THE DEVELOPMENT OF A STABLE SMALLPOX VACCINE*

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(With Plates 1–3)

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INTRODUCTION

This work was undertaken to develop a smallpox vaccine suitable for use under tropical conditions. The glycerinated lymph produced in this country deteriorates rapidly at temperatures above 0° C., and a more stable vaccine would prove invaluable for dispatch to regions without facilities for refrigeration; it would also obviate the need for low-temperature storage during transport. In the United Kingdom, a stable smallpox vaccine could be substituted for the epidemic reserves of glycerinated lymph held at −10° C., which deteriorate slowly and must be replaced at intervals.

The following considerations arose when planning the production of such a vaccine. First, the vaccine must maintain adequate potency for a reasonable period when exposed to tropical temperatures. The rigorous standard adopted was that of compliance with the specification of potency laid down by the British Therapeutic Substances Regulations after storage for at least 1 month at 37° C.

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Secondly, a vaccine destined primarily for tropical use must be in a form which can be used without difficulty by semi-skilled vaccinators. Finally, it must be possible to produce the vaccine economically on a large scale.

Previous work on the preservation of vaccinia virus was recently reviewed in detail (Collier, 1954). The principal methods used are:

(i) Addition of so-called protective substances to liquid suspensions of virus.

(ii) Drying from the liquid state.

(iii) Drying from the frozen state.

Successful results have been reported with all three methods, but in view of certain conflicting reports so far published, further investigation was desirable.

For practical purposes, the best results have been obtained with dried vaccines. Impressive results were reported by Otten (1927, 1932) and by W. A. Collier (1950) with buffalo pulp dried by a method similar to that of Carini (1906). Suspensions of crude pulp are spread in thin layers on large Petri dish lids, and dried slowly *in vacuo* over sulphuric acid. The dried material is scraped off, and distributed into glass ampoules which are then sealed off under vacuum. This method is extensively used in Indonesia, and in view of the good results reported, attempts were made to repeat this work, following Otten's description as carefully as possible. The results were disappointing. Different batches of sheep pulp dried in this way varied considerably in their resistance to storage at 4, 22 and 37° C. No better results were obtained with virus in the form of buffalo pulp, kindly provided by Dr R. Green of the Institute for Medical Research, Kuala Lumpur. There is also a decided risk of scatter of the powdered vaccine during manipulation; moreover, the material is difficult to reconstitute, probably owing to denaturation of the slowly dried protein.

It was accordingly decided to try freeze-drying by the centrifugal method (Greaves, 1944). This is a convenient method for processing large numbers of ampoules, each containing a small volume of liquid, and has the advantage that the product is handled throughout in the container in which it is finally issued. In addition, freeze-dried substances are generally much easier to reconstitute than are those dried from the liquid state. A preliminary account of the initial experiments has already been published (Collier, 1951).

GENERAL PLAN OF PRELIMINARY EXPERIMENTS

In the first instance, the influence of freeze-drying on crude sheep pulp was investigated, and its behaviour on storage compared with that of glycerinated and lanolinated vaccine lymph; tests were also made of the preservative influence of various suspending media on purified virus, both in the liquid state and when freeze-dried. These were screening experiments, designed to select suitable types of virus suspension and suspending media for the more detailed investigations described later.

The usual procedure was to freeze-dry suspensions of sheep pulp, or purified elementary body suspensions (E.B.S.), in ampoules containing 0·3 or 0·5 ml. These were then distributed and held at 4, 22 and 37° C., infectivity titrations being

performed before and after drying, and at intervals thereafter. Samples of the material under test were held at the same temperatures in the liquid state, and were tested in parallel with the homologous dried preparations. It was thought advisable to use two storage temperatures above 4° C. Although the work would have been reduced if only one other temperature had been employed, it appeared that assumptions about the behaviour of a preparation at one temperature from observations made at different temperatures might not be justifiable, and with two media this proved to be so. As a general rule, preparations held at 4 and 22° C. were tested after 1, 2 and 4 weeks, and thereafter at 4-weekly intervals for 6 months. Later tests were done less often, but, if possible, every 8 weeks for a further 6 months, and subsequently at less frequent intervals. Preparations stored at 37° C. were tested less regularly, and usually for shorter periods, as these samples deteriorated more rapidly.

A number of batches were observed for more than 2 years in order to ensure that successful results during the initial period of storage were not transient effects.

MATERIALS

Glycerinated vaccine lymph was prepared by the method in routine use at the Lister Institute (McClean, 1949). The virus used was passaged alternately between rabbits and sheep. The final product was a 1/5 suspension of sheep dermal pulp in 40 % glycerol, containing approximately 0·4 % phenol.

Lanolinated vaccine lymph. Sheep pulp was ground with the minimum quantity of distilled water necessary to produce a smooth creamy emulsion. After tests to exclude the presence of pathogenic bacteria, one part of emulsion was mixed with 8 parts of pure lanolin, which had been heated to 200° C., and cooled to below 40° C. before adding the emulsion.

Glycerinated and lanolinated lymphs were held at −10° C. until required.

Sheep lymph. In this paper, the term signifies a 1/5 suspension of sheep pulp, the crude pulp being ground with twice its weight of distilled water in a machine of the Chalybaus type. After grinding, a further 2 vol. of distilled water were added.

Phenolized sheep lymph. The crude pulp was ground with twice its weight of 1 % phenol in distilled water, and incubated at 22° C. for 48 hr. to reduce the bacterial count. Finally, a further 2 vol. of distilled water were added.

Both phenolized and non-phenolized lymphs were sieved through no. 32-gauge wire-mesh to remove large particles before use.

Elementary body suspensions (E.B.S.). In order to obtain a strain of virus which could be easily purified, and which would show uniform characteristics during repeated passage, the Lister Institute strain (which is normally propagated alternately in the rabbit and the sheep) was converted to a 'homogeneous' strain as described by Beard, Finkelstein & Wyckoff (1937) and by Amies (1938). The starting material was a partially purified elementary body suspension prepared from sheep pulp by differential centrifugation (Pl. 1). This virus was then passed repeatedly on the skin of the rabbit, E.B.S. being prepared from each lot of passage material and used as the seed for the next passage. The E.B.S. were pre-

pared by a method similar to that of Craigie (1932), with Salaman's (1937) modification of fractional resuspension of the virus deposit obtained by angle centrifugation.

The virus obtained after four successive rabbit skin passages was fully homogeneous as judged by dark field and electron microscopy. Pl. 2 shows the changed appearance of the elementary bodies derived from the second rabbit passage. Each animal yielded 10 ml. of concentrated suspension with an egg titre of 10^8 to 10^9. E.B.S. from the 17th to the 52nd rabbit passages were used for these experiments.

Bovine plasma albumen (*Fraction V, Armour Laboratories*) and *normal horse serum* were diluted as required with distilled water.

Plasma-glucose mixture consisted of 70 % human pooled plasma in 0·9 % saline. 7 g. glucose was added per 100 ml.

The above solutions were filtered through Seitz EK pads.

Douglas digest broth was a tryptic digest of horse meat infusion, pH 7·4. It contained 0·0125 % $CaCl_2$ and 0·5 % NaCl.

Peptone broth. This was a horse meat infusion, pH 7·4, containing 1 % peptone and 0·5 % NaCl.

Peptone solutions. 1 and 5 % solutions of Evans Bacteriological Peptone were made in distilled water. The pH was adjusted to 8·0 with 40 % NaOH, after which the solutions were heated to 90° C., and filtered while hot. The pH was then changed to 7·4 with 50 % HCl.

Broths and peptone solutions were sterilized by autoclaving for 15 min. at 15 lb. pressure.

Dextran solution. A 6 % solution in physiological saline as used for human transfusion was further diluted with sterile saline to make a 1 % solution.

METHODS

(*a*) *Freeze-drying apparatus*

An experimental centrifugal freeze-drier of the type described by Greaves (1944) and by Fry & Greaves (1951) was used. Only a brief description of the apparatus will be given as later experiments showed that the results obtained could be duplicated using the standard Model 3PS drier supplied by Messrs W. Edwards and Co. (London) Ltd. It was previously stated (Collier, 1951) that sheep lymph dried in the Edwards apparatus deteriorated more rapidly than when dried in the experimental plant, but this was later found to be due to gross batch variation in the crude lymphs used.

The primary drying chamber accommodated 500 straight-sided ampoules set in solid metal drying heads at 18 degrees to the vertical, in which quantities up to 0·5 ml. could be dried (Pl. 3). The drying heads were mounted on a central shaft which could be rotated at 900 r.p.m. The chamber was evacuated with a 'Megavac' two-stage rotary oil pump. The moisture trap was a mechanically refrigerated coil at the bottom of the chamber, set just above the exit of the vacuum line to the pump. This coil operated at a temperature of -36 to -40° C.

Heat was supplied to the material being dried by electrically heated coils subjacent to the drying heads, input being controlled with a Variac-ammeter circuit. The temperature of the material was measured during drying by copper-constantan thermocouples inserted into sample ampoules. Readings were taken from the thermocouple leads by slip rings rotating outside the chamber. Thermocouples were also used to measure the coil temperature.

