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PRODUCTION OF FORMALINIZED POLIOMYELITIS VACCINE (SALK-TYPE) ON A SEMI-INDUSTRIAL SCALE

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SYNOPSIS

This article describes a semi-industrial method for the production of formalinized poliomyelitis vaccine developed during 1956. The general technique followed that originally devised by Salk with modifications developed locally. The vaccine was tested for safety and innocuity according to the Minimum Requirements laid down by the United States Public Health Service. Seitz filtration was found to be essential for the production of a non-infectious vaccine; filtration through glass filters proved to give inconsistent results. High initial virus titres, proper filtration methods, and the use of a sensitive tissue-culture system for safety testing were considered to be of prime importance for the production of a safe and effective vaccine. Over 60 000 infants and young children were inoculated with this vaccine in a country-wide immunization programme during the winter and spring of 1957. No untoward effects were noted; an immunological evaluation of its performance in those inoculated is in progress.

After the favourable report on the use of formalinized poliomyelitis vaccine in the USA,[3] Israel health authorities were anxious to introduce immunization against poliomyelitis, in view of the high incidence of the paralytic disease in this country.[20] Since no vaccine could be obtained from abroad, it was decided to initiate local production of vaccine on a semi-industrial scale. Work was begun in the winter of 1955-56 with the aim of developing the process and preparing vaccine so that those age-groups most at risk—that is, between 6 and 36 months of age [20]—might be immunized in the winter and spring of 1957.

The development of our method of polio vaccine production was based mainly on the reports of Salk,[10, 11, 13, 14] and on the experience of the Danish workers in this field.[6] In the tests for safety and innocuity we have followed, in general, the Minimum Requirements of the United States Public Health Service and their latest amendments.[18] During this period—

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1955-1956—as a result of the episode in the USA in the spring of 1955 [15] changes in both the production and testing of the vaccine were being constantly introduced and we were compelled frequently to amend our concepts and working methods in order to obtain a non-infectious product.

This report is a summary of our experience and represents a complete description of the method developed for the production and testing of formalinized vaccine; to our knowledge, no such detailed description has yet been published. The major part of the article deals with methods developed for production on a semi-industrial scale, while a brief section covers experimental results and outlines several aspects of importance in the production of polio vaccine.

We believe that the details contained in this report may be of value to workers in other small countries interested in producing a non-infectious and potent poliomyelitis vaccine for local use.

Methods of Production and Testing

Preparation of tissue cultures and virus suspensions

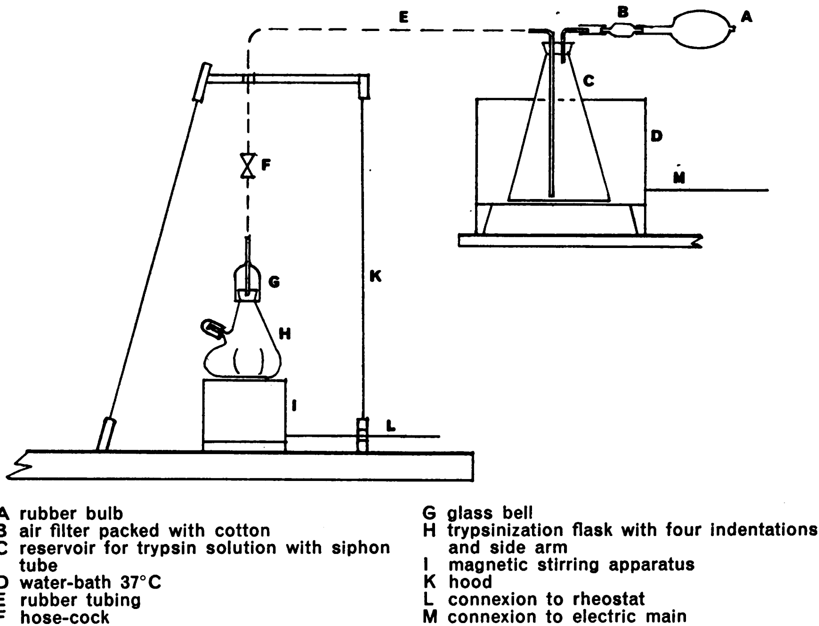
Monkey kidneys were used exclusively in the production of tissue cultures for the preparation of virus suspensions. The monkeys were *Macaca mulatta* imported from India, and weighed on the average 2-4 kg. On arrival, all the monkeys were tuberculin-tested, and 2-14 days before nephrectomy, they were retested with tuberculin by the intrapalpebral method. Only monkeys showing a negative tuberculin test were used, which meant that 2%-3% of the animals had to be discarded.

Usually four monkeys were nephrectomized at a time. They were anaesthetized with 100-150 mg each of sodium thiopentone by the intravenous route, and placed in the prone position for the hair on the back to be shaved. The operation was carried out with all aseptic precautions, as practised in an operating theatre; care was taken to remove the kidneys enclosed in the intact capsule. The nephrectomized monkeys were autopsied and examined for gross pathological lesions, with special emphasis on the lungs.

The kidneys were weighed and decapsulated, and the pelvis was removed. Inside a 50-ml thick-wall centrifuge tube, they were cut into small 3- to 5-mm pieces, which were washed in phosphate-buffered saline (PBS) and placed in a trypsinization flask. The minced tissue in the flask was covered with a 0.25% trypsin solution in PBS and incubated at 37°C for 30 minutes. The pH of the trypsin solution was 7.2.

The trypsinization was carried out in a 500-ml flask with indentations, described by Rappaport [8] for trypsinization by the manual method. A sterile magnetic bar was introduced into the flask, and the flask was placed on a magnetic stirrer under a hood. Trypsin solution was intermittently

FIG. 1. TRYPSINIZATION ASSEMBLY



A rubber bulb
B air filter packed with cotton
C reservoir for trypsin solution with siphon tube
D water-bath 37°C
E rubber tubing
F hose-cock
G glass bell
H trypsinization flask with four indentations and side arm
I magnetic stirring apparatus
K hood
L connexion to rheostat
M connexion to electric main

fed from a 2-litre Erlenmeyer flask kept in a water-bath at 37°C and connected by means of rubber tubing with the trypsinization flask (see Fig. 1). The magnet was allowed to rotate for seven-minute periods at maximum speed, care being taken to avoid foaming. For each run, 75-100 ml of trypsin solution were added. At the end of each run, stirring was interrupted and the supernatant was decanted into a flask kept in an ice-bath. After several runs, when the tissue had become partially exhausted, it was transferred into a 250-ml trypsinization flask and the treatment was continued. After 10-12 runs, most of the tissue became exhausted, as was evident from the appearance of a white mass of connective tissue. So that further treatment might be possible, the remaining tissue was transferred into a 50-ml centrifuge tube, and the connective tissue cut away and removed. The remaining kidney fragments were then incubated with trypsin at 37°C for 30 minutes; thereafter trypsinization was continued, as outlined previously, until the tissue was completely exhausted.

