensure that they have held the vacuum. The commonest method is the use of a high frequency tester (Tesla coil) which causes the residual gases in the evacuated container to glow with a distinct violet colour. Fused-glass seals, whether under vacuum or nitrogen at atmospheric pressure, may be tested by immersing the sealed containers in a dessicator containing water, which may be stained with methylene blue or any other suitable dye. The containers and dye solution in a vacuum dessicator should be subjected to a reduced pressure (20 mm Hg is enough) for a few minutes. When atmospheric pressure is restored, liquid will enter those containers not properly sealed.

2.7 A uniform filling lot is a single suspension or pool of suspensions which is filled in a single working session. A batch of vaccine is material from a uniform filling lot which is dried in a single drying cycle in a single machine. Material from the same filling lot dried in two machines is two batches.

2.7.1 Sampling batches for testing

Final containers for testing must be selected with care. If the containers are closed in the freeze-drying machine, samples should be taken at random from the batch. However, if the containers are removed unclosed from the freeze-drying machine and are subsequently sealed, samples should be taken from the last group of ampoules passing through the sealing machine because of the possibility of absorption of moisture during exposure to ambient atmosphere.

2.8 Testing

Approximately 20 final containers of 0.25 ml fill and 30 containers of 0.15 ml fill will be required from each batch for testing purposes. Three times this number of containers should be set aside for possible repeat testing or testing by an independent laboratory. The number of containers required for testing purposes at WHO reference laboratories is set forth in Appendix 13.

2.8.1 Potency

Potency is best tested by the pock counting technique on the chorioallantoic membranes of 12-day chick embryos (Appendix 3). At least five membranes should be available for counting at each dilution of vaccine tested. Two suitable dilutions should be prepared of the vaccine under test and of a reference vaccine of known satisfactory potency (about 1×10^8 p.f.u. per ml). Enough chorioallantoic membranes are inoculated to ensure at least five countable membranes at each dilution; the volume of inoculum is 0.1 ml. After suitable incubation, usually 42-46 hours at 35-37°C, the membranes are removed, washed and the pocks counted. The potencies of the reference and test vaccines are calculated as pock forming units per ml of undiluted vaccine. The titres are compared. Potencies equal to or greater than 1×10^8 p.f.u./ml indicate a satisfactory vaccine. An international reference preparation for smallpox vaccine is available for the standardization of local working reference preparations.

This test must be done on samples of all batches of final product.

2.8.2 Alternative tests

Under exceptional circumstances only, when eggs are not available for long periods, tests for virus concentration in the final bulk and vaccine potency may be made on the scarified skin of rabbits. A test as described in Appendix 14 including a reference vaccine prepared from the local strain should be done on each batch of the final product.

2.8.3 Stability

To meet the stability requirements of WHO, vaccine must pass the potency test after storage at 35-37°C for four weeks, i.e. its potency must be equal to or greater than 1×10^8 p.f.u./ml.

2.8.3.1 Rapid tests

Various rapid tests of stability have been devised but the commonest is the exposure of ampoules of dried vaccine to 100°C for one hour. To pass this test, the product should retain not less than 10% of its original potency. It has been found empirically that batches of vaccine which pass this very stringent test will pass the standard test as well, although vaccines which fail the more severe 100°C test may still pass the standard test. Vaccines which are dried in excipients other than peptone may not be able to be tested by this method. The rapid test of stability is for emergency use only when vaccine must be released before the standard test can be completed.

2.8.4 Bacteriological tests

The tests prescribed in Appendix 3 are all relatively simple and within the compass of any reasonably equipped laboratory. However, attention must be paid to certain details of these tests, for example in preparing cultures for plate counts of bacterial colony formers, the temperature of the agar which is poured into the Petri dish must not be greater than 44°C, otherwise the viable count may be spuriously low. Incubation of all bacteriological tests should be at 30-32°C.

2.8.5 Moisture content

The quality of potent dried smallpox vaccine is determined by its stability as found by empirical tests and not by its moisture content. However, regular moisture testing may be useful in monitoring the efficiency of the drying plant. Several physical methods are available for estimating the moisture content of dried vaccine. These depend either on drying vaccine to constant weight at a temperature of about 100°C or drying it to constant weight over phosphorus pentoxide *in vacuo* (Appendix 15). The results of estimations by these different types of method are not comparable unless the relationship between them has been determined experimentally; nor are the results obtained by different laboratories using the same method necessarily comparable.

2.8.6 Vaccine stability

Dried smallpox vaccine should be stable for at least three years when it is stored at temperatures below 10°C. Vaccine stored at normal ambient temperature should be used within one month after date of issue.

2.9 Reconstituting fluid

Reconstituting fluid for dried smallpox vaccine should be a solution of 25% (v/v) glycerol in 0.02 M McIlvaine's buffer pH 7.2-7.4 (Appendix 16). Since a small amount of fluid normally adheres to the glass of the container at the time of reconstitution, an additional amount of fluid should be added to ensure delivery of the correct amount. For example, to ensure delivery of 0.25 ml, 0.30 ml may have to be added. If the container of the reconstituting fluid is closed by a fused-glass seal, it may be sterilized after filling and

sealing. If the fluid is in containers closed with rubber stoppers, final sterilization is not possible. In the latter instance particularly, a sterility test must be done on material from the final containers.[3]

3. Labelling

All products shall be clearly identified by labels. The information given on the label on the container or the label on the package must, of course, conform to requirements of the national control authority.

The label should at least show:

the name of the product;

the name of the manufacturer;

the number of the batch;

the condition of storage (less than 10°C) and expiry date.

In addition, the label or leaflet in the package should contain instructions for the use of the product. (A proposed sample leaflet is shown in Appendix 17.)

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

4. Records

4.1 Production protocols and distribution records

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

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

4.2 Records of strain

Records should be maintained of the complete passage history of the vaccine strain.

5. Buildings, equipment and staff

5.1 Buildings

Annex 1 Part A (General requirements for manufacturing establishments) Wld Hlth Org. techn. Rep. Ser., No. 323, should be consulted. Rooms used for animals must have hard, impermeable floors and walls suitable for frequent washing with copious running water. The building must be screened against the entry of insects, and dust should be avoided in every way possible.

The rooms used for preparation of the vaccine should also have washable floors and walls, should not be larger than necessary and should be free of dust.

Structural considerations such as strength of floors, width of doors, and so on, must be taken into account in designing a new building or adapting an existing one. An outline of the requirements for buildings is given in Appendix 18.

5.2 Equipment

If a laboratory is suitable for upgrading to enable it to make freeze-dried smallpox vaccine, it should be equipped to produce at least 500 000 containers a year, each containing 0.25 ml of vaccine. This is equivalent to about 125 litres. Countries not planning to produce this quantity of vaccine annually would be ill-advised to initiate production. The number and type of freeze-driers must be decided individually for each laboratory.

To prepare suspensions of virus for drying, mechanical homogenization is necessary. Types which have proved to be efficient, robust and safe include Silverson Laboratory Mixers of the sealed unit type which, in operation, are immersed in an ice-bath, and the Eppenbach Colloid Mill which has a water-cooled jacket. In the method recommended in this document, the partial purification process applied to the virus includes low-speed horizontal centrifugation. A refrigerated centrifuge is not required. A machine with a capacity of about two litres in a horizontal rotor will be able to handle the centrifugation of virus suspension considerably in excess of 125 litres per year.

For the incubation up to 12 days of fertile eggs for potency testing, an egg incubator will be needed which is able to turn the eggs at a regulated frequency. After the chorio-allantoic membranes have been inoculated, the eggs are incubated stationary at 35-37°C in a standard bacteriological incubator. The incubator should contain a tray of water to maintain sufficient humidity to prevent the membranes from drying. For the bacteriological tests, a separate standard incubator will be needed. Water-jacketed incubators have certain advantages over anhydric types such as great stability of temperature, rapid recovery after being opened for extended periods and a considerable safety margin should there be a power failure since the water in the jacket loses heat relatively slowly.

A water-bath, preferably with an efficient stirrer, will be needed for various purposes such as holding melted agar at 44°C for plate counts of bacterial colony formers and for anaerobic tests which require that the specimens be held at 65°C for one hour before incubation at 30-32°C. If estimations of residual moisture are to be made gravimetrically (Appendix 15) a single-pan substitution balance will be needed with a sensitivity of 0.01 mg and an oven to operate at 100°C with an efficient thermostat.

Other equipment is dealt with in outline in Appendix 18.

5.3 Repair and maintenance facilities

All mechanical and electromechanical equipment is liable to failure. The likelihood of failure is greatly decreased by efficient and adequate maintenance. Ideally, regular preventive maintenance should be available within the laboratory or institute. If it is not, arrangements should be made for the work to be done on a contract basis by a reliable outside body. Loss of production is widely caused by lack of spare parts for machines, especially in developing countries. Spare parts depots should be established, preferably on a regional basis, and efforts should be made to ensure that the commonly required spare parts for all the different pieces of equipment are always available.

5.4 Services

Adequate supplies of water and electricity are essential for the proper functioning of the laboratory. A copious water supply is essential at all times for ensuring the cleanliness of the animal accommodation and may also be needed for cooling refrigerator compressors.

In tropical countries where water is required for cooling refrigerator compressors, the water temperature must be considered and the installation of water-cooling equipment provided where necessary. The performance of the mechanical equipment depends on an unbroken supply of electricity of the correct voltage free of wide voltage fluctuation. Water is sometimes and electricity often a problem. All vaccine-producing laboratories should, therefore, be equipped with standby generators of appropriate output. Current and voltage stabilizers may be necessary.

For sealing of ampoules, a supply of fuel gas is necessary, which, for automatic sealers, must be of constant pressure and calorific value. In order to obtain a thin, stiff and hot enough flame, oxygen admixture is necessary. Some types of hand-held burners and ampoule constrictors may work well enough with compressed air.

Usually a supply of nitrogen of adequate purity (Appendix 11) must also be arranged for. If sufficiently pure nitrogen is not available in the country, commercial, technical nitrogen may be available of a purity which can be easily improved. Up to 1% oxygen can be removed by bubbling through 5% Pyrogallol solution, and humidity can be reduced by passing through a column filled with silica gel or similar dessicant. Vaccine sealed under nitrogen "improved" in this manner should be carefully tested. If unsatisfactory, nitrogen of suitable quality may have to be imported.

5.5 Staff

The staff of a vaccine-producing laboratory should be led by a professionally qualified person with the technical and scientific knowledge necessary for the proper execution of the work. He should have a properly qualified assistant. For the annual production of 125 litres equivalent of dried vaccine, the laboratory will need to be staffed additionally by a senior technician able to carry a fair amount of responsibility and supervise the work of more junior staff. At least three persons of technician or junior technician grade will be needed for the production of vaccinial pulp, the preparation and initial testing of virus suspensions, and running the mechanical equipment - filling machines, freeze-drying plant, etc. Ancillary staff will be needed for animal care, washing up and sterile preparation, and packing and dispatch of vaccine.

No production team for freeze-dried smallpox vaccine is complete without properly trained maintenance staff for all the mechanical equipment.

REFERENCES

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APPENDIX 1

Development and application of the seed virus system

The seed virus system is one of the procedures necessary to ensure that each production lot of vaccine has the same desirable biological characteristics as the parent strain.

The seed virus should be bacteriologically sterile. If it is not sterile, the seed virus system will serve to control the development of phenol resistant bacteria which may evolve with indiscriminate serial passaging of production lots of vaccine.

The seed virus system requires that primary and several secondary seed lots be produced and dispensed in sufficient numbers of containers to ensure an adequate supply of virus for inoculation for long periods of time. The secondary seed virus used for production is not to exceed the fifth serial passage of the primary seed virus; thus, each production lot of vaccine will not be more than six passages removed from the primary seed virus.

The size of a seed virus lot is dependent upon the requirements of the production laboratory. Units requiring large volumes of seed virus during a short interval of time may find it necessary to prepare several passages of secondary seed lots in order to obtain the required volume of inoculum for production lots of pulp. Smaller production units may be able to utilize the second passage of the primary seed as the inoculum in the production of vaccine pulp.