Vacuum measurements were made with a 'Vacustat' (McLeod type) mercury gauge attached to the vacuum line immediately outside the chamber.

The secondary drying system, comprising a small metal desiccator and two manifolds, was evacuated by an Edwards Model 2S 50 two-stage rotary oil pump, the desiccant being phosphorus pentoxide. Vacuum measurements were made with a Pirani gauge.

(*b*) *Drying technique*

The material to be dried was ampouled in 0·3 or 0·5 ml. amounts. The ampoules were closed with caps made of a layer of cotton wool between two layers of gauze. Such caps maintain sterility without interfering with the passage of water vapour.

Primary drying. The ampoules were placed in the upper tier of the primary chamber, which had been cooled to about 4° C. by allowing the refrigerator to run for an hour beforehand. After placing the thermocouples in position, the head was lowered into the chamber, which was then sealed. The drying heads were rotated at 900 r.p.m. and evacuation was begun.

'Snap-freezing' occurred about 15 min. later, when the vacuum had reached 1–2 mm. Hg. The rotor was stopped shortly afterwards, and drying was allowed to proceed for about 5 hr. at a vacuum of 0·05 mm. Hg. During this time, heat was supplied to the drying heads, the total input of watts being approximately equal to the number of ml. of material being dried.

Secondary drying. When the product temperature had risen to 15–20° C., the ampoules were transferred to the secondary desiccator, where they were dried for 18 hr. over P_2O_5. The vacuum ultimately reached in this system was 0·001 mm. Hg.

Constriction and sealing. After secondary desiccation, the ampoules were removed from the chamber, and constricted at the necks in a blow-lamp flame to facilitate subsequent sealing. No ampoule was allowed to remain in contact with the atmosphere for more than 2 or 3 min. during this process; those not actually being constricted were kept in glass desiccators over P_2O_5. They were then attached to the manifolds, and left for a further 2 hr. at high vacuum over P_2O_5 to remove any traces of moisture absorbed during the constriction process, being finally sealed under a vacuum of 0·001–0·003 mm. Hg.

Vacuum testing. The sealed ampoules were held at 4° C. overnight, and were examined next day with a high-frequency tester for retention of vacuum, those failing to give a blue-green fluorescence being discarded. Ampoules passing this test were then distributed for storage at the various temperatures.

Reconstitution. The dried material was reconstituted by adding distilled water to the original volume.

(c) *Infectivity titrations*

At the outset of these experiments, it was necessary to decide which method to use for the estimation of potency of stored preparations. A test of any batch after a given period of storage consisted of four or sometimes six titrations, that is to say, on the dried and liquid material stored at 4 and 22° C. and sometimes on the dried and liquid samples held at 37° C. In view of the number of batches under simultaneous observation, this entailed sixteen to twenty-four titrations a week.

The three possible methods were:

(i) Intracutaneous injection of ascending dilutions of virus in rabbits.

(ii) The scarification method of inoculating ascending dilutions in rabbits.

(iii) Pock counting on the chorioallantoic membranes of embryonated eggs.

The intracutaneous test has a greater reputation for accuracy than the scarification method. Four different preparations may be titrated in parallel on one animal, with a range of six serial dilutions for each titration. The technique is rapid, an important consideration when many tests have to be performed. It was found in practice, however, that the method has serious disadvantages with preparations that have been stored for some time. Anomalous results sometimes occurred, which have been described elsewhere (Collier, 1953*a*). Briefly, these are characterized by poor development of the intracutaneous lesions, and appear to be due to interference by dead virus with the remaining living vaccinia. In the tables referring to infectivity titrations of stored material, such results are merely indicated by the letter 'A', as the titres recorded were thought to be unreliable. Moreover, titrations of sheep lymph of low potency after storage gave unreliable results during the later stages of this work, since intracutaneous injections of low dilutions often produced non-specific reactions due to the presence of adventitious material (Collier, 1953*b*).

The scarification method is more tedious to perform than the intracutaneous titration, but has the same advantage that four preparations may be tested simultaneously, with five or six serial dilutions per titration. In addition, the results of such titrations are usually unequivocal, in contrast to the difficulties sometimes encountered in interpreting the intracutaneous test.

The pock-counting method has the advantage over animal methods that the results are much more reproducible. However, considerations of expense and labour forbade the exclusive use of this test in these experiments, since over 300 eggs a week would have been required.

It was decided at the outset to use the intracutaneous test for the screening experiments, with confirmatory scarification or pock-counting titrations when necessary. Although individual rabbit titrations sometimes gave misleading results due to variation in susceptibility between rabbits, a series of tests over a period gave a reasonably accurate indication of the rates of deterioration of different samples. In later experiments, where more accurate estimations were essential, greater use was made of the pock-counting method.

Rabbit titration techniques

Serial dilutions were made in cold 0·004 M phosphate-citric acid buffer pH 7·2, except for lanolinated lymph, which was diluted with liquid paraffin. Each dilution was made with a fresh pipette, and was thoroughly mixed before transfer to the next tube. The animals were inoculated immediately after the dilutions had been made.

Rabbits. Adult rabbits were used, weighing about 3 kg. The back and flanks were closely clipped before inoculation. Shaving and depilatory pastes were not used.

Use of a standard preparation of vaccinia. Normal susceptibility to vaccinia of all rabbits used was tested by scarification inoculation of 1/1000 and 1/10,000 dilutions of a laboratory standard lymph of known potency. In rabbits of normal susceptibility, the 1/1000 dilution gave rise to a confluent reaction; the 1/10,000 dilution produced a confluent or semiconfluent lesion. Results obtained with the few animals that failed to react normally were discarded.

Intracutaneous titrations. 0·2 ml. amounts of each dilution were injected in rows along the flanks. The end-point was arbitrarily defined as the highest dilution of the original preparation producing a papule of at least 20 mm.^{2}, developing between the 4th and 6th day, and persisting for at least 48 hr.

Scarification titrations. Approximately 0·1 ml. amounts of each dilution were inoculated into well-scarified areas on the flanks of rabbits, each area measuring 3 × 3 cm. The end-point was defined arbitrarily as the highest serial dilution of the original preparation which would produce at least five typical pocks, and which was preceded by a dilution producing twenty or more vesicles. In certain later experiments the highest dilution giving rise to a semiconfluent reaction was taken as the end-point; this has been indicated in the appropriate tables.

Pock-counting titration technique

Fertile eggs incubated for 12 days at 39° C. were inoculated on the chorio-allantoic membrane by the method of Beveridge & Burnet (1946, p. 15).

0·1 ml. amounts of each dilution were placed on the membranes of four to five eggs with a graduated Pasteur pipette; the results were read after a further 48 hr. incubation at 37° C. The mean number of pocks per membrane inoculated with a given dilution was determined; this number was then multiplied by the dilution factor to give the number of pock-producing particles per 0·1 ml. of the original preparation, the titre being expressed as the logarithm of this figure. Secondary foci, which were not common after this period of incubation, were ignored.

Burnet & Faris (1942) pointed out that the relationship between dilution and number of foci per membrane is not linear for vaccinia, since series of the type 300, 62, 10 and 1 foci were obtained with ascending tenfold dilutions. Our experience has been similar, and this raises the problem of the assessment of titre. Thus, for the series quoted, the titre could be taken as 3×10^5, $6{\cdot}2 \times 10^5$ or $1{\cdot}0 \times 10^6$, assuming the dilutions used to have been 1/1000, 1/10,000, 1/100,000 and 1/1,000,000. We therefore adopted the following technique.

A pilot titration was performed by inoculating three groups of two or three eggs with widely spaced dilutions. Groups of five or six eggs were then inoculated with two dilutions differing by a factor of 5 or 10, the lower of which was calculated from the pilot test to give semiconfluent lesions. The higher dilution then gave rise to a convenient number of discrete pocks from which the titre was calculated. This use of the dilution above that giving a semiconfluent reaction provided a standard method for comparing the results of different titrations. With experience, it was often possible to dispense with the pilot test.

RESULTS

I. Experiments with sheep lymph

(*a*) *The stability of glycerinated and lanolinated lymph*

As a background to the experiments on the preservation of vaccinia virus, some information is presented concerning the deterioration on prolonged storage of glycerinated and lanolinated lymph. The latter is issued to certain tropical countries and has the reputation for being more resistant to heat than the glycerinated vaccine. These lymphs were withdrawn at random from batches intended for routine issue, and the results are typical of the loss of potency which may be expected during storage at 4, 22 and 37° C.

Table 1. *Titres after storage of glycerinated vaccine lymph containing* 0·4 % *phenol*

Storage temp. (° C.)	Method of test	Storage time in weeks																		
		0	1	2	4	8	12	16	20	24	28	32	36	40	44	48	52	56	76	108
4	I	6	7	6	.	6	5	5	5	6	.	3	.	4	.	3	.	.	3	5
	S	.	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	2	.	2
22	I	.	7	5	.	A	A	A	2	1	.	0	.	1	.	1	.	.	1	A
	S	.	.	.	4	.	.	.	.	.	.	.	.	.	.	.	.	nil	.	nil
37	I	.	.	5	.	2	.	.	.	nil	.	.	.	.	.	.	.	.	.	.
	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Titres are expressed as the negative logarithm of the endpoint dilution. I = intracutaneous titration; S = scarification titration; A = anomalous result; O = reaction with undiluted material only; Nil = no reaction.