About 20 runs were needed to use up eight kidneys by this method, and the amount of trypsin solution required was approximately 2500 ml. The trypsinized cell suspension was centrifuged as soon as enough was collected to fill two 250-ml centrifuge bottles. Centrifugation was carried out in an international centrifuge at 800-1000 revolutions per minute (r.p.m.) for 10 minutes. The trypsin solution was decanted immediately and the cells

were resuspended in 30-40 ml of medium Mixture No. 199 [7, 12] containing 2% calf serum, 200 units/ml penicillin, 200 μg/ml dihydrostreptomycin and 50 units/ml Mycostatin (nystatin Squibb); the pH of the medium was brought to 7.0 by the addition of a $NaHCO_3$ solution. This medium will be referred to as medium 199-T. The cell clumps were dispersed in the same bottle by repeated drawing back and forth with a 10-ml syringe fitted with an 18-gauge needle. Cell suspensions from two centrifuge bottles were combined, diluted to 200 ml with medium 199-T, and filtered through four layers of sterile gauze. On this cell suspension, cell counts were carried out in a haematocytometer chamber after the cells had been stained with crystal violet. Only nuclei surrounded by protoplasm were counted. The cell suspension was then diluted with medium 199-T to a final concentration of 300 000 cells/ml. From this suspension, bottle and tube cultures were prepared, using 400 ml of cell suspension per 5-litre diphtheria-toxin bottle, 80 ml per one-litre Roux bottle and 1 ml per test-tube.

On the average, 12 litres of cell suspension containing 300 000 cells/ml were obtained from the kidneys of four monkeys. The cultures were incubated at 37°C for 6-8 days—bottles in a horizontal, and tubes in a slanted, position. All cultures were tightly closed with rubber stoppers. At the end of 6-8 days the surface of the bottles and tubes was completely covered with a confluent sheet of cell growth.

Before being seeded with the appropriate virus suspension, the bottles were treated as follows: the fluid covering the cell sheet was pipetted off by means of vacuum. Pre-warmed PBS was added to each bottle (50 ml for the one-litre Roux bottle and 200 ml for the 5-litre bottle), and the bottles were gently rotated so as to wash the cell sheet and bottle. The liquid was pipetted off, and washing with PBS was repeated. After two washings, 150 ml of medium 199 containing 100 units/ml of penicillin and 100 μ/ml of dihydrostreptomycin at pH 7.0 (further referred to as medium 199-M) were added to each one-litre Roux bottle and 750 ml to a 5-litre bottle.

The seed consisted of 0.3-0.5 ml of a 1/25-1/50 dilution of stock virus kept frozen at −20°C. As representative of type 1 poliovirus, the Brunhilde strain was used. This strain was obtained through the courtesy of Dr H. von Magnus in the form of monkey-kidney tissue-culture fluid. In our laboratory it was passed once in monkey-kidney tissue-culture, harvested, distributed in 1-ml amounts, and kept frozen at −20°C until used. The representatives of type 2 and type 3 poliovirus were the MEF-1 and Saukett strains respectively, obtained through the courtesy of Dr J. Salk. These strains were prepared for seed in the same way as the Brunhilde strain.

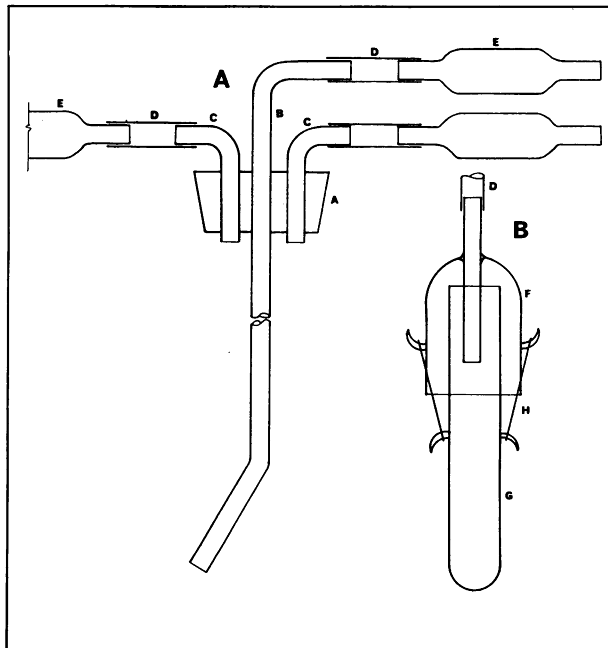
Within 2-3 days after inoculation with either strain, the tissue-culture sheets showed complete and typical degeneration, followed by falling-off of the tissue from the glass. Each culture bottle was examined macroscopically and microscopically, and any showing mould or bacterial contamination was discarded. However, since bacterial contamination could not in every

instance be detected in this way, each bottle was tested individually for sterility by the inoculation of 1-ml amounts into nutrient broth and the plating of several drops on nutrient agar. The agar plates and broth tubes were observed for at least 48 hours. In the meantime the bottles were kept in the cold-room at + 4°C until the results of the sterility tests were recorded. Contents of all bottles found sterile were pooled under a hood into 5-litre Pyrex bottles and placed in the cold-room. In order to allow precipitation of cell debris, the 5-litre pools were kept in the cold for a week or two before processing.

Processing and inactivation of monovalent virus pools

Monovalent pools of 18-19 litres were processed and inactivated in a closed system using calibrated Pyrex carboys of 19 litres. For this purpose, the carboys were fitted with sterile tubing sets consisting of rubber stopper, siphon tube and two short bent tubes (see Fig. 2A). All three glass tubes were protected by air filters packed with cotton. The air filters were attached

FIG. 2. TUBING SET FOR CARBOY (A) AND SAMPLING DEVICE (B)

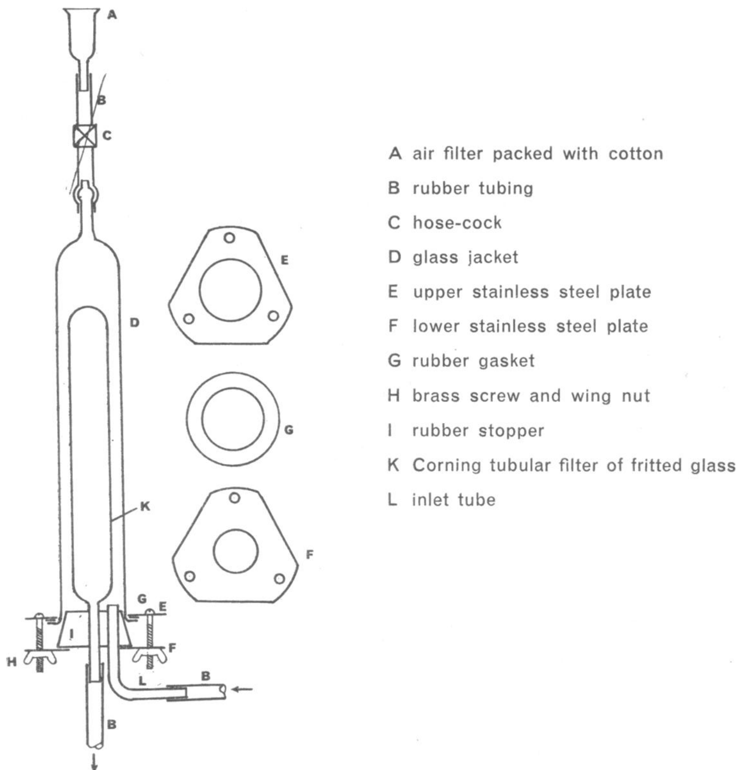


A rubber stopper
B siphon tube
C short bent tube
D short pieces of rubber tubing (in sampling device connected to siphon tube 20 cm in length)
E air filter packed with cotton
F glass bell with hooks
G test-tube with hooks
H rubber bands

by short pieces of Fisher's thick-wall white rubber tubing, so that they could be easily exchanged with sterile rubber tubing or with the sampling device (Fig. 2B)—whichever was needed for a particular operation. The siphon tube serves for transfer of the pool or for sampling; the short bent tubes are used for additions (such as acetic acid or formalin, after removal of the air filters), for application of vacuum or pressure to the carboy, or as release. Carboys, sets, sampling devices and rubber tubings were sterilized separately in the autoclave at 1.5 atm. for 40 minutes.

