THE PRESERVATION OF VACCINIA VIRUS[1]

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"These are the chief methods in use for preserving lymph; and as all occasionally succeed, each has its partisans; and as all occasionally fail, each has its enemies."—James Moore, *The History and Practice of Vaccination*, 1817.

INTRODUCTION

The problem of protecting the living virus in smallpox vaccine against the influence of heat has exercised the ingenuity of vaccinators for a considerable time, and the history of attempts at its solution provides a fascinating record of development in laboratory technique.

It is possible that during the many centuries which saw the practice of variolation established in the East, notably in China and India, primitive attempts were made to preserve the virus from the deleterious influence of the prevalent climatic conditions. Drying as a means of preserving foodstuffs, such as fish and meat, must have been known to the physicians of those times, and the virus of smallpox presented itself naturally as dried vesicle crusts which could be conveniently transferred from one patient to another in whole or powdered form. Since virus in the liquid state is susceptible to the influence of temperature, but will survive for long periods in dried crusts (26), it is quite likely that the value of these as vehicles for the causal agent of the disease was soon appreciated. It is believed, for example, that the ancient Chinese practised variolation by inhalation of powdered scabs through the nose (58).

Whether or not the use of dried crusts was a reasoned attempt to preserve variola virus, the history of the preservation of vaccinia itself is almost coeval with the general use of this agent as a prophylactic against smallpox.

It is not proposed to give a complete account of such experiments, as an exhaustive survey would require a volume to itself. Several short reviews of the subject have been published already (21, 31, 32, 54), but these have been concerned mostly with the history of attempts to preserve the virus by various drying methods. The survey which follows has been planned to cover a rather wider field; a number of phases in the development of the subject have been distinguished, which follow each other in roughly chronological order, and emphasis has been placed on those events which serve as landmarks in our knowledge. Because of its historical interest, however, the very early literature has been dealt with in more detail than is customary in a review of this nature. The reader's attention is drawn not only to the multiplicity of techniques used to solve this problem, but also to the variable results obtained by different workers using similar methods.

[1] This review is based on the introduction to a thesis accepted in December, 1952, for the M.D. degree of the University of London.

THE FIRST PHASE

Preservation on ivory "points" and glass. For many years after the introduction of vaccination at the close of the 18th century, the vaccine virus was either propagated directly from human to human or from animal to human, or was preserved in small quantities for use as required. Writing in 1817, Moore (66) gives the following summary of current methods of preserving the virus:

"When pointed quills or bits of ivory are well and repeatedly moistened with lymph, they preserve the virtues of the Vaccine for a long time. They are more certain, however, the more recent; but when wrapt in lint and secured from air, heat, and moisture, they have sometimes continued efficacious for several months."

And later,

"There are several other methods in use for preserving vaccine lymph. A drop is sometimes inclosed between two bits of square glass: or it may be deposited in a small cavity hollowed out of the centre of a piece of ground glass, and covered accurately with a flat piece of the same size. Lymph desiccated on glass is brought to a proper state for use, by mixing it up with a particle of cold water by the point of a lancet. Vaccine crusts also, when powdered and triturated with cold water, are often efficacious . . . . Crusts have

been transported to the tropical climates, and kept for many months without losing their properties. Ingenious methods have also been devised for attracting the lymph into small capillary glass tubes, and sealing them hermetically. By this means the lymph has been found liquid, and efficacious, after being transported across the Atlantic."

Jenner himself sent lymph dried on threads to de Carro in Vienna, who found it still active on arrival. From Vienna, in 1800, equine lymph of Italian origin was sent "across European and Asiatic Turkey, and over the whole line of deserts to Baghdad", on this occasion sealed in liquid form between glass plates (83). We do not know how long the journey took, but the lymph survived the severe conditions to which it must have been exposed and on arrival was successfully used to vaccinate a child. Seaton (83) states that the virus was then propagated by arm to arm inoculation, and in this manner survived the sea crossing from Basra to Bombay, whence it was distributed throughout India. This assertion has been quoted by later authors, but Moore (66), writing at a time closer to the event, gives a more circumstantial account of the last stage of the journey; he says that after 30 or 40 fruitless attempts to send dried vaccine to Bombay by sea, a batch was eventually received which proved still potent after the voyage.