Storage of glycerinated lymph, batch 1629

Table 1 shows that the titre was well maintained for 24 weeks at 4° C., after which there was a gradual loss of potency. At 22° C. the rate of deterioration was much more rapid, since only anomalous results or low titres were recorded after the 8th week. At 37° C., the intracutaneous titre fell from 10^{-6} to 10^{-2} by the 8th week, and by the 24th week, no reaction could be obtained with the undiluted material.

Comparison of the stability of glycerinated and lanolinated lymph

Glycerinated lymph (batch 1826 in 10-dose capillary tubes) and lanolinated lymph (batch 1393 in 12-dose collapsible metal tubes) were stored and tested in parallel. Both these batches were titrated in fivefold dilution. All titrations were done by scarification, as it is not feasible to titrate lanolinated lymph intra-

cutaneously. In view of the marked initial disparity in titre of these batches, and to facilitate comparison of results, Table 2 does not show the actual titres, but gives instead the fall in potency observed at each test, i.e. the reciprocal of the initial titre divided by the reciprocal of the titre observed after storage.

At 4° C., deterioration rates were similar for both batches, but at 22° C., the glycerinated material lost potency much more rapidly than the lanolinated lymph. Both vaccines were completely inactivated after 1 month at 37° C., as shown by scarification test.

Table 2. *Comparison of deterioration rates of glycerinated and lanolinated vaccine lymph (All titrations done by scarification method)*

Material	Initial titre	Storage temp. (° C.)	Factor by which titre diminished after (weeks) 1	2	4	12	16	20	24	72
Glycerinated vaccine lymph	1:62500	4	5	.	0	0	5	.	25	25
		22	5	.	25	125	2,500	.	12,500	>62,500
		37	62·5	.	>62,500	>62,500	.	.	.	.
Lanolinated vaccine lymph	1:1250	4	5	.	0	5	5	5	5	25
		22	5	.	5	5	25	125	125	125
		37	.	125	>1,250	>1,250	.	.	.	.

(*b*) *Preservative influence of freeze-drying*

Sheep lymph dried without phenol

Table 3 shows the marked preservative influence of freeze-drying on sheep lymph stored at 22 and 37° C. At 4° C., both dried and liquid preparations behaved similarly, undergoing a very slow loss in titre. At both 22 and 37° C., deterioration of the dried suspensions was much slower than of the homologous liquid preparations. After 40 weeks storage at 37° C., a substantial amount of living virus remained in the dried lymph; this was confirmed by pock-counting titration.

Sheep lymph dried with 0·4 % *phenol*

Table 4 shows the results of a similar experiment, using a different batch of pulp containing phenol. The preservative influence of drying was again apparent, although deterioration of both dried and liquid suspensions at 22 and 37° C. was more rapid than with the previous batch. To determine whether this increased deterioration was due to the presence of phenol, or merely to batch variation in the lymphs used, a limited experiment was done using phenolized and non-phenolized samples prepared from the same lot of pulp. Table 5 shows that 0·4 % phenol does in fact hasten the deterioration of dried lymph stored at 37° C. (In this and some subsequent experiments deterioration of potency is given as the ratio of the initial egg titre to the titre after treatment, thus facilitating comparison of different batches.)

The loss in titre which occurs in some phenolized suspensions of sheep lymph during freeze-drying was previously shown to be due to concentration effects during the rapid cooling which takes place before the eutectic temperature is attained (Collier, 1953*c*).

Table 3. *Titres after storage of sheep lymph.* 1/5 *suspension in distilled water, without phenol*

Storage temp. (° C.)	State	Method of test	Storage time in weeks: 0	1	2	4	8	12	16	20	24	28	32	36	40	44	48	52	56	64	80	116
4	Dried	I	5	6	6	6	6	6	6	5	6	.	.	.	3	.	3	.	.	5	.	.
		S	4	.	.	.	.	.	.	.	.	.	4	.	.	.	.	3	.	3	.	.
		P.C.	.	.	.	.	.	.	.	7·04	.	.	.	.	.	.	.	.	.	.	.	.
	Liquid	I	>6	6	7	7	6	6	7	6	7	.	.	.	3	.	5	.	.	5	5	4
		S	.	.	.	.	.	.	.	.	.	.	4	.	.	.	.	5	.	3	.	5
		P.C.	.	.	.	.	.	.	.	7·08	.	.	.	.	.	.	.	.	.	.	.	.
22	Dried	I	.	6	5	5	5	4	5	5	6	.	.	.	2	.	4	.	.	5	5	.
		S	.	.	.	.	.	.	.	.	.	.	3	.	.	.	.	5	.	2	.	.
		P.C.	.	.	.	.	.	.	.	6·15	.	.	.	.	.	.	.	.	.	.	.	.
	Liquid	I	.	5	5	5	6	5	6	5	5	.	.	.	2	.	4	.	.	2	1	.
		S	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.	1	.	Nil	.	.
		P.C.	.	.	.	.	.	.	.	5·11	.	.	.	.	.	.	.	.	.	.	.	.
37	Dried	I	.	.	.	.	5	.	4	.	4	.	.	.	3	.	.	.	.	.	.	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		P.C.	.	.	.	.	5·95	3·48	.	.	.	3·70	.	.	3·79	.	.	.	.	.	.	.
	Liquid	I	.	.	.	.	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		S	contaminated																			
		P.C.	.	.	.	.	<1·00	Nil	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Egg titres are expressed as the log number of pock-producing units per 0·1 ml. of suspension. P.C. = Pock count on chorioallantois. Other symbols as in Table 1.

Table 4. *Titres after storage of sheep lymph.* 1/5 *suspension in distilled water containing* 0·4 % *phenol*

Storage temp. (° C.)	State	Method of test	Storage time in weeks: 0	1	2	4	8	12	16	20	24	28	32	36	40	44	48	52	56	64	80	116
4	Dried	I	6	6	A	6	6	6	3	4	6	.	6	.	3	.	5	.	.	4	4	4
		S	.	.	.	.	4	.	.	.	4	.	.	.	.	.	.	.	4	3	.	5
		P.C.	7·08	.	.	.	.	.	.	5·90	.	.	.	.	.	.	.	.	.	.	.	.
	Liquid	I	7	6	7	7	6	6	3	6	5	.	6	.	4	.	5	.	.	4	3	4
		S	.	.	.	.	4	.	.	.	4	.	.	.	.	.	.	.	4	4	.	5
		P.C.	.	.	.	.	.	.	.	6·70	.	.	.	.	.	.	.	.	.	.	.	.
22	Dried	I	.	6	A	6	6	A	A	2	6	.	5	.	3	.	5	.	.	2	3	2
		S	.	.	.	.	.	.	.	.	3	.	.	.	.	.	.	.	1	3	.	2
		P.C.	.	.	.	.	5·00	.	.	3·15	.	.	.	.	.	.	.	.	.	.	.	.
	Liquid	I	.	6	A	5	6	5	1	2	5	.	A	.	2	.	1	.	.	1	.	.
		S	.	.	.	.	.	.	.	.	1	.	.	.	.	.	.	.	Nil	Nil	.	.
		P.C.	.	.	.	.	5·57	.	.	3·28	.	.	.	.	.	.	.	.	.	.	.	.
37	Dried	I	.	.	.	.	A	3	.	.	3	.	3	.	1	.	.	.	.	.	.	1
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Nil	.	.	Nil
		P.C.	.	.	.	.	3·70	2·94	.	.	.	.	.	.	2·49	.	.	.	.	.	.	.
	Liquid	I	.	.	.	.	A	.	.	.	2	.	.	.	1	.	.	.	.	.	.	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Nil	.	.	.
		P.C.	.	.	.	.	<2·00	Nil	.	.	.	.	.	.	Nil	.	.	.	.	.	.	.

Symbols as in Table 3.

Table 5. *The influence of phenol on dried sheep lymph stored at* 37° C.

Percentage phenol in dried lymph	Log egg titre after: Drying (A)	Storage for 1 month (B)	Storage for 2 months (C)	Fall in titre after: Storage for 1 month (A/B)	Storage for 2 months (A/C)
Nil	7·62	6·93	6·84	4·9	6·0
0·4	7·36	5·70	5·68	45·7	47·8

Table 6. *Comparison of behaviour on storage of two batches of dried sheep lymph*

Batch	Log egg titre after: Drying (A)	1 month at 22° C. (B)	1 month at 37° C. (C)	Reduction in potency after storage at: 22° C. (A/B)	37° C. (A/C)
2031	6·32	3·74	1·34	380	95500
2093	6·51	4·72	3·23	62	1905

(*c*) *Variation in stability of different batches of dried lymph*

Different batches of dried sheep lymph were liable to considerable variation in stability. An illustrative experiment is recorded in Table 6. Two batches of sheep lymph (2031 and 2093) were freeze-dried under identical conditions in an Edwards Model 3 apparatus. Storage tests showed that batch 2031 deteriorated much more rapidly at both 22 and 37° C.