Virus suspensions from several 5-litre bottles were collected by vacuum into a 19-litre Pyrex carboy, care being taken to transfer the supernatant only, without disturbing the sediment. The pool was passed in three separate steps through a series of filters by applying pressure of 0.2-0.3 atm. gauge on the carboy containing the unfiltered pool, and vacuum of 60-65 cm of mercury on the carboy receiving the filtrate. The compressed air used was freed of oil and water by means of a separator, and sterilized by passing through an activated carbon filter (Koby Corporation, USA) and a cotton

FIG. 3. ASSEMBLY OF TUBULAR FRITTED GLASS FILTER FOR FILTRATION IN CLOSED SYSTEM



air-filter. Corning tubular filters of fritted glass (No. 35000, length 8 inches (20.3 cm)) were used. The maximum pore sizes of the filters are as follows: coarse, 40-60 μ; medium, 10-15 μ; fine, 4-5.5 μ; ultra-fine, 0.9-1.4 μ. Assembling of the filters for continuous filtration is shown in Fig. 3.

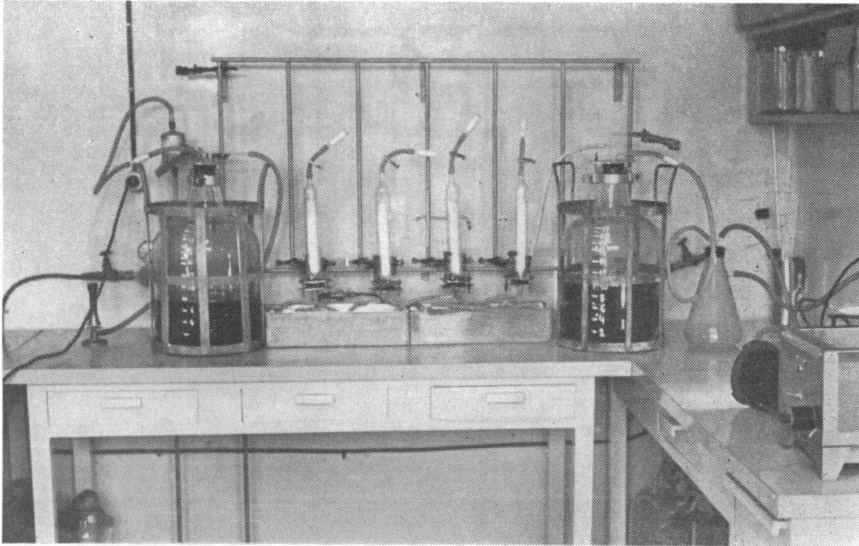
The filtrations were carried out in the following steps (von Magnus,[6] and personal communication):

(*a*) cotton filter and tubular filter, porosity coarse;

(*b*) tubular filters, porosities medium and fine (this filtration was carried out with two pairs of filters, so that through each pair not more than 10 litres of virus suspension were passing);

(*c*) tubular filters, porosities fine and ultra-fine, in pairs as in step (*b*); see Fig. 4.

FIG. 4. FILTRATION THROUGH CORNING TUBULAR FILTERS IN CLOSED SYSTEM



Two pairs of filters are shown, each pair consisting of filters of fine and ultrafine porosities.

The usual time needed for each step was as follows: step (*a*)—30 minutes; step (*b*)—1-2 hours; step (*c*)—4-5 hours.

The carboy containing the ultra-filtrate was then fitted with a set as shown in Fig. 2A (see page 1005), but with the siphon tube ending in a glass-ring aerator. The pool was aerated in the incubator at 37°C with sterile air (Koby and large cotton filters) for about 16 hours in order to expel the CO_2 originally present in medium 199-M. After aeration was completed, the pH of the pool rose above 8.0. The pH was adjusted to 7.0 by addition

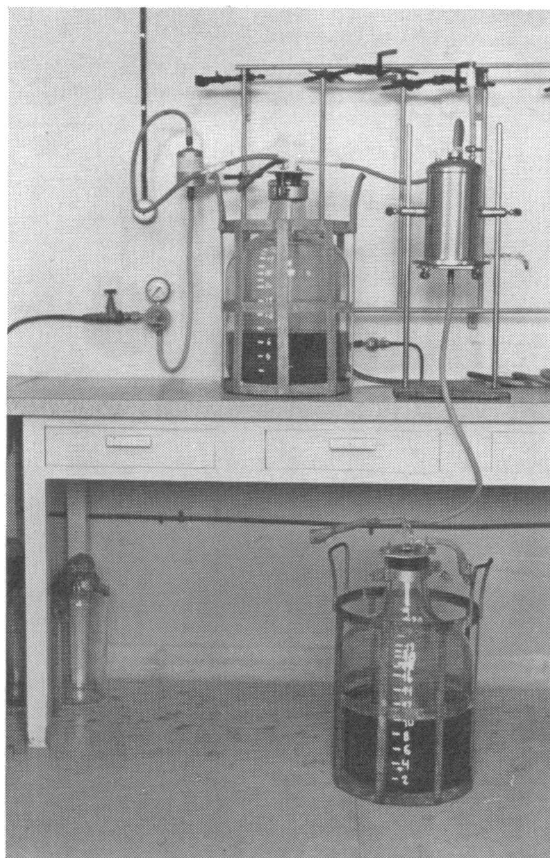
of 0.5 N acetic acid. This was followed by Seitz filtration according to the Minimum Requirements, USPHS, amendment No. 4.[18] A Hercules filter, model 14, equipped with an ST-1 Seitz filter pad (diameter 14 cm, porosity 0.3-0.5 μ) and backed by a sheet of filter paper, was used. Before sterilizing the filter, one litre of demineralized water was passed through the pad under slight pressure. After sterilization, 1-2 litres of sterile Hanks' salt solution, adjusted to pH 7.8 with sodium carbonate, was passed through the filter (as recommended by B. Hampil, personal communication).