The primary and secondary seed virus lots should pass the tests for identity, safety and bacterial content as described in Appendix 3. The potency of the primary and secondary seed virus should be as high as is practicable and assayed periodically (every three months) to ensure adequate potency following long-term storage. Seed virus with a potency less than 5×10^8 p.f.u./ml should not be used.

Ideally, the primary and secondary seed lots should be freeze-dried in ampoules and stored at 4°C or lower. However, an adequate supply of potent seed virus can also be maintained by the use of freeze-dried primary seed virus and the preparation of secondary seed virus as glycerolated suspensions (50% glycerol in 0.004 M McIlvaine's buffer) which will retain adequate potency for one year when stored at -20°C.

A seed virus system can be developed by expanding the following basic scheme to meet the requirements of the individual unit. The outline is based on the following factors:

1. Inoculum to be applied is 1 ml of virus suspension on each 200 cm^2 (14 cm x 14 cm) of scarified skin.

2. The yield of pulp is approximately 3 to 8 g per 100 cm^2 of inoculated skin.

3. A 10% suspension of pulp will contain approximately 10^9 p.f.u./ml following treatment to reduce the bacterial content to 500 colony formers or less.

Individual containers of the secondary seed virus should be filled to the volume that is required to inoculate a single animal, in the ratio of 1 ml for 200 cm^2 of skin to be inoculated.

To obtain seed virus with minimal levels of bacterial contamination, it may be advantageous to inoculate selected sites of skin, such as the upper portion of the side of the vaccinifer between the shoulder and hip. These areas are less subject to contamination during incubation. In addition, these less extensive sites may be washed more thoroughly prior to harvest. The addition of an antibiotic powder to the inoculated area as well as use of sterile loose dressings changed daily during the incubation period may be beneficial in obtaining "clean" pulps.

Appendix 1

The basic system described in the table illustrates the use of two serial passages from the primary seed lot to obtain sufficient seed virus to inoculate up to 1200 vaccinifers. Laboratories using sheep or inoculating areas less than 10 000 cm^2 per calf will be able to inoculate proportionally more animals with the same total quantity of secondary seed virus. Production units requiring larger quantities of seed virus may elect to produce a third or fourth passage seed virus lot or prepare several lots of second passage seed virus for use on an individual lot or combined lot basis.

PREPARATION OF PRODUCTION SEED LOT FROM PRE-PRIMARY SEED VIRUS

	Pre-primary seed	Primary seed lot	Secondary seed lots	
			Passage 1	Passage 2
				(Production seed lot)
Inoculum	Parent seed of acceptable strain	Pre-primary seed	Primary seed	Secondary seed (passage 1) (a) (b)
Volume of inoculum	2 ml	15 ml	15 ml	15 ml 50 ml
Area inoculated	400 cm^2	3 000 cm^2	3 000 cm^2	3 000 or 10 000 cm^2
Approximate yield of pulp	12 g	100 g	100 g	100 or 300 cm^2
Approximate pulp suspension	120 ml	1 000 ml	1 000 ml	1 000 or 3 000 ml
Volume available for seed[a]	100 ml	980 ml	980 ml	980 or 2 980 ml
Number of 50 ml vials			19 or	19 or 59
Number of 15 ml ampoules	6	65	65	

[a] 10-20 ml of pulp suspension to be used for required testing.

APPENDIX 2

Concentration of seed virus by pervaporation

Cellulose dialysis tubing of about 25 mm diameter is used. Approximately 1.2 m will hold about 500 ml of suspension. The tubing is boiled for 10 minutes in distilled water to sterilize and soften it. When it has cooled enough to be handled, a tight double knot is tied close to one end and a sterile funnel is introduced into the other end and the virus suspension is poured in carefully. At least 20 cm of tubing should be left unfilled to permit a knot to be tied. The filled tube is allowed to hang freely in the stream of air from an electric fan. Water evaporates through the cellulose and the loss of fluid with its latent heat of evaporation keeps the material in the tubing cool. The process may be speeded up by doing it in an incubator. The more rapidly water is lost the cooler the remaining suspension becomes.

When the reduction in volume is judged to be sufficient, the concentrated seed virus suspension is collected, e.g. by cutting the bottom of the tube over a sterile beaker or by drawing it into a sterile syringe of appropriate size. Suspensions containing phenol should not be concentrated by this method.

With this method, the seed virus may readily be concentrated tenfold or more.

APPENDIX 3

Testing of vaccine and seed virus

In the course of vaccine production, the following tests are indicated as a minimum. Additional tests may be employed at other stages of production to evaluate and monitor the production process.

A Final bulk

1. Potency test
2. Bacterial colony count

B Final containers

1. Potency test
2. Stability test
3. Bacterial colony count (Bacillus anthracis)
4. Test for Escherichia coli
5. Test for haemolytic streptococci, coagulase-positive staphylococci, B. anthracis
6. Test for Clostridium tetani and other pathogenic spore-forming anaerobes
7. Innocuity test

C Seed virus

1. Potency test
2. Bacterial plate count (B. anthracis)
3. Test for Escherichia coli
4. Test for haemolytic streptococci, coagulase-positive staphylococci, B. anthracis
5. Test for Clostridium tetani and other pathogenic spore-forming anaerobes
6. Innocuity test
7. Identity test

These tests are described below:

1. Potency test

Material required:	Final bulk:	1 ml
	Final containers:	4 containers of 0.25 ml fill; 7 containers of 0.15 ml fill; (these should be pooled)
	Seed virus:	1 ml

Procedure

(1) Eggs are incubated at 38°C for 12 days. The eggs are candled and dead embryos discarded. Sufficient eggs should be prepared to permit counting of pocks on at least five membranes at each dilution used. Additional eggs will, of course, be required for simultaneous titration of the reference standard. On all eggs selected for potency assay, the site of inoculation is pencil-marked on the shell, carefully marking an area which is not in the proximity of any blood vessels.

(2) A hole is cut into the air sac of each egg and a window of about 4 mm x 4 mm is made at the previously marked inoculation site.

(3) Two drops of 0.004 M McIlvaine buffer pH 7.2 are placed on top of the shell membrane and the membrane penetrated with a needle. Care must be taken not to damage the chorio-allantoic membrane.

Appendix 3

The buffer, acting as a wedge, flows by capillary action between the shell and the chorioallantoic membrane. Suction produced at the air sac hole with a rubber mouthpiece or teat, drops the CAM completely. The eggs are now ready for inoculation. The time interval between dropping the CAM and inoculation should not exceed four hours.

(4) Dilutions of the material to be tested are prepared. 0.004 M McIlvaine buffer is used as diluent. Each egg is inoculated with 0.1 ml. The eggs are incubated at 35-37°C for 42-46 hours after which the chorioallantoic membranes are taken out for pock counting. If counting is to be done within an hour or so, the membranes are kept in distilled water. If a longer delay is anticipated they may be fixed (see preparation of fixative below). Thereafter, the pocks are counted. A magnifying lens for examining pocks is very useful.

Fixative

Mercuric chloride ($HgCl_2$)	15.0 g
Distilled water	600.0 ml
Dissolve completely	
Add absolute alcohol	300.0 ml

Criteria for acceptance

Final bulk: Normally should be about 1×10^9 p.f.u./ml

Final containers: Should contain at least 1×10^8 p.f.u./ml in the reconstituted vaccine

Seed virus: Should exceed 5×10^8 p.f.u./ml

2. Vaccine stability

Material required: Same as potency test

Procedure: Two vials from each filling lot are incubated at 35-37°C for four weeks and tested for virus concentration by the pock count method.

Criteria for acceptance

Final containers: The vaccine passes the test if the titre is greater than 1.0×10^8 p.f.u/ml

3. Tests for total bacterial content

Material required: Final bulk: 1 ml

Final containers: 4 containers of 0.25 ml fill;
7 containers of 0.15 ml fill;
(these should be pooled)

Seed virus: 1 ml

Dilutions (1:10; 1:100; 1:1000) of 1.0 ml of the final bulk or seed virus are made in 0.004 M McIlvaine buffer. At least three 1-ml samples of each dilution are cultured on nutrient-broth-agar poured plates as well as a control sample of the buffer.

Appendix 3

For testing of the final containers, 1.0 ml of vaccine diluted 1:10 and 1:100 in 0.004 M McIlvaine's buffer is cultured on nutrient broth-agar-plates. Three samples of each dilution are cultured. The plates are incubated for 72 hours between 30°C and 32°C.

From the number of colonies appearing on the plates the number of living bacteria in 1 ml shall be calculated. If this number exceeds 500, the final bulk or seed virus shall be subjected to further treatment or be discarded. If the number exceeds 500 in the final container, the batch shall be discarded.

If a greater number of colonies appears on the test plates in the higher dilutions, it suggests the plate count has been influenced by the inhibitory action of preservative present.

4. Test for the presence of Escherichia coli

Material required: (same material may be used as in Test 3 above)

At least three 1-ml samples of a 1:10 dilution of vaccine reconstituted in 0.004 M McIlvaine's buffer from the final containers are cultured on McConkey's agar or other media which are suitable for differentiating E. coli from other bacteria. The plates are incubated for 48 hours at 30°C to 32°C. If E. coli is detected, the batch is discarded. If detected in the seed virus, further treatment with phenol may be effective in removing these organisms.

The presence of E. coli in this test generally indicates heavy faecal contamination.

5. Test for the presence of beta-haemolytic streptococci and coagulase-positive staphylococci[1]

Material required: (same material may be used as in Test 3 above)

At least three 1-ml samples of a 1:10 dilution of vaccine reconstituted in 0.004 M McIlvaine's buffer are cultured on poured plates of blood agar. The plates are incubated for 48 hours at 30°C to 32°C and the colonies appearing are examined.

If either of the organisms mentioned is detected, the batch is discarded. If they are detected in the seed virus, further treatment with phenol may be effective in removing these organisms.

6. Test for the presence of Clostridium tetani and other pathogenic spore-forming anaerobes

Material required: Final containers: 4 containers of 0.25 ml fill;
or 7 containers of 0.15 ml fill;
(these should be pooled)

Seed virus: 1 ml

A total volume of not less than 1 ml of vaccine reconstituted in 0.004 M McIlvaine's buffer is distributed in equal amounts into 10 tubes, each containing not less than 10 ml of thioglycollate broth. The tubes are held at 65°C for one hour in order to reduce the content of vegetative organisms, after which they are incubated for at least one week between 30°C and 32°C.

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[1] Any colony seen on any of the plates which morphologically resembles B. anthracis is examined. If the organisms contained in the colony are non-motile, further tests for the cultural character of B. anthracis are made, including pathogenicity tests in suitable animals. If B. anthracis is found to be present, the final bulk or seed virus must be discarded.

Appendix 3

From every tube showing growth, subcultures are made on blood agar plates which are incubated anaerobically at 30°C to 32°C. All anaerobic colonies are examined and identified and if C. tetani or other pathogenic spore-forming anaerobes are present the batch or seed virus is discarded.

Organisms resembling pathogenic Clostridia found in tube culture from which the subculture was made may be tested for pathogenicity by inoculation into animals as follows: groups of not less than five mice are used for each tube culture to be tested. Injections of 0.2 ml of the cultures mixed with 0.1 ml of 4% calcium chloride solution are made intramuscularly into each of the mice at the root of the tail. The animals are observed for one week. If any animal develops signs of tetanus, or if any animals die or develop necrotic lesions at the site of inoculation, the batch or seed virus is discarded. A group of five mice protected with 100 units of tetanus antitoxin should be used as a control for each isolate tested.

7. Inocuity test

Material required: Final containers: 2 containers of 0.25 ml fill;
or 4 containers of 0.15 ml fill;
(these should be pooled)

Seed virus: 0.5 ml

A total volume of 0.5 ml of vaccine reconstituted in 0.004 M McIlvaine's buffer is inoculated intraperitoneally into each of two guinea-pigs. If the guinea-pigs are alive and well one week later, the vaccine can be considered to be acceptable.