(*d*) *Influence of prolonged secondary desiccation*

Although freeze-drying exerted a marked preservative influence on sheep lymph, deterioration at 22 and 37° C. was still too great to permit the use of such material as a dried vaccine. The influence of reduction in the residual moisture by prolonged desiccation over P_2O_5 on its keeping properties was therefore observed.

Drying procedure. A 1/5 phenolized suspension of pulp no. 2142 was freeze-dried. After 17 hr. secondary desiccation, the ampoules were constricted at the necks, divided into two lots, and placed on the vacuum manifolds.

Lot D1 was sealed off under a vacuum of 0·005 mm. Hg after a further 2 hr. *in vacuo* over P_2O_5. Lot D2 was left on the secondary manifold for a further 173 hr. at a vacuum of 0·001 mm. Hg.

The total periods of secondary desiccation were therefore 19 and 190 hr. respectively. Lot D1 was left in the drying laboratory until the second group had been sealed, in order to expose both sets of ampoules to the same temperatures before storage.

Moisture determinations. These were performed by Messrs Edwards and Co., using an apparatus of their own devising (Beckett, 1954). I am indebted to this company for permission to publish the results of these determinations.

The method depends on the evolution of moisture from the dried material *in vacuo*, and its condensation in a cold trap. The ice so formed is then allowed to evaporate within a vacuum enclosure of known volume, and the resulting pressure

rise is measured by an oil manometer. From a knowledge of the ambient temperature, the pressure rise and the volume of the system, the mass of water within the system can be calculated. (1 g. mol. = 22·4 l. at N.T.P.)

The estimations were done on each lot in duplicate, using different ampoules. Lot D 1, dried for the shorter period, contained 4·6 times as much residual moisture as did lot D 2 (Table 7).

Table 7. *Moisture content of dried lymph after normal and prolonged secondary desiccation*

Lot	Secondary drying time (hr.)	Test no.	Weight of sample (g.)	Weight of residual moisture (g.)	Percentage residual moisture by weight	Mean percentage residual moisture
D1	19	1	0·0116	$3·7 \times 10^{-5}$	0·319	0·32
		2	0·0147	$4·7 \times 10^{-5}$	0·320	
D2	190	1	0·0144	$1·07 \times 10^{-5}$	0·074	0·07
		2	0·0120	$0·79 \times 10^{-5}$	0·066	

Table 8. *The influence of prolonged secondary drying on the keeping properties of lymph (All titrations done by scarification method)*

Lot	Secondary drying time (hr.)	Titre: Before drying	Titre: After drying	Storage temp. (° C.)	Storage time (weeks): 5	14	26
D1	19	4	3	22	3	1	1
				37	Nil	Nil	.
D2	190		3	22	2	0	0
				37	Nil	Nil	.

Titres are expressed as the negative logarithm of the end-point dilution.
0 = reaction with undiluted material only; Nil = no reaction.

Results of infectivity titrations

All tests were done by scarification (Table 8).

Storage at 22° C. Scarification tests after 5, 14 and 26 weeks' storage showed that the titre of lot D 2 diminished more rapidly than did that of the lymph dried for the normal period, giving lower titres on each occasion.

Storage at 37° C. Neither batch showed any activity by the scarification test after 5 weeks.

It was therefore concluded that prolonged secondary desiccation does not improve the survival of virus in dried sheep lymph.

(*e*) *Influence of the atmosphere of storage on the stability of dried lymph*

The freeze-dried suspensions considered previously were all sealed under high vacuum. This process is tedious as it involves preliminary constriction of the ampoule necks; without thus reducing the lumen, the ampoule wall collapses when the sealing flame is applied. It is possible, however, to seal tubes whose contents are under atmospheric pressure in a semi-automatic apparatus without the necessity for this constriction, or for sealing off each ampoule on a vacuum

manifold by hand. This would represent an important saving of labour in the large-scale production of a dried vaccine.

Although many workers have prepared dried smallpox vaccine in various forms, no reference has been found to comparative experiments with dried vaccinia stored under vacuum, and under an inert gas such as nitrogen at atmospheric pressure. It was, however, noticed by Achalme & Phisalix (1909) that dried lymph stored under the vacuum produced by a water pump retained its potency better than did that sealed in glass ampoules under air at atmospheric pressure. Otten (1927) stressed that dried lymph should be stored *in vacuo*, and maintained that certain poor results with his dried vaccine were due to defective sealing of ampoules. He stated, however (Otten, 1932) that storage pressure could be safely increased from 0·01 to 0·25 mm. Hg.

Several experiments in this laboratory showed that dried vaccinia (E.B.S. or sheep pulp) tended to deteriorate more rapidly at 22 and 37° C. (but not at −10° C.) when stored under 'dry nitrogen' than when sealed under vacuum. In these experiments, the ampoules were plugged tightly with cotton-wool after secondary drying in a desiccator for 18 hr. over P_2O_5 at approx. 0·003 mm. Hg. They were then returned to the desiccator which was re-evacuated to 0·003 mm. Hg. Nitrogen was then run into the desiccator, passing first through a long silica gel drying tower. When atmospheric pressure was attained, the ampoules were removed in groups of three or four, and sealed with a semi-automatic device, the cotton-wool plugs preventing diffusion of air into the tubes. A small flow of nitrogen was allowed to pass through the desiccator while it still contained ampoules awaiting sealing. The 'dry' nitrogen was supplied by the British Oxygen Company, and was stated to contain less than 10 parts per million of oxygen. It was thought, however, that the rapid deterioration might have been due to traces of moisture or oxygen in the nitrogen, or which gained access to the ampoules during the sealing process.

The following experiment was devised to test this possibility.

A 1/5 suspension of phenolized sheep lymph was prepared from pulp no. 2111, and freeze-dried in the usual way. Secondary desiccation was carried out for 18 hr. over phosphorous pentoxide in the secondary drying chamber, at a vacuum of 0·001 mm. Hg. The ampoules were then divided into three lots.

Lot E 1 ampoules were plugged firmly with cotton-wool as before, and returned to the desiccator, which was then re-evacuated to 0·004 mm. Hg.

Ampoules in lots E 2 and E 3 were constricted and placed on two separate vacuum manifolds, which were pumped down to 0·004 mm. Hg. and then isolated from the vacuum line. Subsequent treatment was as follows:

Lot E 1. Nitrogen was run into the desiccator directly from the cylinder, passage through the silica gel drying tower previously described being omitted. When atmospheric pressure was attained in the desiccator, the ampoules were removed and sealed as previously described.

Lot E 2. Nitrogen was passed from the cylinder into the appropriate manifold through the vacuum tight system to be described, which was dried over phosphorus pentoxide *in vacuo* before use. The nitrogen passed first through a drying tower containing P_2O_5 in order to remove traces of moisture, and then traversed

a cylinder containing red hot copper turnings to remove traces of oxygen. The nitrogen finally passed through a U-tube in an ice-water bath to cool it before it entered the manifold. To prevent the ampoules being blown off the rubber nipples of the manifold, a valve was included in the system, so arranged that the gas pressure was not allowed to rise above that of the atmosphere. When the ampoules had been filled in this manner with dry, oxygen-free nitrogen, they were sealed off *in situ*.

Lot E3. These ampoules were sealed on the manifold under a vacuum of 0·004 mm. Hg.

Samples of each lot were then stored at 22 and 37° C.

Table 9. *Comparison of keeping properties of dried lymph stored under different atmospheres*

(a) Results of scarification titrations

Lot	Dried lymph sealed under	Titre: Before drying	Titre: After drying	Storage temp. (° C.)	Storage time (weeks): 4	8	36
E1	'Cylinder nitrogen'	4	4	22	2	1	Nil
				37	Nil	.	.
E2	O_2 free nitrogen		4	22	3	1	Nil
				37	Nil	.	.
E3	Vacuum		4	22	3	2	1
				37	0	.	.

Titres are expressed as the negative logarithm of the end-point dilution.
0 = reaction with undiluted material only; Nil = no reaction.

Table 10. *Comparison of keeping properties of dried lymph stored under different atmospheres*

(b) Results of egg titrations

Lot	Dried lymph sealed under	Log egg titre: Before drying (A)	Log egg titre: After drying (B)	Log egg titre: After 1 month at 37° C. (C)	Reduction in potency due to: Drying (A/B)	Reduction in potency due to: Storage at 37° C. (B/C)
E1	'Cylinder nitrogen'	7·26	6·34	1·90	8	27,540
E2	O_2 free nitrogen		6·30	< 1·00	9	> 199,500
E3	Vacuum		6·32	3·11	9	1,622

Storage at 22° C. (Table 9). No egg titrations were done on material stored at this temperature, but scarification tests at 4, 8 and 36 weeks showed that the vacuum-sealed lymph deteriorated less rapidly than did the two nitrogen packed lots; the latter did not differ significantly in the rate at which they lost potency.