The pool was filtered continuously by introducing it into the filter through the central opening in the filter-lid (originally intended for the application of pressure). Sterile air under pressure at 0.3 atm. gauge was applied to the carboy which served as a feeding vessel. The Seitz filtrate was collected into another sterile carboy fitted with a stopper and the usual set of tubings (see Fig. 5); filtration proceeds at a very slow rate (2-2.5 litres of filtrate per hour). The Seitz filtrate was left overnight at room temperature (20°C) or in the cold-room. Next day, the pool was pre-heated in a water-bath to 37°C and formalin was added to a final concentration of 1/4000. A 1/200 dilution of formalin (37% formaldehyde) was added at the rate of 50 ml per 950 ml of virus suspension under vigorous shaking of the carboy, and to assure complete mixing, the pool was transferred immediately into a second carboy. From the latter, a sample of 200 ml was withdrawn and distributed in equal volumes into eight 30-ml pilot bottles. The formalin-containing pool (after removal of the set of tubings) and the pilot bottles were closed with rubber stoppers, sealed with taps, and placed in the incubator room at 37°C.

For the next four days, the inactivation of the pool was followed by determining the virus titre in the pilot bottles at regular intervals of time. The first pilot bottle was taken immediately on addition of the formalin; 0.5 ml of a 1/16 dilution of 35% concentrate of sodium bisulfite was added to the 25-ml sample, shaken well and placed in the cold-room. Further pilot bottles were removed from the incubator twice daily, and treated in the same manner. Each neutralized sample was dialyzed against at least 20 vol. of PBS at 4°C for four hours; PBS was then changed and dialysis was continued overnight. The dialyzates were titrated for virus content in monkey-kidney tissue-culture tubes, and the rate of inactivation was determined. Virus titrations were carried out in one-log steps, using ten tubes per dilution and 0.5 ml per test-tube. End-points were calculated according to the method of Reed & Muench.[9] During this four-day period and until the end of the inactivation, the main pool was shaken manually every six hours.

On the fourth day of inactivation, a second Seitz filtration was carried out in the same manner as previously described. Here, the average filtration rate is 5-5.5 litres of filtrate per hour. In order to ensure proper mixing, the pool was transferred on days 6 and 8 of inactivation into clean sterile carboys.

FIG. 5. CONTINUOUS FILTRATION THROUGH SEITZ FILTER*



* Diameter of pad 14 cm

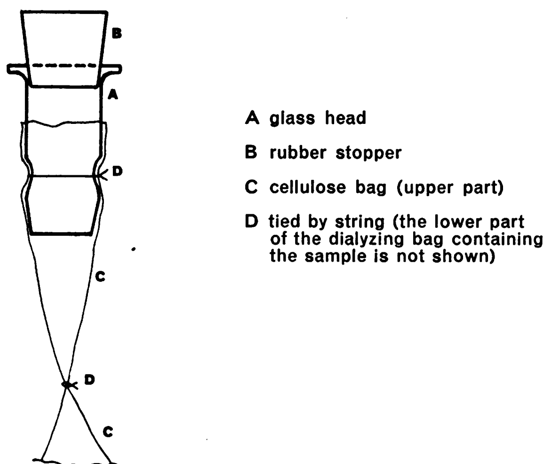
The total time of inactivation in routine production of vaccine was 12 days. Large samples for safety testing in tissue culture were withdrawn twice directly from the carboy. The first sample was taken three days before the end of the inactivation period, and the second sample on the last day of inactivation. For this purpose, a sterile tubing set was inserted into the carboy and a glass sampler connected to the siphon tube (see Fig. 2B page 1005). By means of a rubber bulb (attached to the air-filter of one of the short bent tubes) slight pressure was applied on the carboy, and the desired volume of sample was collected in a sterile bottle. Usually 800 ml were withdrawn on the ninth day. Thereafter, the tubing was withdrawn, and the carboy was sealed with a rubber stopper and returned to the incubator room. Three days later, another sample was withdrawn in a similar manner.

Thereupon, the carboy was sealed and placed in the cold-room, where it remained awaiting the results of the tissue-culture safety tests.

Safety testing of monovalent pools in tissue culture

The samples underwent dialysis before being tested in tissue culture. Dialysis was carried out against PBS (pH = 7.4) in the cold for three days. 100-ml aliquots were placed in dialyzing bags made of seamless cellulose tubing (size $1^1/_8$-inch (28.6-mm), and a glass head, as shown in Fig. 6, has been found suitable for filling the bags and inserting them into glass cylinders. The samples were dialyzed against at least four volumes of PBS, the latter being changed once daily. At the end of the third day of dialysis, the dialyzate was removed from the bag and tested in tissue culture, preferably without delay. The dialyzates were pre-warmed to 37°C and distributed in 50-ml amounts in 6-8-day-old monkey-kidney tissue-culture Roux bottles of one-litre capacity, each containing 100 ml of safety-testing medium. This volume of sample was adopted so as to achieve a ratio of at least 1 ml of vaccine per 3 cm^2 tissue area, according to the Minimum Requirements, USPHS.[18]

FIG. 6. GLASS HEAD FOR DIALYZING BAG



The safety-testing medium consisted of medium 199 to which 0.05% bovine albumin (Armour Laboratories), 200 units/ml penicillin, 200 μg/ml dihydrostreptomycin, and 50 units/ml Mycostatin had been added.[16] The pH of the safety-testing medium was adjusted to 7.9-8.4 by the addition of $NaHCO_3$ and NaOH solutions (J. L. Melnick, personal communication).

The original cultures were observed microscopically every two to three days for at least 10 days, and incubated at 37°C for a period of about 30 days. Subcultures were made on days 7, 14, and 21-28. The 7- and 14-day subcultures were made by transferring 10-ml amounts of culture fluid from the original cultures into new bottles containing 100 ml of the safety-testing medium. These bottles were observed microscopically for cell degeneration every two to three days during a period of 8-10 days, at the end of which subcultures were made by either of two methods: (*a*) in tubes—4 ml from each bottle were distributed in 1-ml amounts into monkey-kidney culture tubes and observed for one week; (*b*) in bottles — 5-ml samples from each subculture were withdrawn, pooled and inoculated in 15- to 50-ml amounts into one or more monkey-kidney tissue-culture bottles.

Subcultures on days 21-28 are made similarly to those of the 7- and 14-day sub-subcultures. Since falling-off of tissue was often observed, it became routine procedure to subculture such material in monkey-kidney tubes in order to distinguish between non-specific and poliovirus degeneration.

A vaccine pool was considered non-infectious if no degeneration typical and proven for poliovirus could be established. In case of doubt, subcultures were made and typing of the cytopathogenic agent against monkey antisera to the three poliovirus types was carried out. Sometimes subcultures were made in human amniotic tissue cultures, in addition to the monkey-kidney tissue-culture tests.

Preparation and testing of the trivalent vaccine pools

After the tissue-culture safety tests on the monovalent pools had been completed, the preparation of the trivalent pool was carried out. Monovalent vaccine pools of types 1, 2 and 3 poliovirus were pooled together in a 45-litre Pyrex carboy; the volumes of each type were approximately equal, but in general a somewhat larger volume of type 1 vaccine was added to the trivalent pool. In order to ensure proper mixing, the pool was transferred back and forth into another carboy of the same size. After thorough mixing, a 4-litre sample was withdrawn for testing. Two litres were immediately distributed in 100-ml aliquots in dialyzing bags, and dialysis was carried out as indicated for monovalent pools. The other 2-litre sample was intended for testing at another laboratory, and, since transport was sometimes involved, sodium bisulfite and parabens were added and the sample treated in the same manner as described below.