8. Identity test

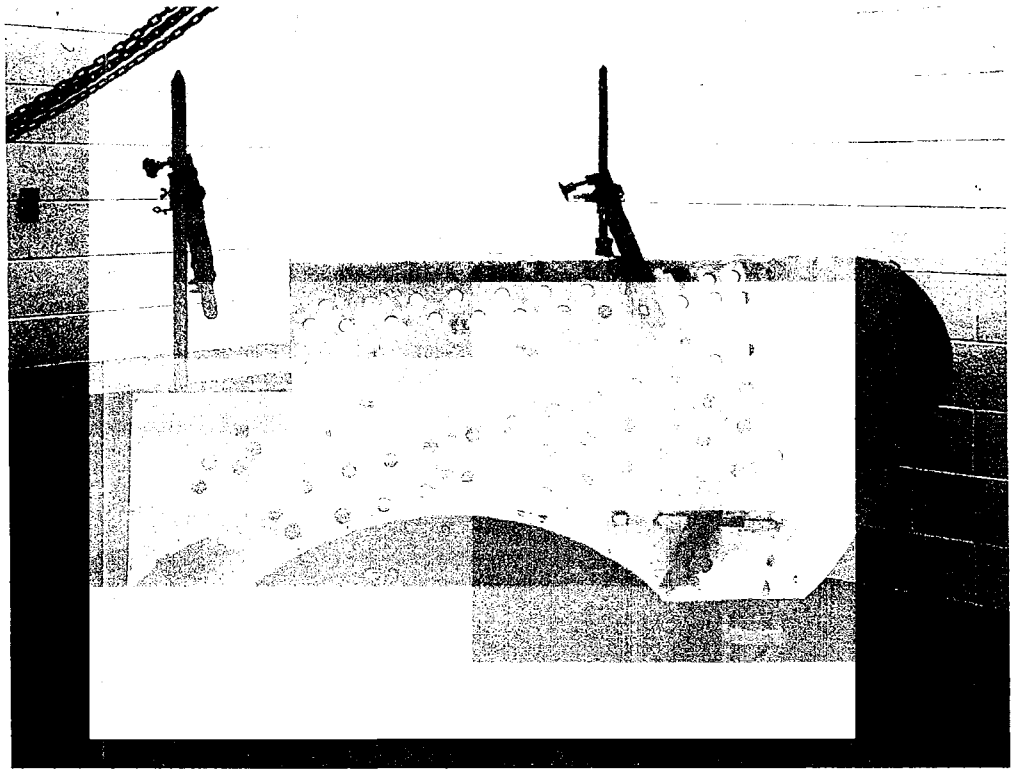
Seed virus should produce characteristic vaccinia pocks when grown on chorioallantoic membrane and when scarified into the skin of a rabbit should not produce necrotic lesions. Strains which produce atypical or haemorrhagic pocks on chorioallantoic membrane or necrotic lesions on rabbit skin should be replaced by more suitable strains.

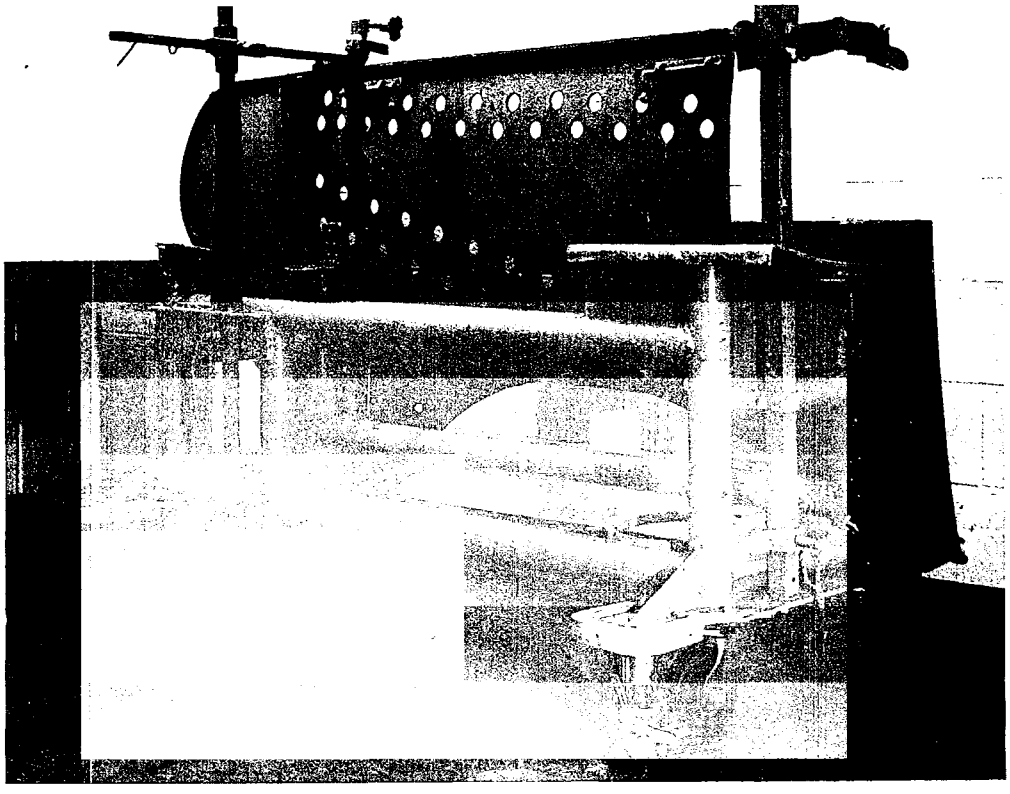
APPENDIX 4

TABLE FOR HANDLING OF ANIMALS DURING SCARIFICATION AND HARVEST

(Specifications to permit fabrication can be obtained from WHO on request)

WHO 80784





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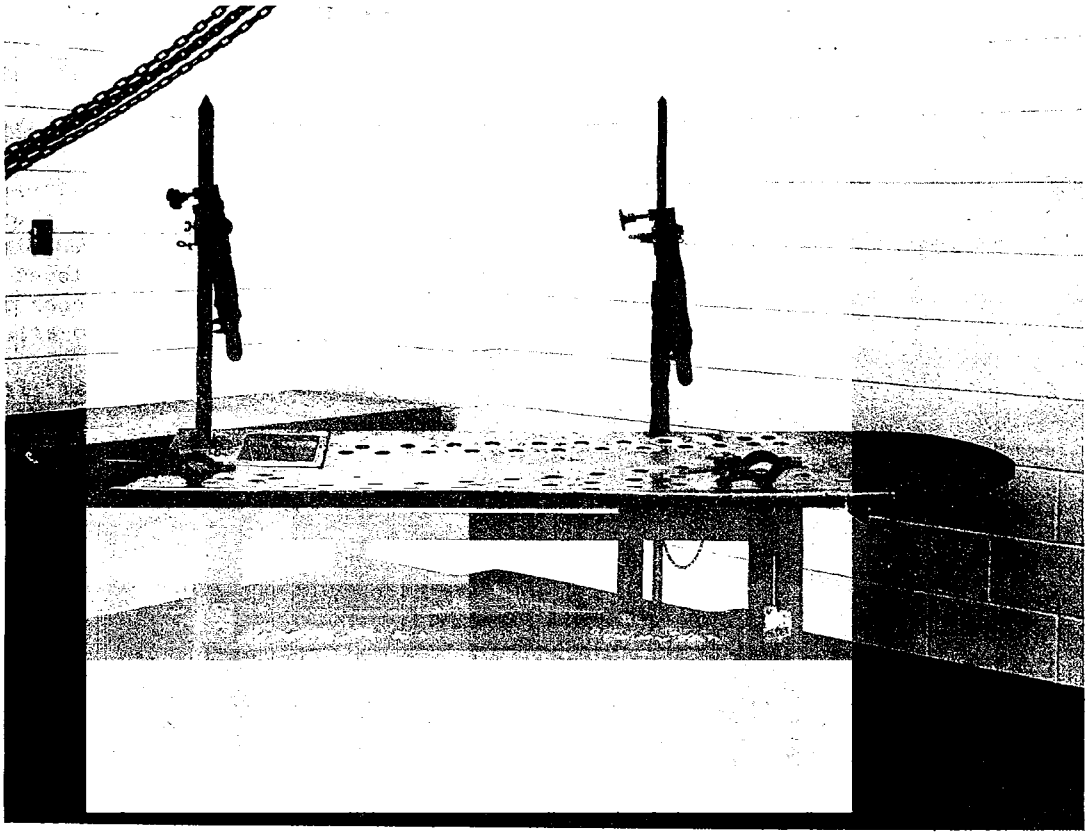
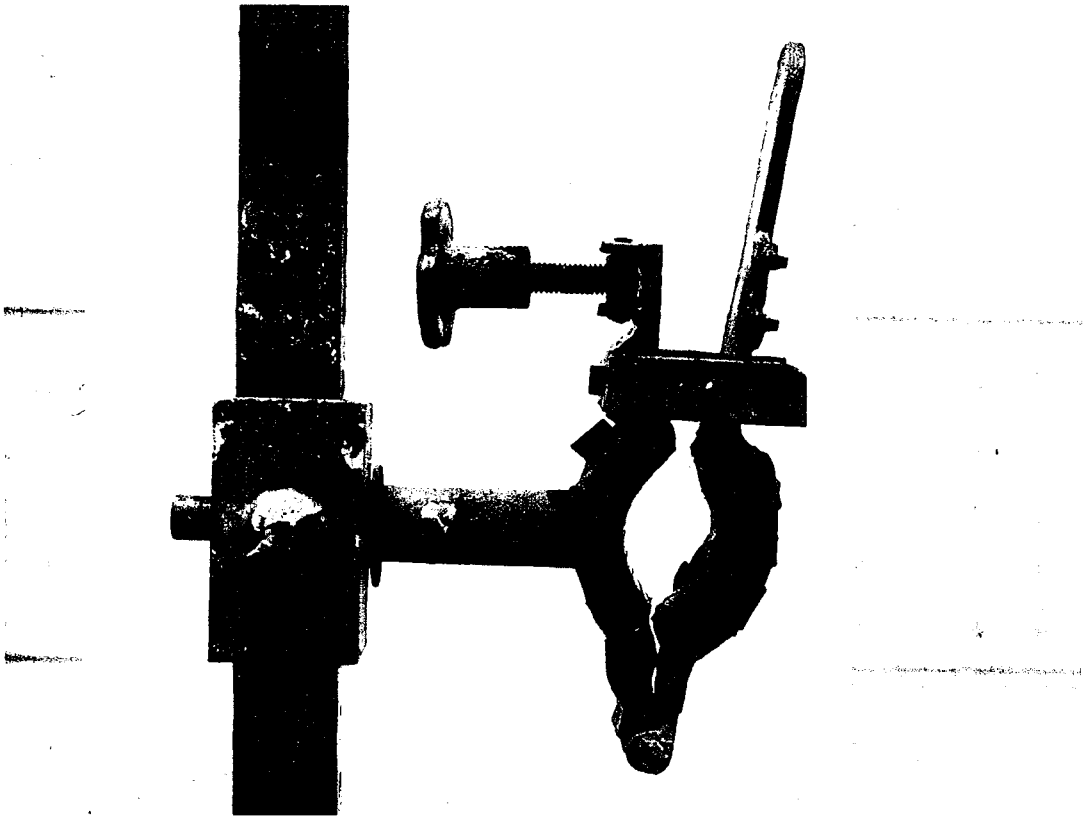
WHO 80785