Storage at 37° C. (Tables 9 and 10). Both scarification and egg tests after 1 month at 37° C. showed that the vacuum-sealed lot again had an advantage over material stored under 'cylinder nitrogen' or oxygen-free nitrogen.

On the basis of this and other experiments, it was decided that vacuum sealing was the safest method to adopt for preserving dried vaccinia virus.

II. EXPERIMENTS WITH ELEMENTARY BODY SUSPENSIONS (E.B.S.)

The experiments described in Part I showed that although freeze-drying exerted a considerable preservative influence on crude sheep lymph, such preparations still fell short of the standard required of an efficient dried vaccine, and that different dried batches were liable to variation in their resistance to storage at the higher temperatures. The behaviour of purified suspensions of virus was therefore investigated in the hope that they would prove more uniform, and that a suitable suspending medium could be found. Several media were tested, some of which have been claimed by previous workers to be effective as preservative agents (Collier, 1954).

The merits of the different media, both liquid and dried, were assessed against the behaviour at various temperatures of 'unprotected' virus stored in 0·004M McIlvaine buffer, pH 7·2, in the liquid state, due allowance being made for differences in initial titre. This solution was selected as being a simple inorganic medium in which the virus would remain dispersed for long periods, maintaining its titre for many months at 4° C. Distilled water was not used for the control suspension, since virus suspended therein flocculates rapidly, and loses potency more rapidly at 4, 22 and 37° C. than when suspended in buffer.

It may be mentioned here that attempts to dry vaccinia suspended in weak buffer, saline or distilled water alone resulted in complete or almost complete inactivation.

Table 11. *Titres after storage of* E.B.S. *in* 0·004M *phosphate-citric acid buffer* pH 7·2

Storage temp. (° C.)	Method of test	Storage time in weeks																	
		0	1	2	4	8	12	16	20	24	28	32	36	40	44	48	52	56	108
4	I	7	7	6	6	6	5	6	.	5	.	5	.	6	.	6	.	.	5
	S	4	.	.	.	.	.	.	4	.	.	.	.	.	.	.	.	4	4
	P.C.	.	.	.	.	.	.	.	.	.	.	6·73	.	.	.	.	.	.	.
22	I	.	7	6	6	6	4	3	.	3	.	A	.	A	.	A	.	.	.
	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Nil	.
	P.C.	.	.	.	.	.	.	.	.	.	.	4·48	.	.	.	.	.	.	.
37	I	.	.	.	3	Nil	.	.	.	.	.	.	.	.	.	.	.	.	.
	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P.C.	.	.	.	4·18	< 1·00	.	.	.	.	.	Nil	.	.	.	.	.	.	.

Symbols as in Table 3.

(a) *Stability of 'unprotected'* E.B.S.

E.B.S. *in* 0·004M *McIlvaine buffer* pH 7·2

Preparation of batch. 0·1 ml. of concentrated E.B.S. no. 33 was added to 100 ml. buffer, and stored in the liquid state only.

pH *after storage.* The pH values of suspensions held at 4, 22 and 37° C., tested after storage for 1 year were 7·3, 7·3 and 7·1 respectively.

At 4° C. the titre remained virtually unchanged for 2 years, but showed a definite fall after 12 weeks at 22° C.; at 37° C. inactivation was complete by the 8th week (Table 11).

A similar experiment with virus suspended in slightly acid buffer (0·004M, pH 6·8) gave almost identical results.

The influence of phenol on 'unprotected' E.B.S.

As it is an advantage to be able to store liquid E.B.S. in the presence of phenol, in order to avoid bacterial contamination, the action of this chemical on the virus was investigated over a long period.

Preparation of batch. This was as for the previous batch, using the same lot of virus, except that phenol was added to a concentration of 0·25 %.

pH *after storage.* After storage for 1 year, values of 7·1, 6·8 and 7·0 were obtained for suspensions stored respectively at 4, 22 and 37° C.

At 4° C. no difference was observed between this batch and the non-phenolized suspension, the titres of both being much alike at all times up to 2 years (Table 12). At both 22 and 37° C., however, there was a more rapid diminution in titre than when phenol was omitted.

Table 12. *Titres after storage of* E.B.S. *in* 0·004M *phosphate-citric acid buffer* pH 7·2, *containing* 0·25 % *phenol*

Storage temp. (° C.)	Method of test	Storage time in weeks																	
		0	1	2	4	8	12	16	20	24	28	32	36	40	44	48	52	56	108
4	I	7	7	6	5	6	5	6	.	3	.	5	.	6	.	6	.	.	4
	S	.	.	.	.	.	.	.	4	.	.	.	.	.	.	.	.	4	4
	P.C.	.	.	.	.	.	.	.	.	.	.	6·78	.	.	.	.	.	.	.
22	I	.	7	5	4	6	5	4	.	2	.	A	.	0	.	A	.	.	.
	S	.	.	.	.	.	.	.	3	.	.	.	.	.	.	.	.	Nil	.
	P.C.	.	.	.	.	.	.	.	.	.	.	2·70	.	.	.	.	.	.	.
37	I	.	.	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	P.C.	.	.	.	2·60	< 1·00	.	.	.	.	.	Nil	.	.	.	.	.	.	.

Symbols as in Table 3.

(*b*) *Selection of suspending medium*

As previously stated, the virus suspended in liquid 0·004M McIlvaine buffer, pH 7·2 (without phenol) was regarded as the standard preparation against which were assessed the preservative properties of the various suspending media, both in dried and liquid state. The assessment of the protection afforded by various treatments is given in Table 15. For reasons of space, it is not possible to quote the full results obtained with each preparation. The only findings given in full are those with digest broth, which exerted a preservative influence in the dried state at 22° C., but not at 37° C., and those of a preliminary experiment with 1 % peptone, which showed a marked preservative influence at both these temperatures in the dried state. The same lot of virus was used for both batches; 0·1 ml. concentrated E.B.S. was added to 100 ml. medium.

E.B.S. *in Douglas digest broth* (Table 13)

Storage at 4° C. The dried suspension maintained a high titre for a considerable period. Intracutaneous and pock-counting titrations showed no significant loss in titre after 36 weeks, and intracutaneous and scarification tests showed titres of

10^{-4} and 10^{-3} respectively after more than 2 years storage; the liquid suspension deteriorated rather more rapidly.

Storage at 22° C. The influence of freeze-drying was well demonstrated at this temperature. After the 8th week, the liquid suspension had undergone a considerable loss of potency, and at the 12th week showed only minimal activity. The dried preparation, on the other hand, maintained its activity as well as the homologous liquid suspension held at 4° C., and was not completely inactivated after storage for 84 weeks.

Table 13. *Titres after storage of* E.B.S *in digest broth*

Storage temp. (° C.)	State	Method of test	Storage time in weeks: 0	1	2	4	8	12	16	20	24	28	32	36	40	44	48	52	60	84	116
4	Dried	I	5	5	6	5	5	5	4	4	A	5	.	5	.	5	.	4	.	3	4
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	4	.	3
		P.C.	6·49	.	.	.	.	6·11	.	.	.	.	.	6·28	.	.	.	.	.	.	.
	Liquid	I	5	4	4	5	5	5	5	A	A	4	.	5	.	A	.	5	.	4	3
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	2
		P.C.	.	.	.	.	.	6·04	.	.	.	.	.	4·88	.	.	.	.	.	.	.
22	Dried	I	.	5	5	5	5	5	5	4	A	5	.	5	.	3	.	4	.	3	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.
		P.C.	.	.	.	.	.	4·53	.	.	.	.	.	4·48	.	.	.	.	.	.	.
	Liquid	I	.	4	5	5	4	1	<1	<1	Nil	A	.	0	.	Nil	.	Nil	.	.	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		P.C.	.	.	.	.	.	1·40	.	.	.	.	.	Nil	.	.	.	.	.	.	.
37	Dried	I	.	.	.	<1	.	.	.	.	.	.	.	.	Nil	.	.	.	.	.	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		P.C.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Liquid	I	.	.	.	<1	.	.	.	.	.	.	.	.	Nil	.	.	.	.	.	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		P.C.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Symbols as in Table 3.

Storage at 37° C. The encouraging results obtained with the dried suspension stored at 22° C. were not reflected in the behaviour of this batch at 37° C., both liquid and dried preparations being rapidly inactivated, a result which will be considered in the Discussion.

E.B.S. *in* 1 % *peptone* (Table 14)

Storage at 4° C. Both liquid and dried material behaved similarly, undergoing comparatively slow deterioration over a long period. After about a year, intracutaneous titres of 10^{-4} and scarification titres of 10^{-2} were obtained with both preparations. The titre of the liquid suspension remained at this level until the 64th week, after which no further material remained for test; the dried material still showed some activity after more than 2 years.