Safety tests were run on the 2-litre sample after dialysis according to the methods described for the monovalent pools. Whereas 1700-1800 ml were tested in the usual manner in monkey-kidney tissue-culture bottles, additional tests for safety were carried out on a 200- to 300-ml sample in human

amnion tissue-culture bottles, subcultures from the latter being carried out in both human amnion and monkey tissue-culture bottles.

The trivalent pool was divided into from two to three filling lots. To these 0.26 g of sodium bisulfite per litre of vaccine were added as an 8.8 % aqueous solution under vigorous shaking of the carboy; this treatment neutralizes the bulk of formaldehyde present in the vaccine. Immediately thereupon, methyl paraben 1.5 g/litre of vaccine, and propyl paraben 0.2 g/litre of vaccine were added in powdered form. The carboy was shaken vigorously, placed in a 37°C water-bath for from two to three hours, and shaken from time to time to accelerate the solution of the parabens. Then, although a part of the parabens remained undissolved, the carboy was placed in the cold-room and shaken vigorously once daily for seven days. Shortly before the pool was distributed into vials, it was filtered through a fritted glass filter, porosity fine, in order to separate undissolved parabens.

Each filling lot was separately distributed into vials, and all equipment was changed after each process. During the operation, random samples were put aside at a rate of 1 %-2 % of the total number of vials, to serve for the testing of sterility and pyrogenic substances, and for the monkey safety-test. Sterility tests were carried out on the vials themselves at room temperature and at 37°C, and by inoculation of the contents from the vials into bottles containing large volumes of fluid thioglycollate medium and nutrient broth. The sterility tests were read at the end of seven days' incubation.

The monkey safety-test was carried out according to the Minimum Requirements, USPHS, amendments Nos. 3 and 4.[18] In addition, two blood samples taken from the inoculated monkeys on the fourth and eighth days after inoculation of the vaccine were tested for the presence of virus in monkey-kidney culture tubes.[16]

Potency tests were carried out in guinea-pigs and chicks. The guinea-pig potency test was based on the method developed by Gard et al.[4] Undiluted vaccine, and vaccine diluted 1/10, 1/100 and 1/1000, were inoculated subcutaneously in 1-ml amounts each into groups of 10-12 guinea-pigs weighing 250-300 g each. Two weeks later, an inoculation of the same amount and dilutions was repeated, followed one week later by bleeding of the animals from the heart. Individual sera were stored at −20°C until tested. Before the neutralization test, which was carried out in monkey-kidney tubes, the sera were inactivated at 56°C for 30 minutes. Equal volumes of the virus dilution and of undiluted guinea-pig serum were combined and incubated at 37°C for 30 minutes. 0.2 ml of virus-serum mixture was inoculated into each tissue-culture tube, using two tubes for each serum. Sera of guinea-pigs vaccinated with Salk's reference " J " vaccine served as controls of the neutralization test.

32-100 $TCID_{50}$ of types 1, 2 and 3 polio virus were used to test each serum. The tissue-culture tubes were observed microscopically for from six to seven days after inoculation of the virus-serum mixtures, and the

protective effect of the sera was recorded. A serum was considered positive if the serum in at least one tube had a neutralizing effect against this amount of virus.

The chick potency test was carried out in 10-day-old chicks according to a method communicated to us by Dr. J. L. Melnick. The same schedule as in the guinea-pig tests was used for immunizations and bleedings. The dose was 0.5 ml, given by the intramuscular route, and the neutralization test was identical with that described for guinea-pig sera.

Other tests

Animal inoculations for innocuity were carried out according to the Minimum Requirements, USPHS.[18] Samples of ultra-filtrates of the monovalent pools, before inactivation, were each tested for the presence of B virus and tubercle bacilli by inoculation into rabbits and guinea-pigs.

Three or four rabbits were inoculated intracutaneously and subcutaneously with 10 ml of the sample each and observed for a period of from three to four weeks; five guinea-pigs were inoculated with 5 ml each of the ultrafiltrate by the intraperitoneal route, observed for a period of from seven to eight weeks, killed and autopsied.

Samples of the trivalent vaccines were each tested for the presence of lymphocytic choriomeningitis virus by intracerebral inoculation in 24-36 adult mice. In addition, 16-32 infant mice were inoculated by the intraperitoneal and intracerebral routes with samples of the trivalent vaccines. A 50-ml sample was also tested for the presence of tubercle bacilli by direct culturing.

Tests for pyrogens were carried out in another laboratory. Tests for total nitrogen were made on a sample of the final product according to the method of Lanni and co-workers.[5] For comparison, total nitrogen was determined in samples of medium 199-M.

Experimental

While the preceding sections describe methods adopted for the production and testing of polio vaccine, here certain results obtained in the course of preparation of vaccine pools are outlined. Some aspects of primary importance in the preparation of non-infectious and potent vaccine pools are stressed—namely, the preparation of virus suspensions of high initial titre, the importance of adequate filtration procedures, and the use of large samples and a highly sensitive tissue-culture system for the detection of the smallest traces of live poliovirus.

Preparation and titration of virus suspension

Our principal aim in the preparation of virus suspensions for processing into vaccines was to obtain crude suspensions with high virus titres in order

to start the inactivation process with a filtered suspension having a titre of at least $10^{-6.0}$ per 0.5 ml.[18] From the practical point of view it was important to prepare large volumes of such virus suspensions. To achieve this, experiments were carried out in which virus fluids were harvested at different times after inoculation of the culture bottles with the appropriate seeds, as outlined on page 1004, while varying the volumes of the maintenance medium 199-M used for virus synthesis. To determine the most adequate time of harvest, samples were withdrawn 24, 48, 72 and 96 hours after inoculation with virus, and titrations were carried out in monkey-kidney tube cultures using 0.5 ml inocula and 10 tubes per log dilution. It was observed that high virus titres were reached a day or so before full degeneration of the cell sheets occurred, slight differences being observed between the three poliovirus types. Practically, however, it was found most convenient to harvest the virus suspensions when full degeneration of the tissue was evident. This usually occurred on the second and third day after inoculation of the culture bottles.

No significant differences in virus titre were noted when minor changes in the volume of the suspending medium were made. An increase in volume of maintenance medium from 100 to 150 ml in the one-litre Roux bottles, and from 500 to 750 ml in the larger 5-litre diphtheria toxin bottles, did not influence markedly the virus titres obtained. Occasionally even higher titres were recorded for the larger volumes. Therefore, volumes of suspending fluids adopted for the preparation of crude virus suspensions were 150 ml and 750 ml for one-litre and 5-litre Roux bottles respectively.

The procedures and time schedules adopted for seeding with virus, the volume of suspending fluid used, and the time chosen for harvest resulted in crude virus suspensions having titres which ranged from $10^{-7.0}$ to over $10^{-8.0}$ per 0.5 ml. These high initial virus titres were essential and adequate since they permitted the losses encountered during the filtrations which followed.