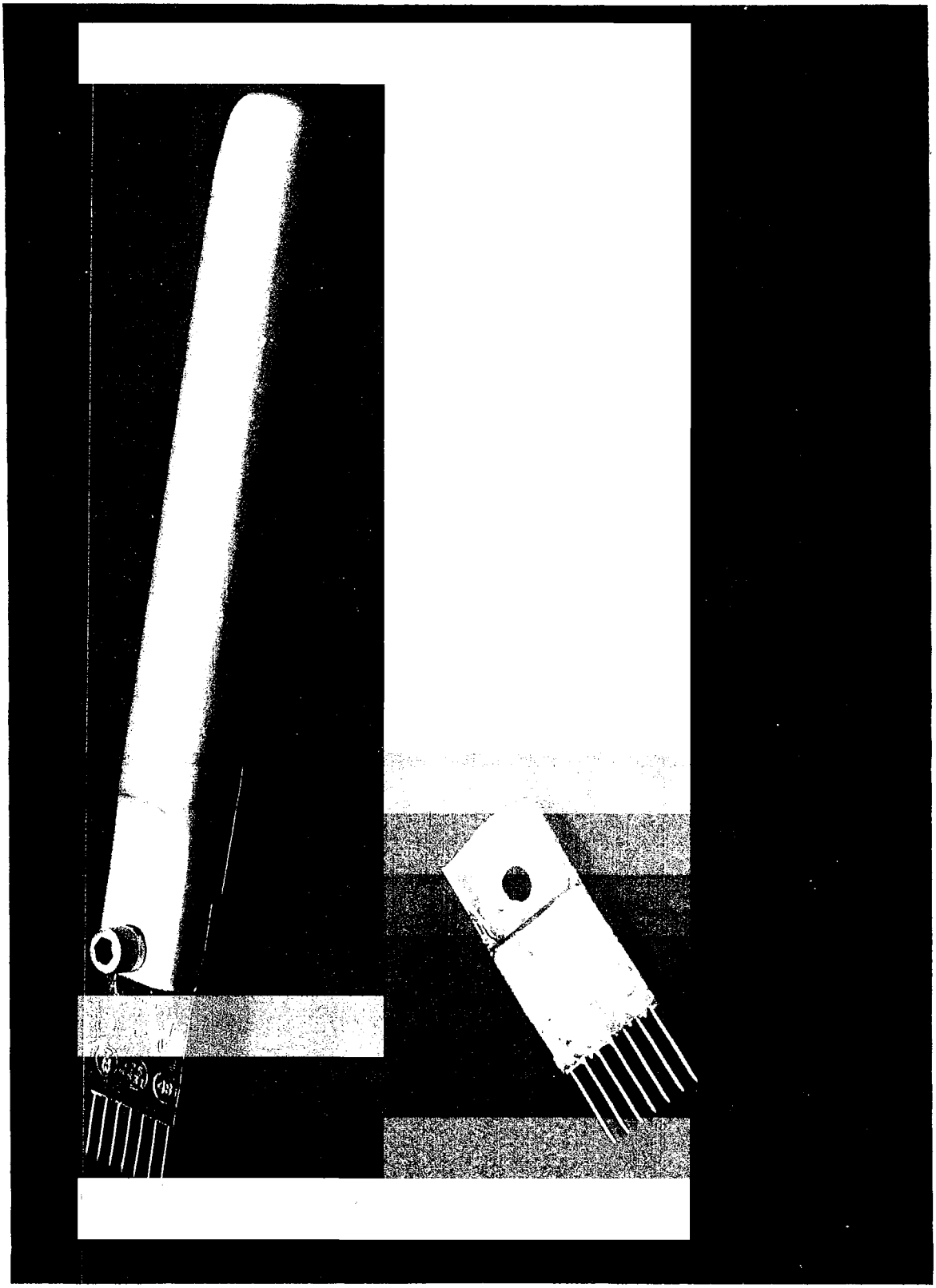




APPENDIX 5

Instrument for scarification of vaccinifier (Available from WHO on request)