Storage at 22° C. A considerable number of anomalous results were obtained with both preparations held at this temperature, but it is obvious from Table 14 that freeze-drying protected well, active virus being demonstrable in the dried suspen-

sion after more than 2 years storage, whereas the liquid showed little activity after the 16th week.

Storage at 37° C. The freeze-dried virus maintained some activity for a remarkably long period. A scarification titration done at the 116th week gave a semi-confluent reaction with the undiluted material, and survival of virus was confirmed

Table 14. *Titres after storage of* E.B.S. *in* 1 % *peptone*

Storage temp. (° C.)	State	Method of test	Storage time in weeks 0	1	2	4	8	12	16	20	24	28	32	36	40	44	48	52	64	116
4	Dried	I	6	6	A	5	5	A	3	4	2	.	A	.	A	.	4	A	4	1
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.
		P.C.	5·26	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Liquid	I	6	6	4	5	4	4	3	4	3	.	0	.	A	.	4	3	4	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2	.	.
		P.C.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
22	Dried	I	.	6	A	4	4	A	A	A	A	.	0	.	0	.	4	A	4	3
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1	.	1
		P.C.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	Liquid	I	.	5	6	4	2	2	2	A	A	.	Nil	.	0	.	Nil	Nil	A	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Nil	.	.
		P.C.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
37	Dried	I	.	.	.	4	.	3	A	.	.	.	.	.	2	.	.	.	.	A
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	0
		P.C.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	2·34
	Liquid	I	.	.	.	<1	.	Nil	.	.	.	.	.	.	Nil	.	.	.	.	.
		S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		P.C.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Symbols as in Table 3.

Table 15. *Preservative influence of various suspending media on vaccinia virus stored at* 22 *and* 37° C.

Preparation	22° C. Dried	22° C. Liquid	37° C. Dried	37° C. Liquid
1 % bovine plasma albumen (fract V)	0	0	0	0
5 % bovine plasma albumen (fract V)	±	0	0	0
10 % normal horse serum	±	N.T.	+	0
Plasma-glucose mixture	0	–	0	–
Douglas digest broth	+	0	0	0
1 % peptone	+	0	+	0
5 % peptone	+	N.T.	+	N.T.
1 % peptone broth	+	0	+	0
1 % dextran	0	0	0	0

\+ = marked preservative influence;
± = slight preservative influence;
0 = no preservative influence;
N.T. = not tested for sufficiently long period, due to contamination;
– = not stored in liquid state.

by a pock count on the chorioallantois of log 2·34. By contrast, the liquid suspension stored at the same temperature was inactivated within the first month.

Table 15 shows the assessment of the protective action exerted by a number of suspending media tested in this way.

(c) *Confirmatory experiments with* E.B.S. *freeze-dried in peptone*

The preliminary results with virus dried in peptone suggested further experiments, making more use of the pock counting and scarification methods of titration to assess deterioration rates more accurately. 5 % peptone seemed to give better results than the 1 % solution. Thus the batch of virus in 1 % peptone described above had an initial egg titre of log 5·26, which fell to log 2·34 after storage for 116 weeks at 37° C. A batch of virus dried in 5 % peptone with an initial titre of log 5·30 still showed a titre of log 4·26 after the same period at 37° C., i.e. a fall of only 11-fold.

Trial of different peptones

Hitherto, only Evans Bacteriological peptone, batch C12590 had been used for these experiments. To determine that other peptones were equally effective, four aliquots of an E.B.S. were suspended respectively in this batch of Evans peptone, Evans Bacteriological peptone batch K12580, Difco Proteose peptone, and Difco Bacto peptone, each in 5 % concentration, prepared as described under 'Materials'.

Table 16. *Protective influence on vaccinia virus of different batches of peptone.*

(a) *Results of scarification titrations*

	Scarification titre* after			
		Storage at 37° C. for		
Batch of peptone	Drying	4 weeks	12 weeks	52 weeks
Evans C12590	1/16,000	1/8,000	1/4,000	1/640
Evans K12850	1/16,000	1/8,000	1/4,000	1/640
Difco proteose	1/16,000	1/16,000	1/2,000	1/1,280
Difco Bacto	1/16,000	1/8,000	1/4,000	1/640

* End-point: highest dilution giving semiconfluent reaction.

Table 17. *Protective influence on vaccinia virus of different batches of peptone.*

(b) *Results of egg titrations*

	Log egg titre after				Fall in titre after storage at 37° C. for		
		Storage at 37° C for					
Batch of peptone	Drying (A)	4 weeks (B)	12 weeks (C)	52 weeks (D)	4 weeks (A/B)	12 weeks (A/C)	52 weeks (A/D)
Evans C12590	7·38	7·15	7·11	6·42	1·7	1·9	9·1
Evans K12850	7·15	7·26	7·23	6·30	0·0	0·0	7·1
Difco proteose	7·20	6·97	7·15	6·30	1·7	1·1	7·9
Difco Bacto	7·34	7·43	7·18	6·46	0·0	1·4	7·6

The four suspensions were freeze-dried in 0·3 ml. amounts, stored at 37° C. and titrated in eggs and by scarification after 4, 12 and 52 weeks. Tables 16 and 17 show that there was no marked difference in the preservative influence of the various peptones.

Trial of dried vaccine in the field

A number of experiments similar to the last had shown that purified virus freeze-dried in 5 % peptone, pH 7·4, did not fall in titre by more than a factor of 2 when stored at 37° C. for 1 month. Thereafter, the deterioration rate was even slower. The method thus seemed suitable for the production of a heat stable vaccine, and a batch was used for vaccinating groups of children after storage under various conditions. In this and all subsequent experiments the material was dried in 0·25 ml. amounts (25 doses) in an Edwards Model 3PS centrifugal drier, as it was hoped to use this apparatus for eventual large-scale production. The main differences in technique consisted in constricting the ampoules immediately after the primary drying, and carrying out secondary desiccation on the manifolds alone; cotton-wool plugs were inserted in the ampoules during this stage to prevent contamination from the nipples of the manifolds.

Table 18. *Vaccination success rates with vaccinia dried in* 5 % *peptone and stored at* 22 *and* 37° C

Storage temp. (° C.)		Time stored (weeks)						
		0	4	8	12	16	24	52
22	Log egg titre	7·28	.	.	.	.	6·63	6·04
	No. vaccinated	23	.	.	.	.	22	12
	No. positive	23	.	.	.	.	21	12
37	Log egg titre	.	6·89	6·40	6·30	6·28	.	.
	No. vaccinated	.	23	18	22	21	.	.
	No. positive	.	22	17	21	21	.	.

Vaccinations were done by the same operator, using the following technique. Each 25-dose ampoule was reconstituted with 40 % glycerol buffered at pH 7·4. A needle was flamed, and after cooling was used to transfer a small quantity of vaccine from the tube to the vaccination site. A linear scratch, $\frac{1}{4}$ in. (0·6 cm.) long was made through the drop of vaccine, and the site covered with a piece of Elastoplast dressing strip. Results were recorded on the 7th day after vaccination. The children were aged from 3 months to 6 years, and none had been vaccinated previously.

Table 18 shows that after 1 year at 22° C., the vaccine had fallen in egg titre by a factor of 17, and still gave a full quota of successful vaccinations. After 4 months at 37° C., the titre had fallen by a factor of 10, and all vaccinations were positive.

Typical primary vesicles were produced in all cases recorded as positive. The inflammatory areola was smaller than that produced by glycerinated lymph, and in some cases was absent. This was probably due to the relative freedom of dried E.B.S. from adventitious material.

(*d*) *Experiments with partially purified virus derived from sheep*

At this stage, attention was paid to the possibility of producing this vaccine on a large scale. In view of the need for large quantities of E.B.S., attempts were made to see if these results could be reproduced using virus derived from sheep pulp,

which is readily available. It was known that different batches of crude pulp were liable to considerable variation in their keeping properties when dried, so that some measure of purification was desirable. Initially, observations were made to see whether constant results could be obtained with virus only partially purified.

As a routine procedure, 25 g. crude pulp were ground in a mortar with 80 ml. phosphate-citric acid buffer, 0·004M, pH 7·2, and 1·0 g. powdered neutral glass. This crude suspension was then clarified by three cycles of low-speed centrifugation, after which the pooled supernatants containing the virus were centrifuged for 2½ hr. at 3000 r.p.m. in the angle head. The resulting deposit was resuspended in 15 ml. of the same buffer, containing 0·5 % phenol. This suspension was clarified by low-speed horizontal centrifugation for 2 min. The supernatant was saved, the deposit resuspended in a further 15 ml. of phenol-buffer, and clarified again. The pooled supernatants constituted the final E.B.S., which was then incubated at 22° C. for 48 hr. to reduce bacterial contamination.