As outlined on pages 1005-1008, two kinds of filtration preceded the inactivation of the monovalent virus pools—namely, filtration through a series of fritted-glass filters and through a Seitz filter pad. Table I shows the effect of these successive filtrations on the titres of the three different poliovirus serotypes. The total loss during filtration usually amounted to approximately one log of virus, fairly equally distributed between filtration through glass filters and that through the Seitz filter. Thus, the titres of the Seitz-filtered virus suspensions, before the addition of formalin, ranged from $10^{-6.0}$ to $10^{-7.4}$ per 0.5 ml.

Inactivation of virus suspensions

The rate of inactivation of the monovalent virus pools was followed during the first four days using pilot bottles, as described on page 1008. Table II presents typical results of titrations of pilot bottles at different

TABLE I. LOSSES IN VIRUS TITRES DURING SUCCESSIVE FILTRATIONS OF CRUDE VIRUS SUSPENSIONS

Type of virus suspension	Monovalent pool								
	type 1			type 2			type 3		
Crude	7.5 *	8.0	7.7	8.0	7.9	7.8	7.0	7.5	7.5
After ultra-fine glass filtration	7.1	7.5	7.5	7.6	7.4	7.7	ND	7.2	7.4
After ST-1 Seitz filtration	6.8	7.0	7.0	7.0	6.8	7.4	6.0	6.4	7.0
Total loss	0.7	1.0	0.7	1.0	1.1	0.4	1.0	1.1	0.5

* Expressed as negative log titre per 0.5 ml ND = not done

times during the inactivation of Seitz-filtered pools of the three poliovirus types. At 68 hours after the addition of formalin (final concentration 1/4000), no virus could usually be detected when 5 ml (10 tubes × 0.5 ml) of the undiluted sample were tested. However, when subcultures were made from these negative tubes in new monkey-kidney tube cultures, poliovirus was often recovered.

In general, the rate of inactivation of the monovalent pools was similar to that reported by Salk et al.[13] It is to be stressed that Seitz filtration resulted in vaccine pools which were consistently non-infectious when submitted to rigid safety tests using large samples of vaccine (up to 800 ml each) at the end of the inactivation period and three days previously.

TABLE II. FORMALDEHYDE INACTIVATION OF MONOVALENT SEITZ-FILTERED POOLS

Sample	Hours after addition of formalin (approximate)	Type 1			Type 2			Type 3		
1	0	7.4 *	7.0	6.8	6.8	7.1	6.9	6.8	7.4	7.5
2	23	3.5	3.5	3.0	4.5	3.7	4.2	4.6	4.4	4.2
3	43	2.2	2.0	1.0	1.6	1.6	1.6	2.0	1.8	1.5
4	50	1.3	0.5	0.3	1.5	1.4	0.9	ND	1.1	ND
5	68	0.4	0.0	0.0	0.2	0.5	0.5	0.5	ND	0.0
6	73	0.0	0.0	0.0	0.1	0.2	0.0	0.3	0.1	0.0
7	91	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	ND

* Expressed as negative log titre per 0.5 ml ND = not done

Table III shows the results of virus titrations of samples during the inactivation process of pools filtered through glass filters only. The rate of inactivation did not differ essentially from that of Seitz-filtered pools.

TABLE III. FORMALDEHYDE INACTIVATION OF FRITTED-GLASS FILTERED POOLS

Sample	Time after addition of formalin (hours)	Type 1			Type 2	
		I	II	III	IV	V
1	0	6.9 *	7.4	7.4	6.7	6.0
2	23	3.6	4.5	3.8	3.5	2.6
3	43	1.3	1.2	1.7	1.4	0.7
4	50	0.5	0.8	1.4	1.0	0.5
5	68	0.0	0.5	0.2	0.2	0.0
6	73	0.0	0.5	0.0	0.0	0.0
7	91	0.0	0.0	0.0	0.0	ND
	9-12 days	0	0	+ **	+ **	+ **

* Expressed as negative log titre per 0.5 ml ND = not done
** Virus was found in the safety tests

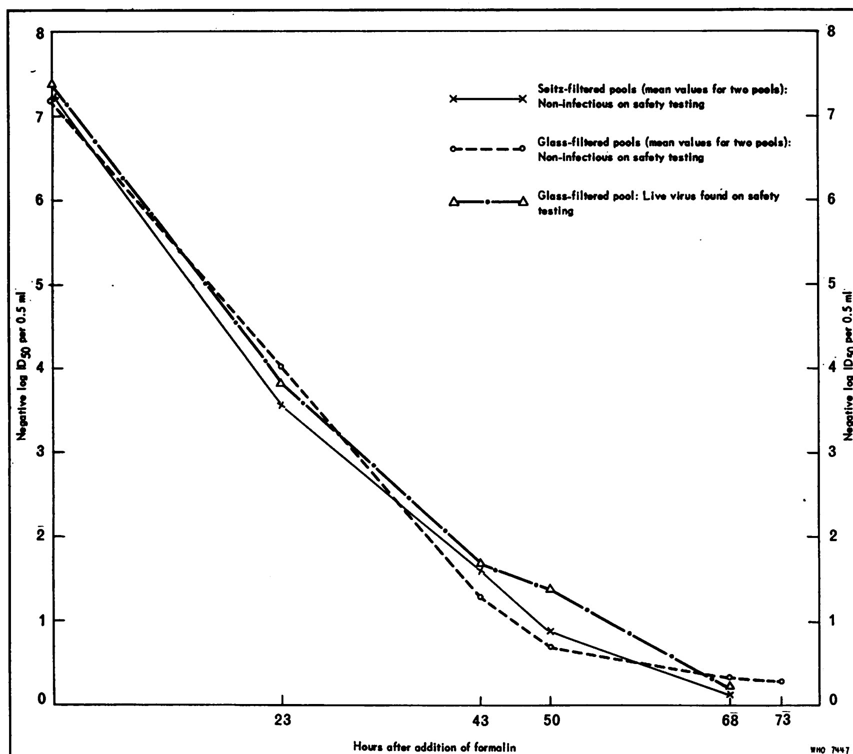
Sometimes virus could not be recovered 68 hours after the addition of formalin (pools I and V). However, these pools, although submitted to an additional filtration on the fourth day of inactivation through a fine and an ultra-fine filter, frequently showed traces of live virus when submitted to safety testing (Table III, pools III, IV and V); occasionally, non-infectious pools were obtained using glass filters only (Table III, pools I and II). Thus, glass filtration alone proved inadequate, as was shown by the recovery of live poliovirus from monovalent vaccine pools prepared in this manner.

Whether a virus-free monovalent vaccine pool will ultimately be obtained could not be predicted from the inactivation curve. The inactivation curves of glass-filtered virus pools in which live poliovirus was detected in the safety tests, of glass-filtered pools which were negative in the safety tests, and of Seitz-filtered pools, were strikingly similar and practically identical (see Fig. 7). These observations are discussed on page 1021.

Safety testing

The methods used in safety testing of the monovalent and trivalent pools have been described on pages 1010-1013. As indicated previously, the reliability of the safety tests was based on the use of a highly sensitive

FIG. 7. TYPICAL INACTIVATION CURVES OF MONOVALENT POOLS (POLIOVIRUS TYPE 1)



tissue-culture system and on testing large samples of vaccine. The sensitivity of the tissue-culture system used was ascertained by the use of reference virus with known titre.