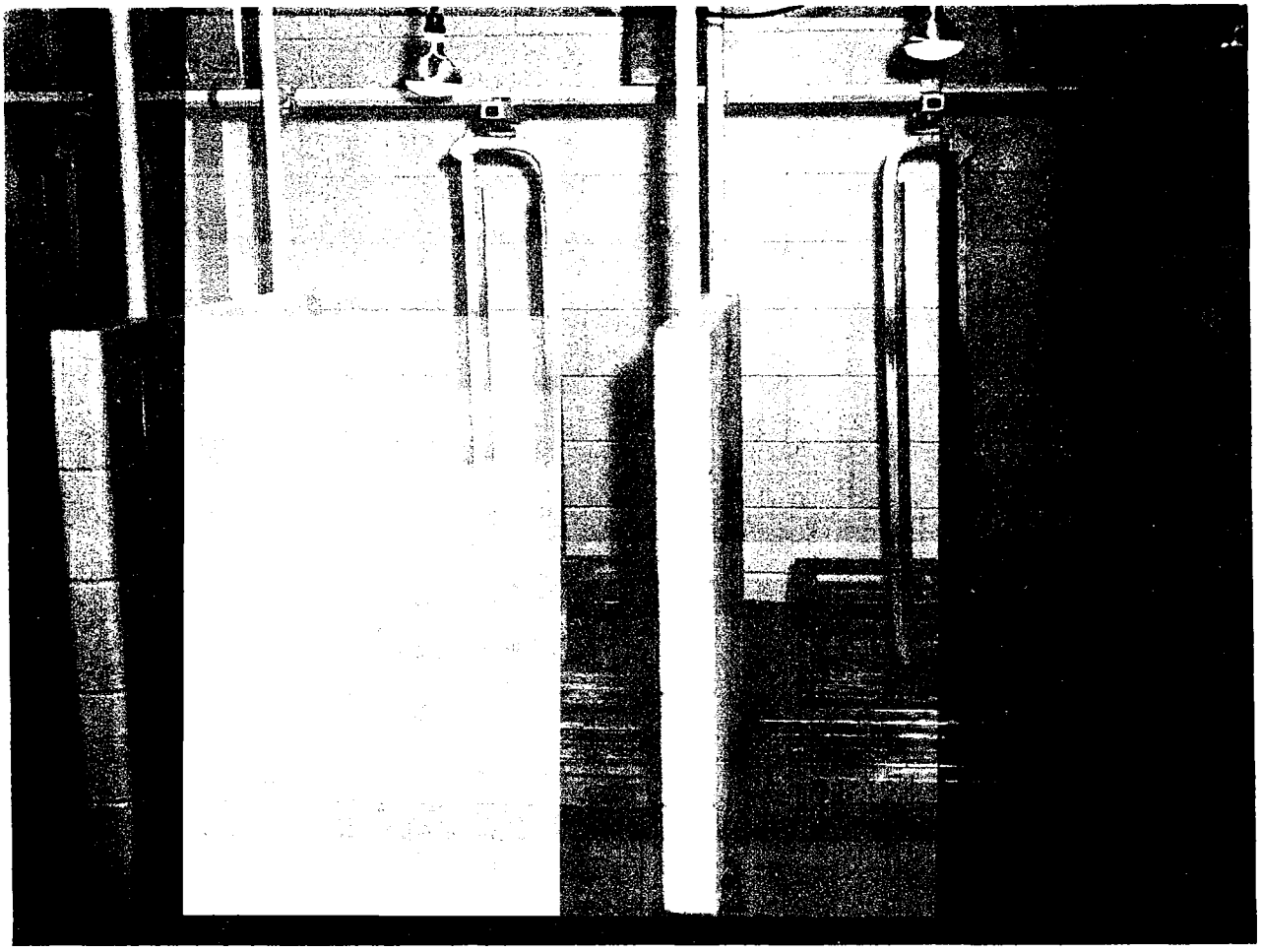
WHO 80782

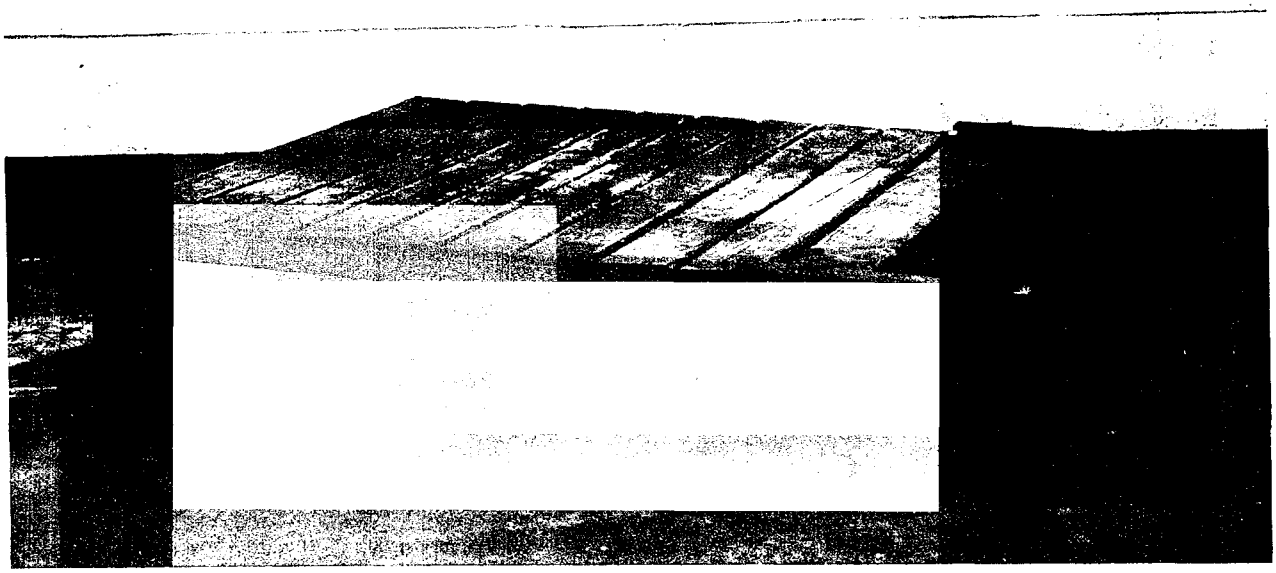


APPENDIX 6

Wooden platform for animals during incubation period

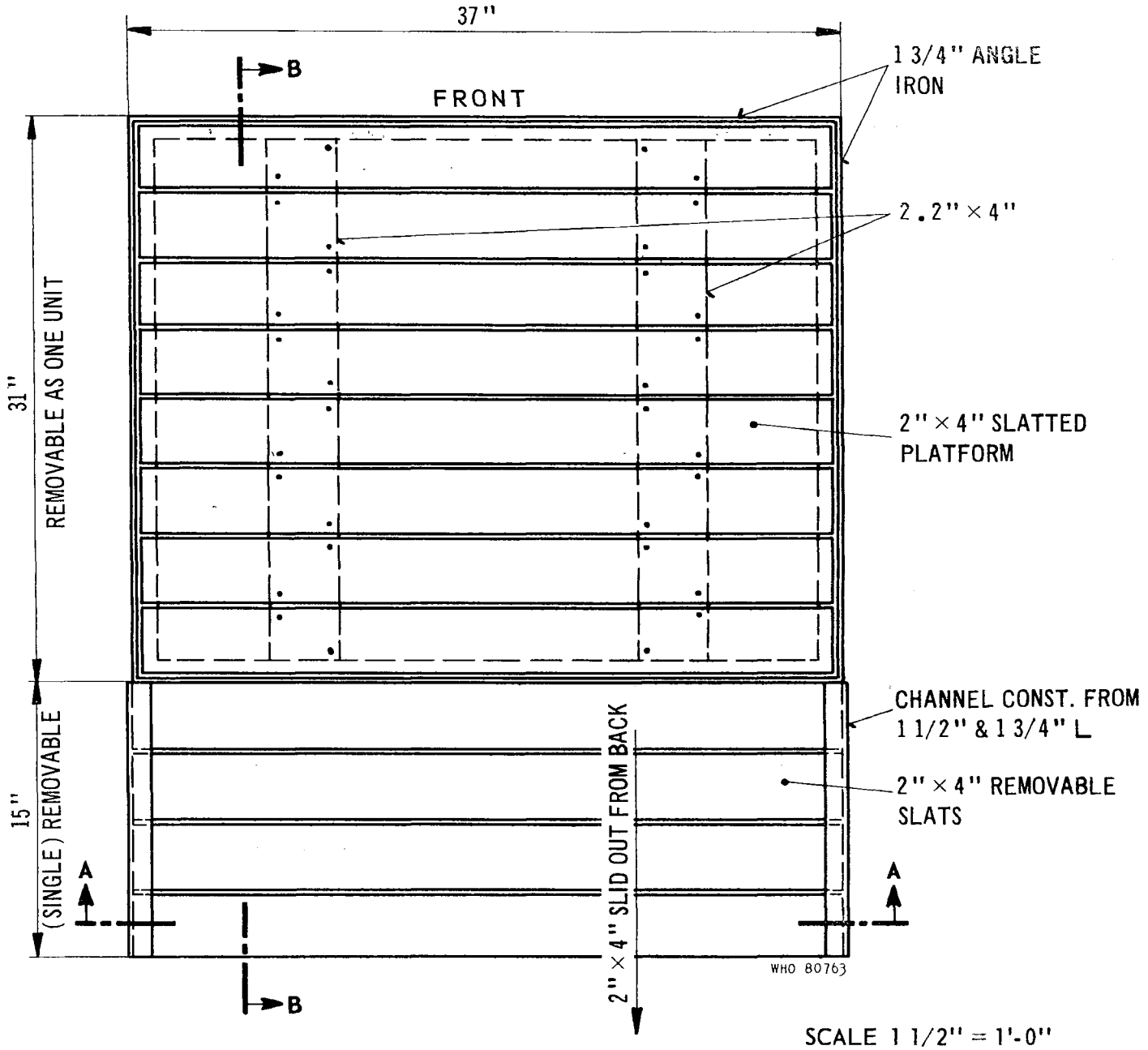
WHO 80783





Appendix 6

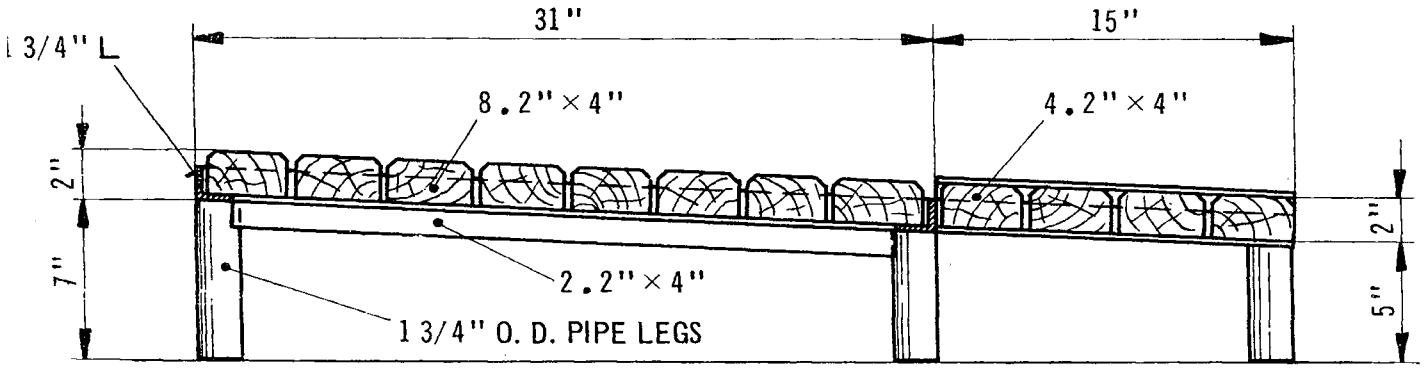
PLAN VIEW SLATTED PLATFORM



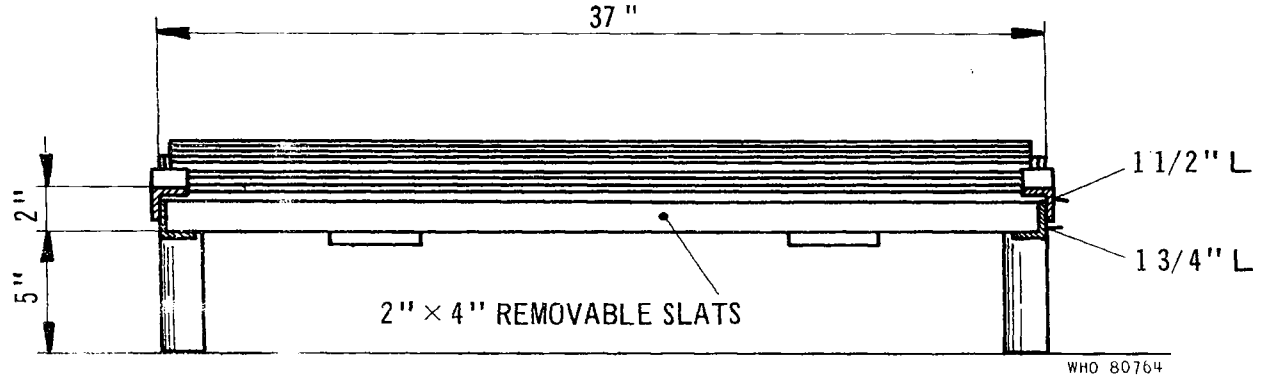
SCALE 1 1/2" = 1'-0"

Appendix 6

SECTION B-B



SECTION A-A



SCALE 1 1/2" = 1'-0"

APPENDIX 7

Preparation of .004 M McIlvaine buffer (pH 7.2-7.4)

1. Make up Stock solution A

   Dissolve 2.1 grams of analytical grade citric acid ($C_6H_8O_7.H_2O$) in 100 ml of distilled water.

2. Make up Stock solution B

   Dissolve 35.6 grams of analytical grade disodium phosphate ($Na_2HPO_4.2H_2O$) in 1000 ml of distilled water.

3. Mix 1 ml of Stock solution A with 9 ml of Stock solution B.

4. Add distilled water to mixture of solutions A and B to make 5000 ml.

5. Determine pH. Normally, this will be between pH 7.2 and 7.4 If it is above pH 7.4, a very small amount of Stock solution A will lower the pH to the proper range. If pH is below 7.2, discard.

Note: Stock solutions as well as buffer should be kept refrigerated. At refrigerator temperatures, the phosphate in solution B will crystallize out of solution. Before using, the solution must be warmed until crystals redissolve.

APPENDIX 8

Preparation of phenol for treatment of virus extracts

Analytical-reagent grade phenol must be used. A convenient amount to prepare at one time is 100 g. Crystals of phenol are weighed and heated in a borosilicate glass beaker to melting point (100.5°C). The molten phenol is then transferred, by careful pouring, into a hot borosilicate measuring cylinder. Enough hot (90°C) glass-distilled water is added to the molten phenol to ensure that it remains liquid in the form of a solution of water-in-phenol. Before addition to the molten phenol the water should be used to rinse the beaker. When the solution and its container have cooled to room temperature, cold glass-distilled water is added to dilute the solution so that approximately 1.0 gram phenol is contained in 1.5 ml. The solution is now transferred to a clean dry borosilicate reagent bottle with ground-glass stopper, and appropriately labelled.

For the treatment of for example 1600 ml of suspension with 0.5% (w/v) phenol, 8 g phenol will be needed. If the stock solution has been adjusted so that 1.5 ml contains 1.0 g of phenol, then 12.0 ml of stock solution must be measured out. The 1600 ml suspension should be added rapidly to the 12.0 ml water-in-phenol solution giving a solution of 0.5% (w/v) phenol in water. For the purpose for which it is being used the small inaccuracy introduced by increasing the final volume of phenolized suspension may be disregarded. During the treatment period the phenolized suspension should be mixed constantly.

APPENDIX 9

Preparation of peptone solution

To make a litre of 10% (w/v) solution of peptone for freeze-drying smallpox vaccine, add 100 g peptone to about 800 ml glass-distilled water and stir gently but steadily while warming to about 90 °C. When the peptone is dissolved the solution is acidified to pH 5.0 by addition of N-HCl. Under these conditions, phosphates will be precipitated. When no further precipitation occurs, the solution is filtered hot through Grade 1 Whatman filter paper. The pH value of the dephosphated solution is adjusted to 7.0-7.4 by addition of N-NaOH. When it has cooled to room temperature, the volume of the solution is adjusted to one litre by addition of glass-distilled water. It may now be distributed into individual bottles and sterilized at 121 °C for 15 minutes. Any peptone solution left in a bottle after it has been opened must be discarded and not kept for use on another occasion. The adjustments of pH are best made by adding the acid or alkali slowly to the constantly stirred solution while monitoring the pH value on a pH meter equipped with a glass electrode immersed in the solution.

APPENDIX 10

Final containers (ampoules, vials and vampoules)

Material

Containers for vaccine and reconstitution fluid should be made of a glass of the quality of neutral glass as defined in, for example BP, or according to USP XVII, Type 1 (United Kingdom or United States of America standards).

However, the basic test methods for classification do not consider the possibility of absorption of virus to the glass and appropriate tests should be performed to assure that this does not occur.

To enable ampoules to be sealed in automatic machines and to facilitate sealing of them by hand, the glass should, furthermore, have a long temperature interval between its softening point and its working point; in the case of ampoules manufactured on automatic machines, the manufacturer will have considered this, but when ampoules are produced locally by hand this point will need to be specially checked.

A. Ampoules (for nomenclature see Fig. 1)

1. Shapes

Ampoules are produced in a great variety of shapes. The types of freeze-dryer and the sealing technique to be used impose certain limits to the types acceptable in each particular case.

(a) For centrifugal type primary dryers, the basic model is cylindrical, tubular with round bottom (as B.S. 795 type L) (Fig. 2) from which is derived a similar type but with a flat bottom and, to facilitate sealing off the manifold of the secondary dryer, a constriction at the sealing point, (Fig. 3).

(b) Ampoules for use in shelf-dryers should have a flat bottom; if they are to be connected subsequently to a manifold (secondary dryer or vacuum line) a cylindrical stem (Fig. 4) would be most practical, otherwise the usual shapes of stems as shown in Figs. 5 and 6 are fully acceptable. These ampoules may be made with a constriction just above the shoulder to facilitate opening by the vaccinator.

2. Dimensions and tolerances

(a) For centrifugal type dryers, the maximum outside diameter and over-all length of the ampoules are determined by the size of holes drilled in the carrier plates and the number of layers in the centrifuge assembly respectively. The mouth diameter is determined by the connecting ports on the secondary dryer, which are usually conical rubber nipples requiring quite close tolerances ($\pm$ 0.2 mm) on the inside diameter of the mouth. Dimensions of ampoules as shown on Figs. 2 and 3 would fit Edwards centrifugal and secondary dryers, 3OP and 3OS.

(b) Shelf-type dryers do not impose any tolerances on diameters. For maximum capacity of the machine, the outside body diameter should be the smallest possible which permits a liquid height of 10 mm or less. If the height of the liquid vaccine fill is greater than this, drying will be retarded. The height of the body should be three to four times the liquid height to allow convenient space for reconstitution. This means that the capacity of an ampoule, when intended for freeze drying, is only one-quarter to one-third of its usually stated liquid capacity.

The over-all height should not be more than the free distance between two shelves in the dryer less 10 mm to (preferably) 20 mm to allow free escape of vapour. The minimum over-all height will depend on the method of sealing intended. For hand-sealing and for sealing on machines using the draw-off method, there must be about 25 mm to 30 mm between the mouth and the sealing point. Tip-sealing should not be used for smallpox vaccine. Special tolerances are required for ampoules to be sealed in automatic sealing machines and these will be different for different types of machine. The most important of these are the diameter and the wall thickness at the sealing point of the stem. Good quality ampoules will be delivered, graded according to gauged diameters at this point, see Fig. 1, point (e), and should not be mixed in the processing. If specifications are based as far as possible on one or another of the existing national standards for ampoules, such as British Standard (B.S.) 795 or German Standard DIN 58377 (Figs. 5 and 6), dimensions and tolerances are more certain to be those required since these are taken into consideration by manufacturers of ampoule-handling machines, at least in the respective countries. Use of standard ampoules will also result in lower prices and more rapid deliveries.