Table 19. *Fall in titre of* E.B.S. *dried in* 5 % *peptone during processing*

Batch no.	Log egg titre of parent E.B.S. (A)	Log egg titre of dried vaccine (parent E.B.S. diluted tenfold) (B)	Fall in titre (A/(B × 10))
S 19	9·04	7·70	2·2
S 23	8·85	7·76	1·2
S 24	8·93	8·00	0·0
S 27	8·85	7·64	1·6
S 29	9·08	7·72	2·3
S 32	8·90	7·40	3·2

The E.B.S. was then plated to determine the bacterial count, and titrated in eggs for virus content. It was not used unless the bacterial count was less than 1000 organisms per ml. and the virus titre more than 5×10^8. After passing these tests, 1 vol. of E.B.S. was diluted 10 times with 5·5 % peptone, made up as described under 'Materials'. The suspension was then ampouled in 0·25 ml. amounts and freeze-dried in the Edwards 3PS apparatus. The final product therefore contained not more than 100 organisms per ml., i.e. 10 times less than the statutory maximum. The virus titre in eggs, allowing for dilution with peptone and losses due to freeze-drying, did not fall below 10^7; experiments, not reported here, showed that this titre was the minimum necessary to secure 100 % successful primary vaccinations, by both multiple pressure and scratch techniques.

Loss in titre due to freeze-drying. Table 19 shows the loss in titre which occurred in six batches between completion of preparation of the parent E.B.S., and completion of freeze-drying. The fall in egg titre varied from nil to about threefold. These observations were selected from sixteen similar determinations by reference to a set of random numbers.

Storage at 37° C. Table 20 shows the deterioration of eight batches of vaccine similarly chosen at random from twenty-seven batches stored at 37° C. The Therapeutic Substances Regulations stipulate that smallpox vaccine shall produce a confluent reaction on the rabbit skin when diluted 1/1000, and at least ten

vesicles at a dilution of 1/10,000. All these batches conformed to this standard after storage for 3 months at 37° C. Indeed, only two batches out of the twenty-seven failed to pass the test at 3 months, but both of these passed after storage for one month at this temperature.

Table 20. *Storage at* 37° C *of sheep virus dried in* 5 % *peptone*

Batch no.	Scarification titre* after: Drying	Scarification titre* after: Storage for 1 month	Scarification titre* after: Storage for 3 months	Log egg titre after: Drying	Log egg titre after: Storage for 1 month	Log egg titre after: Storage for 3 months
S 10	1/10,000	1/10,000	1/10,000	7·34	7·42	7·20
S 14	> 1/20,000	1/10,000	1/10,000	7·48	7·62	7·15
S 19	1/40,000	1/10,000	1/20,000	7·70	7·20	7·30
S 21	1/40,000	1/20,000	1/10,000	7·97	7·83	7·18
S 23	1/40,000	1/10,000	1/20,000	7·76	7·59	7·32
S 25	1/40,000	1/20,000	1/10,000	7·60	7·49	7·46
S 28	> 1/40,000	1/20,000	1/10,000	7·85	7·64	7·20
S 29	1/40,000	1/10,000	1/20,000	7·72	7·53	7·49

* End-point: highest dilution giving semiconfluent reaction.

Table 21. *Storage at* 45° C *of sheep virus dried in* 5 % *peptone*

Batch no.	Scarification titre* after: Drying	Scarification titre* after: Storage for 1 month	Log egg titre after: Drying	Log egg titre after: Storage for 1 month
S 27	1/40,000	1/10,000	7·64	7·30
S 28	> 1/40,000	1/20,000	7·85	7·73
S 29	1/40,000	1/20,000	7·72	7·66
S 30	1/20,000	1/20,000	7·79	7·85

* End-point: highest dilution giving semiconfluent reaction.

Table 22. *Results of bacteriological tests on batches of sheep* E.B.S. *dried in* 5 % *peptone*

Batch no.	Colonies grown from 1·0 ml. of dilution 10^{-2}	10^{-3}	10^{-4}
S 10	1	1	1
S 11	1	2	0
S 17	0	0	0
S 20	0	0	0
S 23	0	0	0
S 28	1	0	0

Storage at 45° C. Preliminary results on four consecutive batches show that all passed the Therapeutic Substances test after 1 month. The egg titre was also well maintained (Table 21).

Bacteriological purity. This falls well within the statutory limit of 1000 organisms per ml. Table 22 shows the results of plate counts done on a random sample of batches of dried vaccine. All counts were done within 8 weeks of drying. Pathogenic aerobic or anaerobic organisms were not found in any batch.

DISCUSSION

Preliminary experiments showed that crude sheep lymph, either lanolinated or dried, did not withstand storage sufficiently well for use in tropical areas. Lanolinated vaccine, which has been advocated as having good keeping properties at high temperatures (King, 1920), does not in fact maintain its potency significantly better at 37° C. than glycerinated lymph. These findings agree with those of Cunningham (1922) and Cunningham & Cruickshank (1924). Even though the stability of crude vaccine pulp was markedly increased by freeze-drying, such material did not conform with the criteria for a vaccine intended for use in hot climates. One experiment indicated that no better results were obtained by prolonging the desiccation of crude pulp, thus reducing the residual moisture content. It is interesting that Fry (1951) and Fry & Greaves (1951) consider that over-drying is deleterious to some bacteria, and advocate the addition of glucose to the suspending medium in order to retain a modicum of moisture. Although we were unable to show that a plasma-glucose mixture protected vaccinia virus, it does appear that over-drying impairs subsequent survival, even though the titre immediately after drying is not diminished. A possible explanation is that since water vapour will continue to be evolved from material stored under high vacuum until equilibrium is attained with the atmosphere in the ampoule, over-dried preparations with an initially satisfactory moisture content may be depleted beyond a critical level during storage. This process would of course be more rapid at higher temperatures.

Further experiments with dried crude pulp suspensions showed that nitrogen packing does not give as good survival of the virus as does storage under vacuum, and that this is not due to traces of moisture or oxygen entering the ampoules during the sealing process. This contrasts with the findings of Proom (1951) in a somewhat similar experiment with dried cultures of *Neisseria meningitidis*. He stated that when the plugged ampoules were filled with nitrogen and exposed to air before sealing, the survival after heating at 80° C. for 1 hr. was less than when they were first evacuated on a manifold, and then filled with nitrogen and sealed off *in situ*. Cultures sealed on the manifold under nitrogen, however, survived as well as when sealed under vacuum. As nitrogen is chemically inert, it is difficult to see why vaccinia is more rapidly inactivated in its presence than when stored *in vacuo*; it may be that some impurity other than oxygen or water contributed to this effect.

The batch-to-batch variation in keeping properties at 22 and 37° C. is a serious disadvantage of dried crude sheep lymph. This may be due to differences in the protein content of the menstruum provided for the virus by different batches of pulp, or to variations in homogeneity which effect subtle alterations in the drying process.

The experiments with E.B.S. showed that all the suspending media tested afforded protection against the lethal influence of freeze-drying. They varied greatly, however, in their ability to preserve the virus during storage at higher temperatures. None of the media protected in the liquid state at 22 or 37° C. In

the dry state, 10 % horse serum protected the virus better at 37° C. than at 22° C., while the converse held true for digest broth. In this connexion, it was noticed that digest broth (dried or liquid) becomes progressively darker on storage at room temperature, and this change is even more pronounced at 37° C.; the dried suspensions also become more difficult to reconstitute. This is probably an example of the 'browning reaction' which is due to combination of reducing sugars with the free amino groups of amino-acids or proteins (Henry, Kon, Lea & White, 1948; Lea and Hannen, 1950; Lea, 1950). This reaction has a high temperature coefficient (Lea, 1950), and can occur in dried human plasma (Lea, Hannen & Greaves, 1950), the rate being largely dependent on the amount of water present, and on the temperature of storage. The amino-sugar reaction also leads to decrease in solubility of the dried material; although the free amino-nitrogen and sugar content of the digest broth used in our experiments was not estimated, the parallel between our findings and those of Lea *et al.* is obvious. It is possible that this reaction is connected with the greatly increased deterioration at 37° C. of virus dried in broth, and that differences in preservative action of various media are due to chemical changes which can occur even in 'dry' preparations stored *in vacuo*.

The best results at both 22 and 37 ° C. were obtained with peptone, and it is of interest that broth containing 1 % peptone also protected well at 37° C. Better survival at 37° C. was obtained by raising the concentration of peptone from 1 to 5 %; results with 1 and 5 % bovine plasma albumen were also rather better with the higher concentration.

The reason for the excellent preservative properties of peptone remains to be shown. It may be that preservative action is related to the molecular size of the suspending agent, since peptone, with comparatively small molecules, protected the virus better than such substances as serum or bovine plasma albumen.

5 % peptone protects sufficiently well at high temperatures to make it a suitable stabilizing agent for dried smallpox vaccine. The addition of other substances, such as sugars or salts, to the peptone solution was not investigated, since peptone alone gave perfectly satisfactory results for practical purposes, and it was desired to keep the medium as simple as possible for routine production. In this connexion, an important advantage is that the results can be reproduced with peptones from different sources. Partially purified virus dried in 5 % peptone will withstand storage at 37° C. for several months with very little diminution in titre; indeed, active virus is still present after more than 2 years at this temperature, while deterioration rates at lower temperatures are of course even slower. It is noteworthy that partially purified suspensions of sheep virus are even more stable than the highly purified rabbit adapted virus used in the earlier experiments (cf. Tables 18 and 20). Preliminary experiments have indicated that satisfactory stability is shown even at 45° C. Using partially purified virus, these results are highly reproducible; such suspensions can be made on a large scale by differential centrifugation, and are freed from contaminants comparatively easily with the aid of phenol. Dried vaccine prepared in this way also offers the important advantage of easy reconstitution, even after prolonged storage at tropical temperatures. The vaccine is now in routine production at this Institute.