In the early 19th century, therefore, the transmission of vaccine for long distances was attended by many difficulties and hazards. This situation saw no improvement for many years to come since the methods of preservation used in 1868 (83) were almost identical with those detailed a half century previously by Moore. Some contemporary opinions of these various methods are available. Thus Seaton (83) stated that lymph kept in capillary tubes in liquid form was better preserved than when dried on glass squares or on ivory "points". The latter method, however, was advocated by Warlomont (89) who coated the ivory with gum arabic to prevent it absorbing the lymph. He claimed that vaccine thus prepared lasted much longer than glycerinated lymph which only retained its potency for 5 or 6 days. The same author drew attention to a danger in the method whereby lymph dried on glass was "reconstituted". It seems that the necessary moisture was often provided by the vaccinator or "sage femme" breathing on the glass; Warlomont rightly pointed out the danger of infection from such a procedure.

The first use of glycerol. At about the middle of the 19th century, glycerol was adopted as a vehicle for vaccine lymph. Although the method is said to have originated in Germany, the earliest reference I have found to its use was in 1850 by Cheyne (17), an English physician. He advocated mixing lymph with glycerol to prevent decomposition and claimed successful results with lymph treated in this way and stored for two months, presumably at room temperature. On the other hand, Seaton (83) in 1868 condemned the method in the following manner: "I think it scarcely necessary to advert, except for the sake of deprecating it, to the proposal which has been made of keeping lymph liquid for a longer time by mixing it with glycerine. What effect, if any, glycerine might have in retarding the decomposition of lymph I have not thought it worth while to enquire: because it is with *lymph, pure lymph*, as pure, as concentrated, and as pungent as can be, that we should vaccinate, not with mixtures and compounds. But I happen to be acquainted with some results of using the mixture, and they exhibited, as might be expected, a large relative amount of failure."

Today, a century after Cheyne's experiments, glycerol is used extensively in the manufacture of smallpox vaccine; as will be shown presently, however, these early misgivings found more recent expression on less empirical grounds.

THE SECOND PHASE

Drying of lymph from the liquid state in bulk. In the century after Jenner's discoveries, preserved lymph was largely put up as individual doses, dried, as we have seen, on glass, threads or ivory. In 1881, however, Reissner (73) published the first description of a method for drying vaccine in bulk, his object being to facilitate the distribution and use of vaccine derived from animals. He dried calf lymph on glass microscope slides in a desiccator and ground the product to a fine powder which was kept in boxes or in small tubes. To make it serve for more vaccinations, the dried vaccine was diluted by the addition of an inert powder; quite good results were obtained with material which had been stored for two weeks (74). This lymph was used by Hager (43, 44) who thought it

possible that drying the infective agent in association with the dermal cells of the host might be responsible for the preservative effect. Schulz (82) also obtained good results with this material, but Bauer (4), although acknowledging its stability on storage, thought that the necessity for reconstitution before use was a drawback; he also stated that it caused severe vaccination reactions when used undiluted. Schoen (81) particularly advocated the use of dried lymph rather than arm to arm propagation, drawing attention to the danger of transmitting other diseases by the latter method.

Although at this time increasing emphasis was being laid on the use of lymph derived from animals, humanized vaccine was still employed, and Warlomont (89), 1883, mentions several workers, himself included, who dried pustular material from human vaccinations in bulk. Successful results were obtained with material stored for 90–130 days. He also gives the first account of a vacuum drying method, used by Verardini, who processed 300 human pustules at a time for 5–6 days under a vacuum of 10–15 mm Hg. This dried vaccine survived best at 0 C to 6 C. Unfortunately, no reference to a publication by Verardini was quoted.

The introduction of potency testing in animals. Until the beginning of the present century, the potency of lymph could only be assessed by observation of the success rate with human vaccinations. Calmette and Guérin (14), 1901, then published a method of potency testing by rabbit inoculation; later the technique was improved enormously by Guérin (42) who introduced the inoculation of serial dilutions of virus. Vaccine lymph could now be titrated with some accuracy, and many subsequent reports of tests on stored vaccine acquired much greater value.

In 1902, the first investigation in England of a method for drying lymph in quantity was reported, and animal tests of stored material were used. Blaxall (7) dried calf lymph in porcelain dishes over sulfuric acid *in vacuo*. After 24–48 hours, the product was ground in a mortar and then dried for a further period to get rid of any absorbed moisture; the loss in weight due to drying was about 68%. The lymph was stored at room temperature and was tested by inoculating calves with the undiluted material. Positive reactions were obtained after storage for 5 months.

Carini (16), 1906, described a similar method of drying lymph with the refinement of storage of the dried material *in vacuo*. He compared the deterioration rates of dried and glycerinated lymph on storage at 37 C and found that, whereas the latter was inactivated after 20 days, the dried lymph still produced lesions in the calf after 30–37 days.