3. Closing

Ampoules are always closed by a fused glass seal. In the case of machines performing the primary and secondary dryings in separate units, this is the last step in the secondary process, carried out by sealing off the ampoules one by one from the manifold with a hand-held burner. The same method is also used for sealing ampoules which have been dried more or less completely in a shelf-dryer and subsequently attached to a manifold. This process requires some skill, particularly if the ampoules are sealed under vacuum rather than dry oxygen-free nitrogen. It is also time-consuming and not adaptable to mechanization as might be necessary in industrial scale production.

Standard ampoules which have been dried to the required final stage in a shelf-dryer may be sealed by usual methods, with certain precautions to prevent their taking up excess moisture from the air:

(a) They should be filled with dry nitrogen before the chamber is opened.

(b) Exposure of the ampoules to ambient atmosphere should be as brief as possible and the relative humidity of the ambient atmosphere should be controlled and brought down to the lowest practical values (30% relative humidity).

(c) While awaiting sealing by hand, the ampoules should be kept in vacuum desiccators and only a handful removed at a time for sealing within a few minutes.

In larger scale production the desiccators might be replaced by vacuum conservation cabinets divided in separate compartments, each compartment not larger than the number which can be sealed on a machine within half an hour.

B. Vials (Fig. 7)

1. Vials are used in shelf-dryers only. They are not produced in a great variety of shapes, but rather by two different techniques, i.e. blown in a mould (moulded vials) or formed from tubing (tubular vials). The latter type is preferred because the glass walls and particularly the bottom can be made thinner, or more uniform thickness and nearly flat, giving a better heat-transmission in freeze-drying. Tubular vials are also free of irregularities at the mouth, which might interfere with the closure.

Appendix 10

2. Dimensions and tolerances

As regards outside body diameters, the considerations are similar to those mentioned above for ampoules for use in shelf-dryers, with the additional point that the minimum outside body diameter must be slightly more than the diameter over the rim of the mouth and the brim of the rubber stoppers. For various technical reasons, a minimum outside body diameter of 13.5 to 14 mm is required. The over-all height is limited by the fact that the vial with its rubber stopper loosely inserted must pass freely between a shelf and its stoppering plate in raised position. The minimum height is governed by the necessity for the stoppering plate, when near its lowest position, to press the stoppers completely down to the brim.

It is important for proper closing, that all vials in a charge or at least on the same shelf, are of the same height; the maximum tolerable variation in height depends to some extent on the nature of the stoppering mechanism but should not be more than ± 0.75 mm preferably ± 0.50 mm or less.

Furthermore, to secure a reliable, tight seal of the vials, the inside diameter of the neck must be kept within close tolerances (± 0.2 mm) and for proper fitting of aluminium seals the outside diameter of the rim, the vertical height of this and the angle of its lower edge is important and the aluminium capping machine must be constructed to fit these dimensions.

3. Closing

In shelf-dryers which are equipped with stoppering plates, vials are closed permanently in the freeze-drying chamber itself by means of special slotted or fluted stoppers, which must be made of butyl rubber (the rate of diffusion of gases and water vapour through ordinary rubber is such that considerable deterioration of the contents occurs in a relatively short time). These stoppers (example shown in Fig. 8) must be made to very close tolerances, of a suitable hardness and with a smooth surface. These should be closely matched to the vials intended for use. They must be made so that they can easily be inserted in the vials in a half-closed position and remain in that position during the drying process without offering too much hindrance to the free escape of vapours from the content of the vials; on the other hand they should be able to slide completely down to the brim under the pressure of the stoppering plates. (A slight coating of the stoppers or the mouths of the vials with a silicone-emulsion might be a help.) Closure of the vials in the chamber may be done under vacuum (50 microns or less) or with an oxygen-free dry nitrogen gas fill. After the stoppered vials are removed from the dryer the closure must be secured by sealing collars (caps) of aluminium.

A disadvantage of the vials is that irrespective of how small the fill of vaccine the diameters of the vials cannot be less than about 14 mm in diameter. When small fills are processed, the output is low in proportion to the shelf area.

C. Vampoules

1. These are hybrids of vials and ampoules and are provided with cutting and sealing constrictions. They are so far commercially produced only in the shape shown in Fig. 9.

2. Dimensions and tolerances

Dimensions and tolerances for the vampoule as well as for the special slotted stoppers are as critical as indicated above for vials. However, the smaller over-all diameter allows a larger number to be dried in a batch than of vials.

Appendix 10

3. Closing

This is done in two steps. As a first step they are closed by stoppering plates in the drying chamber itself under oxygen-free dry nitrogen by the same techniques as used for ordinary vials. This closure is temporary but permits the containers to be kept at ambient room atmosphere for sufficient time to allow the final sealing by fusion of the glass at the constriction. Fusion of the glass is done by hand or machine sealing as with ampoules. As the closure with the stopper is temporary, ordinary rubber may be used. The stoppers normally can be reused several times.

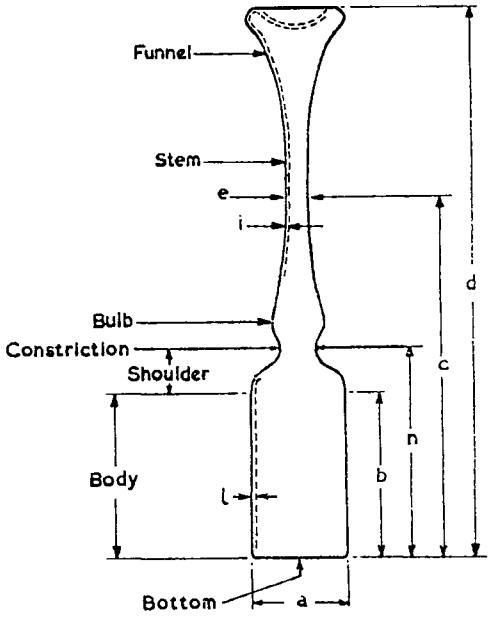


Fig. 1. Nomenclature of ampoules (according to British Standard 795, 1961)

a. Body diameter.

b. Body height (nominal capacity line).

c. Height to gauging point.

d. Over-all height (for open or closed stem ampoules).

e. Stem diameter at gauging point.

i. Glass thickness of stem at gauging point.

l. Glass thickness of body.

n. Height to constriction.

Appendix 10

SPECIAL FREEZE-DRYING AMPOULES

For use with centrifugal primary dryers and manifold secondary dryers (Edwards 30P2 and 30S1)

Internal diameter of open end carefully controlled and glazed to give minimum wear on the rubber bonded nipples of the Secondary Drier.

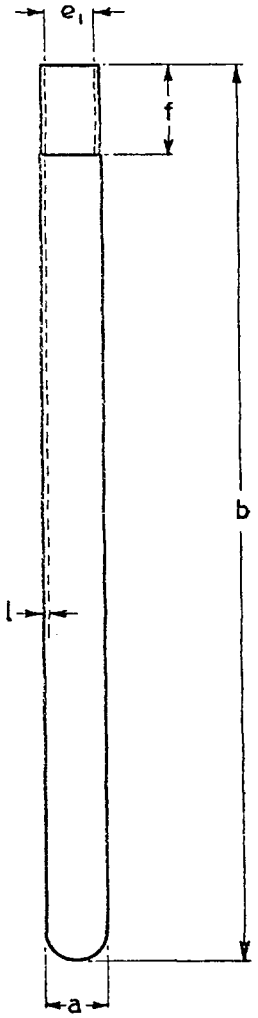


Fig. 2. British Standard 795 Type L (1961)

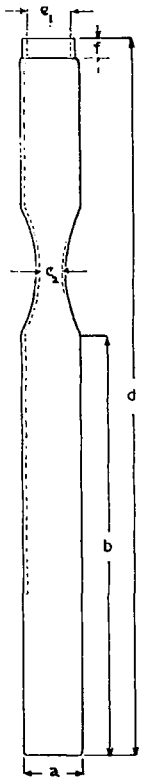


Fig. 3. Johnsen & Jorgensen Ltd., No. 19/H/1007

	Type L	19/H/1007
Diameter of body (a)	7.375 ± 0.375	8.0 - 8.5
Over-all length (d)	100.0 ± 2.0	109.0 - 111.0
Neck internal (e_1)	6.0 ± 0.1	5.8 - 6.2
Constriction internal (e_2)		1.55 - 2.5

Appendix 10

Flat bottom ampoule with wide, cylindrical stem and glazed mouth

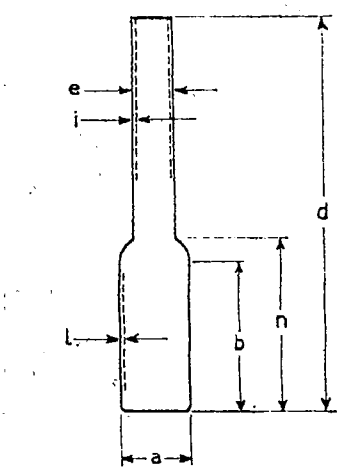


Fig. 4

For drying in shelf-dryer with subsequent secondary drying and/or sealing on manifold. Diameter of stem to fit on or in manifold connectors.

Appendix 10

CONVENTIONAL STANDARD AMPOULES WITH FLAT BOTTOMS, ADAPTABLE TO SHELF DRYING AND SUBSEQUENT SEALING BY HAND OR MACHINE

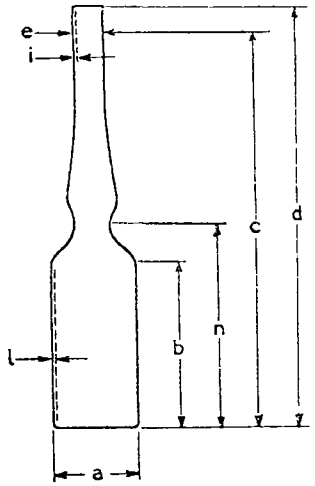


Fig. 5. British Standard 795 Type Q (1961)

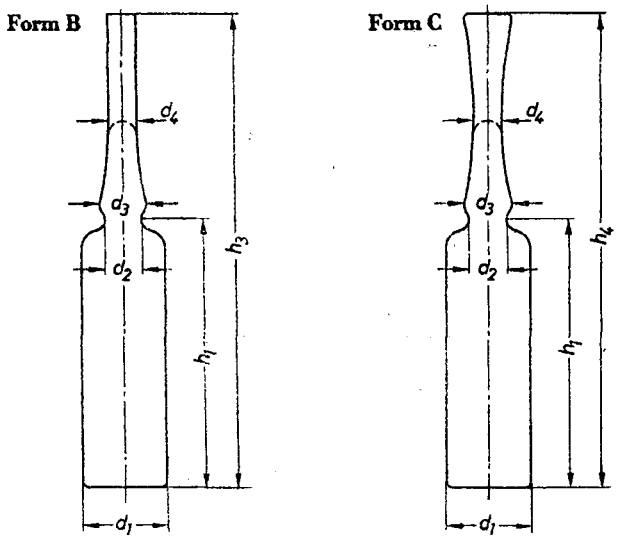


Fig. 6 DIN 58 377 (May 1967)

For drying of 0.15 to 0.25 ml vaccine in a Shelf-dryer, the following ampoules would be suitable:

	British Standard 795, Type Q	DIN 58 377, Type B & C
Nominal capacity in ml	1 ml	1 ml
Outside body diameter	10.0 ± 0.5 mm	10 - 10.5 mm
Body height (to cut-open constriction)	26.0 ± 1.0 mm	25 - 26 mm
Over-all height for draw-off sealing	55 ± 0.25 mm	60 ± 0.5 mm
Outside stem diameter	4.25 ± 0.5 mm	5 - 6 mm

Note: The above dimensions do not take into consideration possible special requirements of any type of ampoule sealing machine. This should be checked in each particular case.