Reference virus obtained from the National Institutes of Health, Bethesda, Md, USA, was used. The reference virus vials were received frozen on dry ice and the titre indicated on the label was $10^{-6.5}$ per 0.5 ml. They were stored in our laboratory at —20°C. When 6-8 monkey-kidney tissue-culture bottles per dilution were used, the titre of the reference virus varied from $10^{-6.8}$ to $10^{-7.5}$ per 0.5 ml. In safety testing, reference virus was titrated along with vaccine samples, the same monkey-kidney cells and the same safety-testing medium being employed for both. It was endeavoured to test the sensitivity of the tissue-culture system at least once during a complete safety test on each monovalent vaccine pool and with the safety testing of each trivalent pool.

Reference virus was also used to investigate the effect of dialysis. In order to ascertain that dialysis of the vaccine samples does not reduce

virus titres, this process was carried out on 50-ml samples of dilute reference virus (dilutions of $10^{-6.0}$, $10^{-7.0}$ and $10^{-8.0}$ per 0.5 ml) in medium 199-M, followed by titration in monkey-kidney tissue-culture bottles. Table IV shows the results of two such experiments. No appreciable loss in titre of reference virus which could be attributed to dialysis was observed, nor any loss in titre of reference virus when titrations were carried out in preparations of vaccine.

TABLE IV. EFFECT OF DIALYSIS ON TITRE OF REFERENCE VIRUS

Experiment No.	Titre of reference virus in Roux bottles per 0.5 ml	
	before dialysis	after dialysis
1	$10^{-7.4}$	$10^{-7.1}$
2	$10^{-7.2}$	$10^{-7.2}$

The pH of the safety-testing medium was brought to 8.0 or higher on the assumption that the system is more sensitive at this pH (J. L. Melnick, personal communication). In our own experience, when comparative end-point titrations were carried out at varying pH, slightly higher virus titres were obtained at alkaline pH. The increase in the sensitivity of the safety-testing system at high pH is possibly due to breaking-up of virus clumps which may contain live virus particles.

The use of human amnion-cell cultures was introduced in our laboratory following the demonstration that these cells are highly sensitive to poliovirus.[21] Since later reports by Fogh & Lund [2] have indicated that titres of poliovirus obtained in human amnion cells are even higher than those in monkey-kidney cell cultures, safety tests were carried out in trypsinized human amnion bottle cultures in addition to the usual monkey-kidney tissue cultures.

Live poliovirus was detected in the safety tests on several occasions, but only in monovalent pools which had been submitted to glass filtration alone prior to, and on the fourth day of, inactivation by formalin. Poliovirus was usually found in one or more bottles four to five days following inoculation with the dialyzed sample. Out of five monovalent pools in which live poliovirus was detected, in one instance only was virus recovered on subculture of samples which were negative in the direct cultures. Poliovirus was detected in samples taken at the end, as well as in samples taken three days before the end, of the inactivation period, and sometimes in both. Reheating of the " positive " monovalent vaccine pools did not result in a non-infectious pool. However, Seitz filtration and reheating of these

virus-containing pools removed all live virus, as was proved by subsequent safety testing.

In no instance among 15 lots did we recover live poliovirus in the safety tests of monovalent pools which had been filtered through an ST-1 Seitz filter pad before and during the inactivation process. All samples, 700-800 ml each, from such pools were found to be consistently negative when submitted to safety tests in tissue culture on days 9 and 12 of inactivation. We have also found all trivalent pools negative for poliovirus both in the tissue-culture safety tests and in the monkey safety tests.

Potency of the vaccine pools

Table V shows typical results of potency tests of a trivalent vaccine pool tested in guinea-pigs by the method of Gard et al.[4] For comparison, results of potency tests of a batch of commercial USA vaccine, of Salk's reference vaccine " J ", and of live poliovirus are presented. The neutralization tests

TABLE V. RESULTS OF POTENCY TESTS IN GUINEA-PIGS

Vaccine	Dilution	Poliovirus		
		type 1 (200 $TCID_{50}$)	type 2 (100 $TCID_{50}$)	type 3 (200 $TCID_{50}$)
Local trivalent pool number 2	(10^0)	7/8 *	8/9	8/9
	10^{-1}	5/10	7/10	8/10
	10^{-2}	2/7	2/6	4/7
Vaccine titre **		1.3	1.8	2.1
Commercial USA vaccine	(10^0)	8/8	8/8	8/8
	10^{-1}	4/8	6/7	6/8
	10^{-2}	2/8	7/10	4/9
Vaccine titre		1.5	$\geq$ 2.3	2.0
Live virus (types 1, 2 and 3 combined in equal parts)	(10^0)	6/6	8/8	ND
	10^{-1}	6/6	6/6	ND
	10^{-2}	2/4	5/5	ND
Titre		$\geq$ 2.3	$\geq$ 2.8	—
Salk's reference vaccine " J "				
Vaccine titre		1.7	2.0	1.8

* Number of sera neutralizing the respective amounts of virus/total number of sera tested.

** Expressed as median effective dose (ED_{50}) of vaccine; values are logs of $1/ED_{50}$, corrected by 0.3, according to Gard.

ND = not done

of the respective guinea-pig sera inoculated with the local vaccine, the commercial USA vaccine, Salk's reference vaccine " J ", and live virus were carried out simultaneously.

Usually, Salk's reference vaccine " J " only was used for comparison, and sera of guinea-pigs immunized with this reference vaccine were included in the neutralization tests when testing the immunogenic effect of the local vaccine.

The results of the potency tests indicated that the local vaccines were not inferior to commercial vaccines currently used in the USA, although in our vaccine a different type 1 strain was included. The vaccine titres for type 1 were in the range of 0.9-1.7 while those of types 2 and 3 ranged from 1.5 to 2.5. Potency of type 1 vaccine was thus usually lower than that of types 2 and 3—a fact that has been well established in the past.[11, 14] No data are presented on the results of the chick potency tests since these conformed in general with the results of the guinea-pig potency tests.

Use of the Vaccine for Immunization

The local vaccine was used for immunization of infants and young children up to four years of age during the winter and spring of 1957. Over 60 000 infants and young children received two inoculations, spaced by one month, of 0.3-0.5 ml each by the intracutaneous and subcutaneous routes respectively. No untoward reactions were noted and the vaccine proved to be safe. Antibody tests on pre- and post-vaccination serum samples are in progress. Preliminary serological tests indicate that children with no pre-antibody to all three types of poliovirus develop types 2 and 3 antibodies to a significant titre after the inoculation of two doses of vaccine. The development of type 1 antibodies in such children is much less striking; only 40%-50% of infants developed measurable type 1 antibodies after receiving two doses of the vaccine. However, the type 1 " booster " effect in children with low type 1 pre-antibody titres was very marked. These preliminary results are in accord with the findings on the serological response of infants and pre-school children to poliomyelitis vaccine reported by Brown & Smith.[1] The results and evaluation of the mass immunization of infants and young children are awaited, and will be reported separately in due course.