SUMMARY

1. Requirements for a heat-stable smallpox vaccine are suggested.

2. Sheep lymph dried from the frozen state retains its potency at 22 and 37° C. better than aqueous, glycerinated or lanolinated suspensions of lymph. Dried crude lymph is not, however, sufficiently stable for use in tropical areas; it is also liable to vary in stability from batch to batch. No better results were obtained by prolonging secondary desiccation.

3. Phenol in low concentration is deleterious to vaccinia virus stored in the dried or liquid state at 22 and 37° C.

4. Dried vaccinia virus stored under nitrogen loses potency more rapidly than when sealed *in vacuo*, even when precautions are taken to rid the nitrogen of traces of oxygen and moisture.

5. A number of suspending media were examined for their preservative influence on purified vaccinia virus. None was effective in the liquid state, but all protected the virus against the lethal influence of freeze-drying. In the dried state, these media varied in their ability to protect the virus during storage at 22 or 37° C. The best results at both temperatures were obtained with virus freeze-dried in 5 % peptone. This medium also protects the virus well at 45° C. Different types of peptone were equally effective.

6. Virus dried in 5 % peptone still gave a full quota of successful primary vaccinations of children after storage for 12 months at 22° C., or for 4 months at 37° C.

7. Using partially purified sheep virus dried in 5 % peptone, a vaccine can be produced which shows a high degree of resistance to heat, is relatively free from bacterial contamination, and is easy to reconstitute after prolonged storage. These results are highly reproducible.

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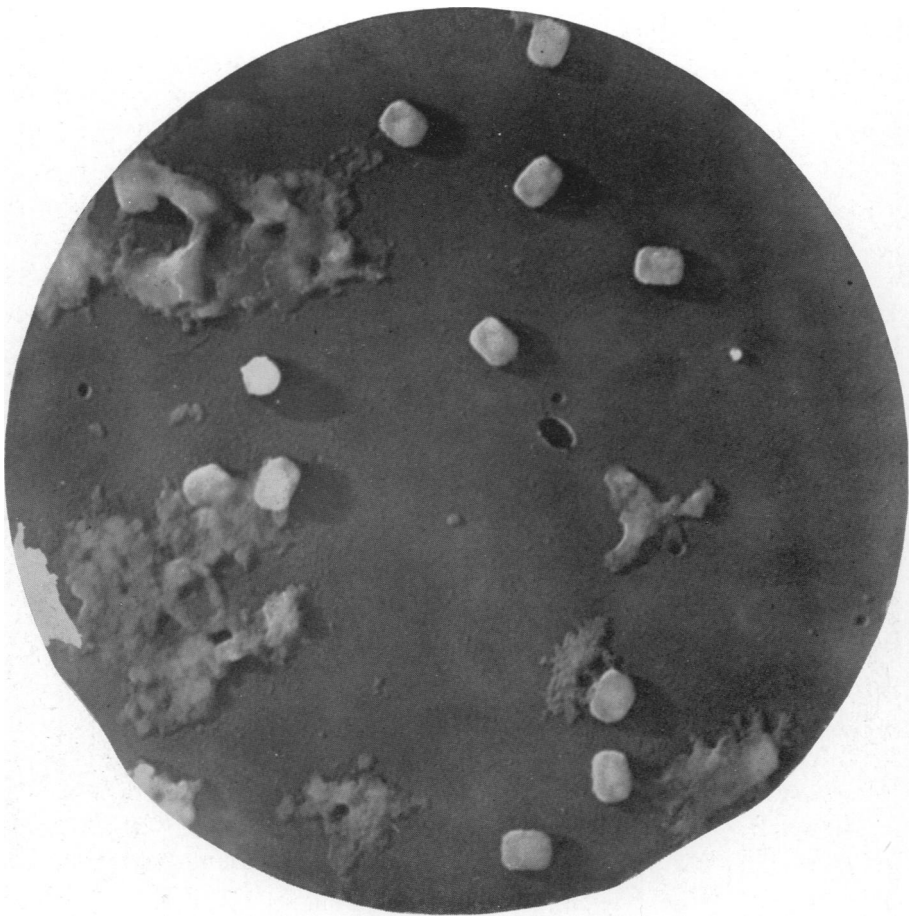

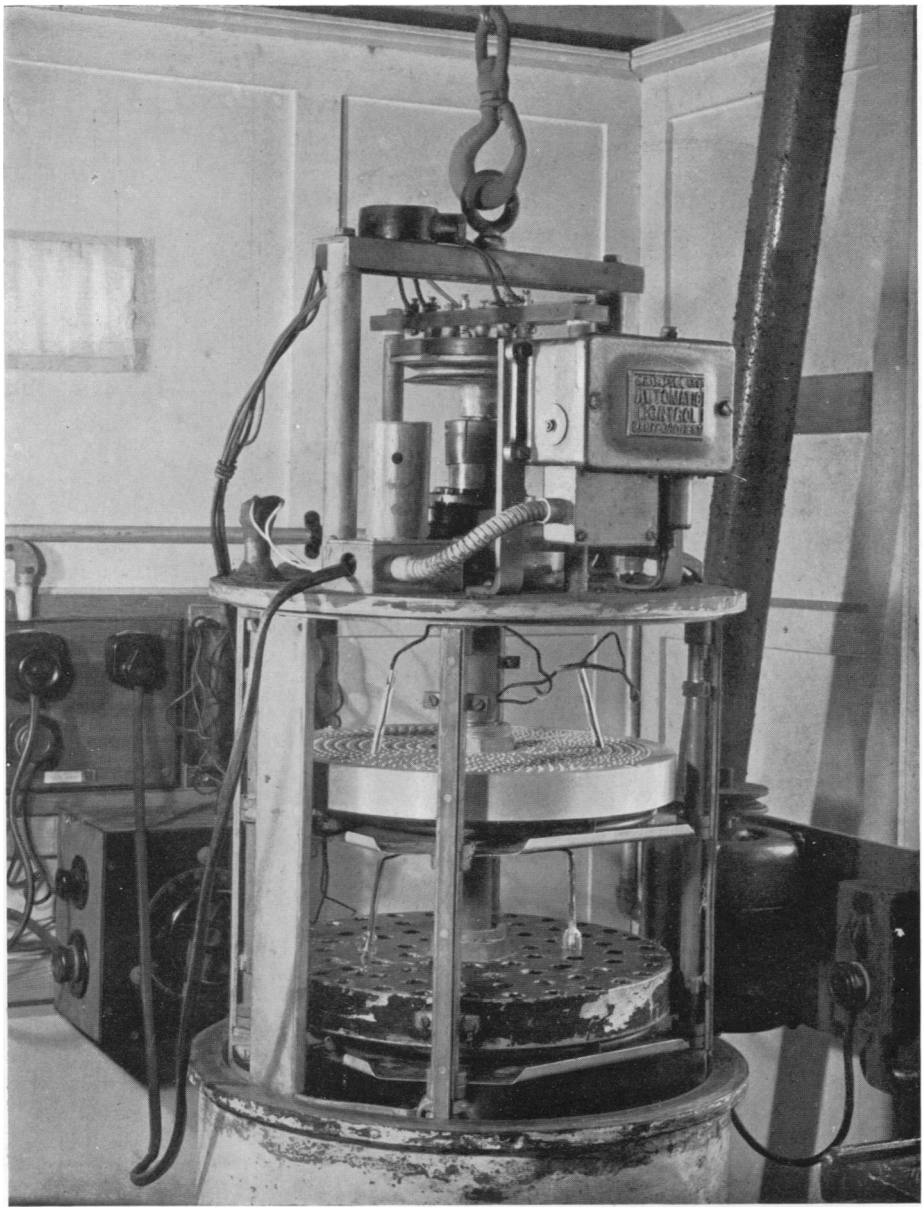
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EXPLANATION OF PLATES 1–3

PLATE 1

Electron micrograph of vaccinial elementary body suspension prepared by differential centrifugation from crude sheep pulp (magnification, × 23,800). Note irregular appearance of elementary bodies, and large amount of amorphous debris.

PLATE 2

Electron micrograph of vaccinial elementary body suspension prepared by differential centrifugation from second consecutive rabbit skin passage (magnification, × 23,180). The elementary bodies are more uniform in size and shape than those obtained from sheep pulp, and there is less background material.

PLATE 3

Experimental centrifugal freeze drying apparatus. Primary drying chamber, open. The two drying heads are mounted on a central shaft, with heater coils and reflector shields immediately below them. Two ampoules, each containing thermocouple junctions, are in position on each tier.

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