Appendix 10

Tubular vials and special stoppers for use in shelf-dryers

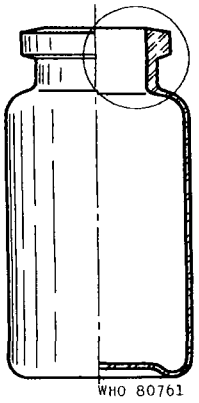


Fig. 7

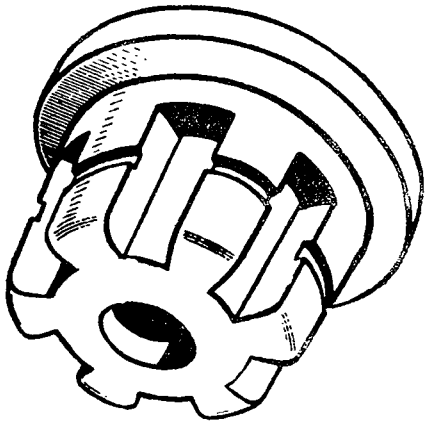


Fig. 8 (one type of rubber stopper)

Dimensions for use in shelf-dryer with stoppering plates:

Outside body diameter	13.5 - 14 mm
Over-all height	42 - 58 mm $\pm$ 0.2
(If vials less than 48 mm in height are desired, some shelf-driers may have to be modified)	
Inside neck diameter	7.0 $\pm$ 0.2 mm
Outside neck diameter over rim	13.0 $\pm$ 0.2 mm
Nominal liquid capacity	3.5 to 6 ml

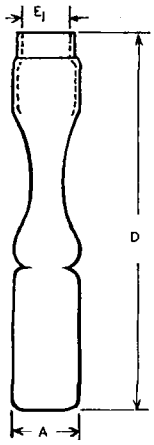


Fig. 9

Vampule

Body diameter	(A)	9.5 - 10.0 mm
Over-all length	(D)	60.0 - 61.0 mm
Controlled neck	(E_1)	7.5 - 7.9 mm

APPENDIX 11

Specification of nitrogen for filling of vaccine containers

Nitrogen more than 99.9% as dry gas.

Limits of Impurities - Volume per Million

Oxygen	less than 10
CO_2	less than 5
CO	nil
Other carbon compounds as CO_2	less than 5
Hydrogen	not more than 20 usually less than 1
Neon	" " " 600 " " " 1
Helium	" " " 160 " " " 1
Argon	approximately 5
Water vapour	not more than 0.01 g/m^3 at 21 kg/cm^2

COMPARISON OF CAPACITIES OF SELECTED FREEZE-DRYERS IN COMMON USE

Freeze-Dryers Make and Model	Capacity per charge — Number of containers(1)			No. of containers processed each week if three charges			No. of containers processed each year if 120 charges/yr. (000's)		
	Vials	Ampoules	Vampules	in Vials	in Ampoules	in Vampules	in Vials	in Ampoules	in Vampules
A) Shelf types	14mm OD	12mm OD	10mm OD						
Edwards 30P2T(S)	2 200	3 000(2)	4 400	6 600	9 000	13 200	264	360	528
" LH0/5 shelves(3)	4 400	6 000	8 800	13 200	18 000	26 400	528	720	1 056
Stokes 12PV	5 750	7 800	11 200	16 250	23 400	33 600	650	936	1 344
Usifroid SM.J	1 250	1 600	2 300	3 750	4 800	6 900	150	192	276
" SM/8 shelves(4)	10 800	14 000	19 200	32 400	42 000	57 600	1 296	1 680	2 304
B) Centrifugal type		8mm OD							
Edwards 30 P2 +		1 140)			3 450			138	
" 30 S2		1 184)							

Notes : (1) The outside diameter (OD) of the various containers is given. Such containers readily accept a 0.25 ml liquid fill of vaccine providing at least 25 doses by conventional vaccination techniques, many more if the bifurcated needle is employed.

(2) Maximum height permissible would be 65-70 mm. when stoppering plates are removed.

(3) Maximum height permissible would be 60 mm. otherwise the number of shelves must be reduced to 4, reducing capacity by 20%.

(4) For use with vials, the machine could take 12 shelves, increasing the capacity by 50%; for vampules the machine could take 10 shelves, increasing the capacity by 25%.

There is no intent to imply that this is a complete list of makes and models available, nor does the use of names of makers and model designations constitute any endorsement or recommendation. The sole purpose of the table is to give some orientation as to outputs to be reasonably expected per week and year for various types of equipment in common use.

APPENDIX 13

NUMBER OF SAMPLES REQUIRED FOR THE TESTING OF FREEZE-DRIED SMALLPOX VACCINE IN A WHO REFERENCE LABORATORY

Test	Required number of vials or ampoules with	
	0.25 ml	0.15 ml
Initial potency	4	10
1 hour 100°C	4	10
Potency and stability	8	20
Bacteriological examination	8	40
Subtotal	24	80
Phenol content	16	40
Moisture content	4	7
Total	44	127

In the Americas, specimens are submitted to Connaught Laboratories, University of Toronto, Canada, after arrangements have been made through the WHO Regional Office for the Americas.

Specimens from other areas should be submitted to: Chief, Smallpox Eradication
World Health Organization
Avenue Appia
Geneva, Switzerland

APPENDIX 14

Vaccine potency testing by the rabbit scarification method

(a) Reference smallpox vaccine. The reference vaccine is prepared from the local vaccine strain and standardized against the International Reference Preparation or by pock count.

(b) Dilution of vaccines for test. Dilutions of vaccine are made using the same diluent for both the reconstituted reference and test vaccines. Starting with not less than 0.5 ml of each vaccine, dilutions including 1:3000, 1:9000, and 1:27 000 are made.

(c) Preparation of the test animals. Rabbits having skin free of blemishes are used. The hair is removed from the areas to be scarified by a method which leaves the skin free of hair and abrasions. No virucidal or virustatic chemical should be left on the skin area to be inoculated. Test sites measuring 2.5 x 5.0 cm are marked off on the denuded skin of each rabbit with care being taken not to stretch the skin. All test sites are uniformly scarified.

(d) Inoculation of the test animals. Dilutions of the reference vaccine are placed on each rabbit along with those of the test vaccine(s). Immediately following thorough mixing, 0.2 ml of each vaccine dilution is applied and rubbed into the appropriate scarified test area. Upon completion of all inoculations on an animal, the sites are air dried (using cool air only) before the animal is returned to its cage.

(e) The test vaccine and the reference vaccine are compared on the fourth or fifth day. The test vaccine passes if its potency is equal to or greater than the reference vaccine as determined by the extent of reactions on the rabbit's skin.

Note: This method is not recommended except in unusual circumstances when potency testing on CAM cannot be performed.

APPENDIX 15

MOISTURE CONTENT DETERMINATION

A. Gravimetric determination of residual moisture

The apparatus needed for this determination is a single pan substitution balance (sensitivity of 0.01 mg) with an arrangement for suspending a load below the balance on a stainless-steel wire passing through a hole in the floor of the balance cabinet (Fig. 1). Although a simple matter, this modification should be made by the manufacturer of the balance or his accredited agent. A balance such as the Mettler type H2O TDG, equipped with a taring device and a removable attachment for weighing below the floor of the cabinet, is to be preferred for convenience and for the precision it permits. Before the balance pan is attached, the wire is passed through the thermometer hole in the top of an oven. The oven must be equipped with a thermostat able to regulate the temperature ± 2°C at 100°C, and should also have a thermometer on the door with its bulb in the cavity of the oven. A sheet of expanded polystyrene 5 cm thick placed between oven and bench or table will effectively prevent the bench and balance being heated.

To determine the moisture in a sample of freeze-dried vaccine, enough containers should be taken to give a total sample weight of at least 50 mg, e.g. a fill volume of 0.25 ml of vaccine in 5% peptone calls for four containers, each of which should contain 12.5 mg of peptone. After the removal of labels the exterior of each container is cleaned with a solvent such as acetone to remove grease etc. deposited by handling. If necessary the necks of ampoules or vampoules should be scored with a file to enable them to be opened later. Containers must be handled only with forceps after they have been cleaned with solvent.

Four containers are placed in a crucible or other suitable vessel and weighed (A). The containers are opened with the aid of forceps and returned to the weighing vessel. The oven is now switched on and brought to 100°C. The containers are reweighed at intervals until the weight is constant (B), when they are taken from the oven. The vaccine is reconstituted and discarded and the containers are washed thoroughly in deionized or glass-distilled water. (Note: any spicules of glass which have resulted from opening of the ampoule must be carefully retained and included in the subsequent weighing.) The washed containers are returned to the oven and dried to constant weight (C).

The percentage of residual moisture is calculated as follows:

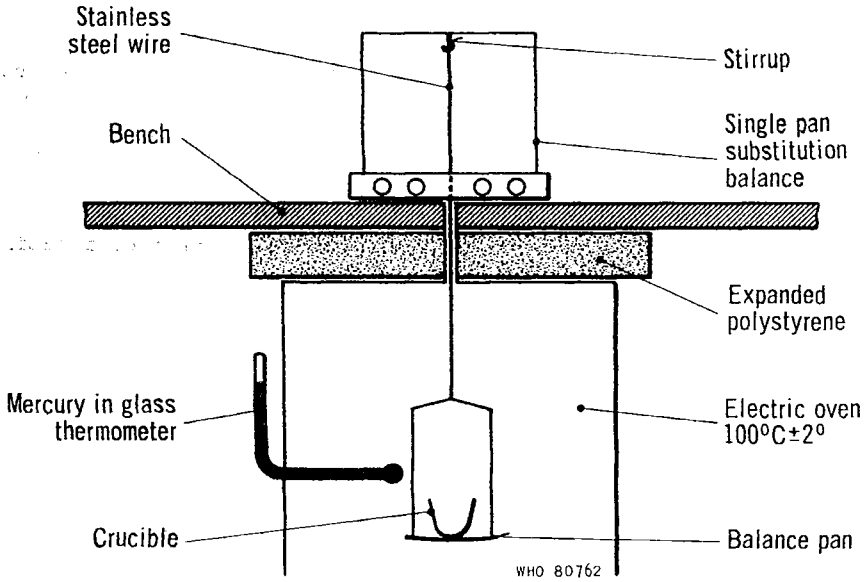
$A - C$ = weight of vaccine in containers

$A - B$ = weight of moisture driven from vaccine

$\frac{A - B}{A - C} \times 100$ = percentage of moisture in vaccine

Strictly speaking, this method measures total volatile substances at 100°C, but for practical purposes these may be equated with water.

Appendix 15



B. Method employing P_2O_5

Material required 12 0.25 ml containers or 20 0.15 ml containers

Apparatus
- Moisture controlled cabinet with sealed arm slots (copy of plans available from WHO on request)
- Vacuum desiccators
- Desiccators (with P_2O_5 as drying agent)
- Analytical balance (to the nearest 0.01 mg)

Procedure

Wipe the vials clean and place them in a desiccator which, in turn, is put inside a desiccator charged with fresh P_2O_5. The sample vials are kept in the desiccator under slight vacuum for 24 hours.

On the second day, charge the moisture controlled cabinet with P_2O_5 by placing several Petri dishes of P_2O_5 inside the cabinet. Transfer the desiccator with the sample vials into the moisture controlled cabinet. It is left inside the cabinet under positive pressure of a mixture of gas (5% CO_2, 21% O_2, 74% N_2) for at least half an hour before it is opened.

After the half-hour period, the desiccator is opened and the cap and rubber stoppers of the sample vials are removed and the vials are weighed immediately on the analytical balance. (This is the wet weight of the vials.) All handling of sample vials is done with forceps.

After weighing, all open vials are returned to the desiccator and are then removed from the moisture controlled cabinet and stored at room temperature at 20-30°C for six days under vacuum (1 mm of Hg).

Appendix 15

After six days of storage (i.e. seven days from the beginning), the moisture controlled cabinet is recharged with P_2O_5. The desiccator with the sample vials is transferred back into the moisture controlled cabinet and, using all previous precautions, the sample vials are reweighed. (This is the dry weight of the vials.)