Comment

The results reported here indicate that it is possible to obtain a safe and effective poliomyelitis vaccine if attention is paid to proper methods of processing and testing. Such poliomyelitis vaccine can be prepared in a consistently reliable manner if certain procedures are observed, the most important, in our experience, being the preparation of virus suspensions of

high initial titre, proper filtration techniques and the use of a sensitive tissue-culture system for the detection of traces of non-inactivated poliovirus in the vaccine pools. Seitz filtration seemed to be of the utmost importance in the preparation of non-infectious vaccine pools. In our hands, Seitz filtration which preceded the inactivation by formalin did not markedly lower the initial virus titre, the loss during this filtration amounting to approximately 0.5 log of virus only. This relatively low loss in virus titre may have been due to the rinsing of the filter pads with Hanks' salt solution before filtration, and to the observation of a proper ratio between filter-pad area and volume of fluid filtered (B. Hampil, personal communication).

Virus pools filtered through Seitz filter pads before the inactivation and on the fourth day of inactivation by formalin were consistently non-infectious when submitted to rigid safety tests in tissue culture. Glass filtration alone cannot be relied upon in the production of safe vaccine pools. Since sediments were never encountered in our work, even when working with glass filters alone, we assume that the inadequacy of glass filtration may be due to its permitting the passage of virus aggregates which do not undergo " complete " inactivation. On the other hand, ST-1 Seitz filter pads, because of their smaller pore size as compared with UF glass filters, apparently do not permit the passage of these poliovirus aggregates.

The discussion in the literature [17, 19] as to whether the inactivation of poliomyelitis virus by formaldehyde runs the course of a first-order chemical reaction or deviates from the linear course appears to us to be a theoretical rather than a practical problem. Since such a high degree of over-treatment with formalin is applied, the inactivation curves seemed to us non-relevant from the practical point of view. The general slope of the inactivation curve is undoubtedly important since it demonstrates whether the inactivation process is proceeding in the desired way. No dependence, however, can be placed on the precise rate of the initial fall in virus titre. The exact slope of the inactivation curves had no bearing on the ultimate outcome of the inactivation process, as was proved by subsequent safety tests carried out on the last day of inactivation and three days previously (see Fig. 7, page 1017). Consequently, the ultimate reliance for the safety of a vaccine must be the absence of live poliovirus as demonstrated by the safety tests of the monovalent and polyvalent pools.

* * *

Since the submission of this article, we have carried out several experiments in which the second Seitz filtration (on the fourth day of inactivation) was either omitted or replaced by filtration through an ultrafine fritted glass filter. This resulted in monovalent pools of a much higher potency. It seems that the ST-1 Seitz filter pad removes large amounts of antigen. So far no live virus was found on safety-testing of four such pools.

RÉSUMÉ

En 1956, le laboratoire des virus du Ministère de la Santé d'Israël a mis au point une méthode de production semi-industrielle du vaccin antipoliomyélitique formolé. Dans l'ensemble, la technique suit celle qui a été conçue initialement par Salk, avec certaines modifications introduites sur place. Le vaccin a été soumis à des essais de sécurité et d'innocuité suivant les prescriptions minimums de l'United States Public Health Service.

Les souches de virus poliomyélitique employées sont Brunhilde (type 1), MEF-1 (type 2) et Saukett (type 3). On cultive le virus sur des cultures stationnaires trypsinées de cellules rénales de singe; les suspensions de virus sont filtrées, avant inactivation, sur des filtres de verre fritté (gros à ultra-fin), puis sur Seitz. Une deuxième filtration sur Seitz a lieu le quatrième jour de l'inactivation. Celle-ci se fait par addition de formol (concentration finale 1/4000) au pH 7,0 et séjour à 37°C pendant 11 à 12 jours. Les épreuves d'innocuité portent sur des échantillons volumineux de matériel dialysé; elles consistent à inoculer les cultures de tissu rénal de singe et, en outre, des cellules amniotiques humaines. Pour vérifier la sensibilité du système de culture tissulaire, on se sert de virus de référence fourni par les National Institutes of Health de Bethesda, Md.

On a constaté qu'il était essentiel de procéder à la filtration sur Seitz pour fabriquer un vaccin non infectant. En filtrant uniquement sur verre, on obtient des résultats irréguliers.

Le produit définitif est soumis à des épreuves d'innocuité sur le singe. Les essais d'activité sont effectués sur des poussins de 10 jours et des cobayes. On neutralise le formol dans le produit définitif et on ajoute des parabens comme agents de conservation. Les mélanges monovalents ont un volume de 15 à 18 litres et les mélanges trivalents un volume de 45 litres environ.

On a observé que pour produire un vaccin inoffensif et efficace, il est capital que les titres initiaux de virus soient élevés, que les méthodes de filtration soient satisfaisantes et que le système de culture tissulaire employé pour l'essai d'innocuité soit très sensible.

Le vaccin ainsi produit a été administré à plus de 60 000 nourrissons et jeunes enfants, au cours d'un programme national de vaccination qui s'est déroulé pendant l'hiver et le printemps de 1957. Aucun effet nocif n'a été enregistré. On procède actuellement à l'analyse immunologique de l'action de ce vaccin chez les nourrissons et les enfants inoculés.

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REFERENCES

1. Brown, G. C. & Smith, D. C. (1956) *J. Amer. med. Ass.* **161**, 399
2. Fogh, J. & Lund, R. O. (1955) *Proc. Soc. exp. Biol. (N.Y.)*, **90**, 80
3. Francis, T. et al. (1955), *Amer. J. publ. Hlth*, **45**, Part 2, No. 5
4. Gard, S. et al. (1956) *Arch. ges. Virusforsch.* **6**, 401
5. Lanni, F., Dillon, M. L. & Beard, J. W. (1950) *Proc. Soc. exp. Biol. (N.Y.)*, **74**, 4

6. Magnus, H. von et al. (1955) *Danish med. Bull.* **2**, 226
7. Morgan, J. F., Morton, H. J. & Parker, R. C. (1950) *Proc. Soc. exp. Biol. (N.Y.)*, **73**, 1
8. Rappaport, C. (1956) *Bull. Wld Hlth Org.* **14**, 147
9. Reed, L. J. & Muench, H. (1938) *Amer. J. Hyg.* **27**, 493
10. Salk, J. E. (1955) *J. Amer. med. Ass.* **158**, 1239
11. Salk, J. E. (1956) *Amer. J. publ. Hlth*, **46**, 1
12. Salk, J. E., Youngner, J. S. & Ward, E. N. (1954) *Amer. J. Hyg.* **60**, 214
13. Salk, J. E. et al. (1954) *Amer. J. publ. Hlth*, **44**, 563
14. Salk, J. E. et al. (1955) *Amer. J. publ. Hlth.* **45**, 151
15. Scheele, L. A. & Shannon, S. A. (1955) *J. Amer. med. Ass.* **158**, 1249
16. Syverton, J. T. et al. (1956) *Amer. J. Hyg.* **64**, 74
17. Timm, E. A. et al. (1956) *J. Immunol.* **77**, 444
18. United States Public Health Service (1955) *Minimum requirements : poliomyelitis vaccine*, Bethesda, Md.
19. Wesslen, T. et al. (1957) *Arch. ges. Virusforsch.* **7**, 125
20. Yekutiel, P. et al. (1955) *Bull. Wld Hlth Org.* **12**, 651
21. Zitcer, E. M., Fogh, J. & Dunnebacke, T. H. (1955) *Science*, **122**, 30