When weighing is completed, the sample vials are removed from the moisture controlled cabinet in the same manner as before. The content of each vial is emptied and the vials are cleaned with water, followed by rinsing with distilled water and finally with alcohol. The empty vials are then dried in an oven at 100°C for an hour and then placed back in the desiccator for 24 hours under slight vacuum.

The desiccator is then transferred back into the moisture controlled cabinet and empty vials are reweighed. (This is the tare weight of the vials.)

Calculation

Weight of sample before drying = wet weight - tare weight

Weight lost in drying = wet weight - dry weight

$$\% \text{ moisture in sample} = \frac{\text{weight lost in drying}}{\text{weight of sample}} \times 100$$

APPENDIX 16

Preparation of reconstituting fluid: 25% (v/v) glycerol in 0.02 M McIlvaine's buffer

1. Make up stock solution A[1] (as in Appendix 7)

   Dissolve 2.1 g of analytical grade citric acid ($C_6H_8O_7.H_2O$) in 100 ml of distilled water.

2. Make up stock solution B[1] (as in Appendix 7)

   Dissolve 35.6 g of analytical grade disodium phosphate ($Na_2HPO_4.2H_2O$) in 1000 ml of distilled water.

3. Mix 10 ml of stock solution A with 90 ml of stock solution B.

4. Add distilled water to make up to 1000 ml.

5. Determine pH. Normally, this will be between pH 7.2 and 7.4. If it is above pH 7.4, a very small amount of stock solution A will lower the pH to the proper range. If pH is below 7.2, discard.

6. Add 25 parts by volume of glycerol (B.P. quality) to 75 parts of diluted buffer solution and mix well.

7. The reconstituting fluid can be sterilized by autoclaving the filled and sealed ampoules at 121 °C for 20 minutes.

---

[1] The stock solutions should be kept refrigerated. At refrigerator temperatures, the phosphate in stock solution B will crystallize out of solution. Before using the solution must be warmed until crystals redissolve.

APPENDIX 17

Instructions for use of freeze-dried smallpox vaccine (from the WHO Manual on Smallpox Eradication)

Freeze-dried vaccine, intended for multiple puncture or scratch vaccination, is prepared with live vaccinia virus purified and then freeze-dried. One ampoule (or vial) contains vaccine, another one the diluent. The number of doses of vaccine in the container depends on the technique. Using the multiple puncture method, one dose requires .002 ml; other methods require .010 ml.

Reconstitution of vaccine

Clean the necks of both the ampoule containing the vaccine and the ampoule containing the diluent and allow to dry. Cut the ampoule with the file provided in the package. Transfer all of the diluent into the ampoule containing the freeze-dried vaccine. A sterile needle and syringe or sterile pipette may be required. The vaccine is easily reconstituted by gentle shaking.

The reconstituted vaccine must be used during the same working day. Unused vaccine should be destroyed.

Skin preparation

No special preparation of the skin is needed. If the site is obviously dirty, a cloth moistened with water may be used to wipe the site.

Vaccination technique

Vaccinate on the outside of the left (or right) upper arm about the insertion of the deltoid muscle. Vaccination must be performed with sterile needle, vaccinostyle, bifurcated or surgical needle, or other suitable instrument.

Multipuncture technique (bifurcated needle)

A bifurcated needle is inserted into the container of reconstituted vaccine. On withdrawal, a droplet of vaccine, sufficient for vaccination, is contained within the fork of the needle. The needle is held at a 90° angle (perpendicular) to the skin. The wrist of the vaccinator rests against the arm. The points are touched lightly to the skin surface permitting the droplet of vaccine to be deposited on the skin. For primary and revaccination, 15 up and down (perpendicular) strokes of the needle are rapidly made in an area of about 5 mm in diameter. The strokes must be sufficiently vigorous so that a trace of blood appears at the vaccination site. Sterilization of needle may be done by: (a) flaming - the needle is passed through the flame of a spirit lamp for about three seconds and allowed to cool completely before use; (b) boiling - the needle is sterilized by boiling for 20 minutes. Subsequently, it must be dried thoroughly to ensure that the fork of the needle does not contain a drop of water when inserted into the vaccine ampoule.

Scratch technique

A small drop of vaccine is placed on the skin and a linear scratch about one-quarter of an inch (6 mm) is made through the vaccine drop. A small trace of blood should be observed at the vaccination site. The vaccine is then rubbed into the scratch with the side of the needle.

No dressing should be used after vaccination

Appendix 17

Contra-indications

In endemic smallpox regions or in areas geographically proximate only individuals who are obviously severely ill are not vaccinated.

Storage of vaccine and labelling

Before reconstitution, vaccine should be kept in a place at a temperature of less than 10°C (refrigerator). If stored below +10°C, it can be expected to maintain full potency for three years. If stored at +37°C, the vaccine is valid for one month only.

Unused reconstituted vaccine should be discarded at the end of a working day.

Ampoules or vials which are cracked or damaged should be destroyed.

APPENDIX 18

Laboratory space needed for annual production of 500 000 containers of freeze-dried smallpox vaccine of 0.25 ml per container

I Rooms

A. Quarantine facilities in building separate from that used for production.

1. Stalls to accommodate 12 animals
2. Room for washing and clipping animals during quarantine period

B. Main production facilities

1. Room for washing and shaving animals immediately before scarification
2. Operating room for making scarifications and harvesting the pulp. (Air conditioned for efficiency of personnel)
3. Incubation room adequate to accommodate six animals. This room should be constructed to prevent extremes of temperature
4. Room for storage of animal feed
5. Room for equipment storage
6. Room for processing of pulp and centrifugation
7. Room for filling (air conditioned for protection of the product)
8. Room for freeze-drying equipment
9. Room for cleaning the glassware
10. Room for drying, packing and sterilization of the glassware
11. Room for labelling, packing and shipping the final vaccine containers
12. Coldroom (+4 °C) for storage of the final vaccine containers prior to completion of stability tests
13. Room for potency and stability testing and for residual moisture content
14. Room for bacteriological testing
15. Room for smaller animals (mice, guinea-pigs, rabbits, etc.)
16. Room for records and personnel

II Equipment

A. Major equipment required (minimum)

1. Table for holding animal during scarification and harvesting
2. Balance for weighing pulp
3. Deep freezers (-20 °C)
   (a) Storage of pulp (two)
   (b) Storage of seed (one)
   (c) Storage of samples (one)
4. Homogenizer of type of Silverson laboratory mixer or Eppenback colloid mill
5. Centrifuge (non-refrigerated) with capacity of about two litres - for low speed centrifugation of pulp suspension

6. Incubators (preferably water jacketed)

   (a) Egg incubator - preferably with device to turn eggs automatically
   (b) 35°-37°C incubator - for incubation of inoculated eggs and for stability testing of vaccine
   (c) 30°-32°C incubator - for bacteriological studies
   (d) 25° incubator - for phenolization of vaccine suspension

7. Waterbath - for holding melted agar at 43°-45°C and for one hour 65°C incubation of cultures for anaerobic tests

8. Machine for filling of containers prior to freeze-drying

9. Machines for freeze-drying

10. Machines for sealing of vaccine containers

11. Standby generator

12. Cages for small animals (guinea-pigs, mice, rabbits)

13. pH meter

B. Other equipment (partial list)

1. Cloths, gloves and boots for the workers and cloths for the animals

2. Material for washing and cleaning the animals and the floor

3. Clipping and shaving material

4. Scarification instruments and spatulas

5. Equipment for killing the animal (needles, knives, tubes, syringes, anaesthetics)

6. Curettes or spoons for harvesting pulp

7. Glass jars for storage of pulp

8. Laboratory glassware

9. Glass containers, freon, homogenizers

10. Glass containers for vaccine and reconstitution fluid

11. Autoclave, drying oven, other equipment for a laboratory kitchen plus packing room

12. Labelling and packing equipment

13. Facilities for bacteriological control, among others tubes, plates, broths, cotton, swabs, pipets

14. Equipment for making holes in the eggs and sealing the eggs, egg candler equipment for opening the eggs and counting

15. Equipment for determination of moisture content

16. Media, chemicals and packaging materials

APPENDIX 19

WHO TRAVELLING SEMINAR ON SMALLPOX VACCINE PRODUCTION

List of Participants

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Appendix 19

List of Working Papers

		Presented by
WP/1	Methodology of Freeze-dried Smallpox Vaccine Production	Dr C. Kaplan & Dr A. C. Hekker
WP/2	The International Reference Preparation of Smallpox Vaccine	-
WP/3	Stability and Preservability of Freeze-dried Smallpox Vaccine with Different Moisture Contents	Dr M. Nomura et al.
WP/4	Relation of the PFU Value to the Volume of Inoculum in the Titration of Vaccinia Virus on the Chorio-Allantoic Membrane of the Chick Embryo	Dr D. Slonim
WP/5	Recommendations Relating to the Manufacture of Smallpox Vaccine	-
WP/6	Status of Freeze-dried Vaccine Production	Secretariat
WP/7	Comparison of Capacities of Various Freeze-Dryers	Secretariat
WP/8	Effect of 1% Phenol on Several Bacterial and Viral Species	Dr D. Slonim
WP/9	Attempt to utilize 1% Phenol for Bacterial Sterilization of Calf Lymph Smallpox Vaccine	Dr D. Slonim
WP/10	Phenolization of Smallpox Vaccine	Dr A. C. Hekker
WP/11	An Alternate Heat Stability Test	Dr A. C. Hekker
WP/12	Vaccine Production Data - Rijks Instituut	Dr A. C. Hekker
WP/13	Stability of Reconstituted Vaccine	Dr A. C. Hekker
WP/14	A Note on the Preservation of the Infectious Activity of Vaccinia Virus by Means of Glycerine	Dr D. Slonim
WP/15	Some Factors Influencing Quality and Quantity of Infectious Pulp Yields in Calf Skin Inoculated with Vaccinia Virus	Dr D. Slonim
WP/16	Vaccinia Virus Stability during Storage in Fluid Suspension at +2°C to +4°C, during Lyophilization Procedure and during Storage in Lyophilized Form at +37°C	Dr D. Slonim
WP/17	The Influence of the Atmosphere of Sealing on the Stability of Freeze-dried Smallpox Vaccine	Dr C. Kaplan
WP/18	Virus Strains for Smallpox Vaccine Production	Dr S. S. Marennikova

Appendix 19

		Presented by
WP/19	To be replaced by WP/25	
WP/20	Production Method for Freeze-dried Smallpox Vaccine	Dr P. Fenje
WP/21	Stability Study on Freeze-dried Vaccine Exposed to Room Atmosphere before Sealing	Dr A. C. Hekker Dr R. Schneider
WP/22	Results of Heat Resistance Testing at 37°C and 45°C of RIV Vaccine 6664 Reconstituted with Diluents of Different Glycerine Contents	Dr A. C. Hekker
WP/23	Stability of Smallpox Vaccine Freeze-dried with or without Stabilizers and Sealed under Different Conditions	Dr A. C. Hekker
WP/24	Information Regarding Vaccine Production - Connaught Medical Research Laboratory	Dr R. J. Wilson
WP/25	Dryvax® - Production and Control Testing Experience	Dr Alan Bernstein
WP/26	Four Extracts from Papers Dealing with Miscellaneous Aspects of Freeze-dried Smallpox Vaccine Production	-
WP/27	Production of Smallpox Vaccine on Calf Skin with Different Strains of Vaccinia Virus	Dr A. C. Hekker
WP/28	Vaccine Production Data - Research Institute of Virus Preparations, USSR	Drs V. N. Milushin & S. S. Marennikova
WP/29	Stability of Dried Smallpox Vaccine at Various Temperatures	Dr P. Fenje
WP/30	Smallpox Vaccine Production in a Limited Number of Passages in One Animal Species	Drs S. S. Marennikova, V. N. Milushin & K. L. Chimishkyan
WP/31	Notes on Smallpox Vaccine Production Methods in South American Laboratories	Dr P. Fenje