It now began to be realized that the advantages gained by drying lymph were partially offset by the fact that it could not be sterilized by a period of contact with glycerol, as was the common practice. Carini (16) therefore recommended the collection of pulp from the calves with aseptic precautions to reduce the number of bacterial contaminants, or the lymph could be reconstituted with glycerin-water and left for 24–72 hours before use.

Achalme and Phisalix (1), 1909, thought that the active agent was killed on storage by liberated enzymes from the dermal cells present in glycerinated lymph and considered that drying, by preventing enzyme action, should preserve the virus from attack. Whether or not this assumption was correct, they successfully preserved calf lymph by drying it on porous plates ("assiettes poreuses") over H_2SO_4 *in vacuo*, evacuation being performed with a water pump. Dried lymph was better preserved as large pieces than in powdered form, and they recommended that it should be kept sealed under vacuum. These authors tested the potency of their lymph by inoculation on rabbits' ears or on the calf. They did not express their results quantitatively but stated that after storage for one year at 37 C, the desiccated material gave a comparable reaction on the calf to that produced by fresh lymph. This procedure was therefore advocated as being the method of choice for preparation of smallpox vaccine in hot countries.

The use of dried lymph in the tropics. The years between the opening of the 20th century and the outbreak of the first World War saw a great increase in activity in this field. Both German and French workers were concerned to find a way of sending lymph to their overseas colonies without loss of potency during the voyage or during subsequent storage under adverse conditions. The difficulties of setting up overseas stations where lymph could be manufactured nearer the place of use were numerous. Apart from the shortage of skilled technicians, it was considered that animals vaccinated under tropical conditions gave poor yields of lymph, and that the dangers of secondary contaminants were great (90). Suitable

vaccinifers were often difficult to obtain because of decimation by trypanosomiasis (75).

Even if lymph could have been produced locally, the problems of internal transport were considerable. Ziemann (92) refers to this difficulty in the Cameroons, and Ross (79), 1913, relates how lymph might have to endure "a month's sojourn in a hot postbag on a porter's head in the sun". The transport of lymph on the Ivory Coast in water cooled cloth bags is described by Sorel and Arlo (86) in 1912. This method was tried in French Equatorial Africa as late as 1929, but fresh lymph sent in this way, or packed in thermos flasks, rapidly deteriorated (11). Similar difficulties were encountered in the Phillipine Islands (80).

It is not surprising, therefore, that there were numerous trials of dried lymph under tropical conditions. Camus (15), 1909, was among the first workers to send bulk-dried vaccine overseas, and he also used the method of drying lymph in thin layers over sulfuric acid; he added 10% gum arabic to the lymph before drying, not to improve its keeping properties, but to facilitate reconstitution which was otherwise difficult. Camus adopted elaborate precautions to protect the virus; it was dried in the dark, and the product was stored under vacuum in glass tubes, protected from "all rays" by thick tinfoil. This material was sent by post to the Sudan during the hot season and gave a vaccination success rate of 86–89%. This appears to be a creditable result, but Camus considered that the lymph had lost too much potency as a result of drying, that the vaccinial eruptions were not as good as those produced by glycerinated lymph, and that the dried material did not give a good reaction on the calf.

Joyeux (51) described the use of Camus' lymph in French Guinea; used on arrival, after a month's journey, 89.5% of 105 vaccinations performed were "successful", but few of the reactions were normal, poor "takes" being observed in most cases. A calf was also inoculated, but although only a sparse eruption occurred, it was later shown to be immune to revaccination.

Carini's method (16) was used in Berne by Tomarkin and Serebrenikoff (87) in 1910 for the large scale production of dried vaccine. This compared favorably on storage at 37 C with glycerinated, lanolinated and vaselinated lymph deriving from the same batch of crude pulp. After 14–18 days all lymph stored in the liquid state was completely or almost completely inactive, while the dried lymph was still potent after being held at this temperature for two months. Even so, irregular results were often obtained with the desiccated material, some batches proving more resistant to heat than others.

Leger (61) reported poor results with lymph dried at the Paris Vaccine Institute by the Achalme-Phisalix technique. He observed only 12% successful vaccinations with this lymph after storage for two months but stated that even this result was better than that obtained with the Swiss lymph prepared at Berne and tested under similar conditions. By contrast, Ross (78) was very favorably impressed with the stability of lymph dried by the Achalme-Phisalix process. At Nairobi he recorded 18/23 successful vaccinations with desiccated lymph which had been stored at the prevailing temperature for 14 months. He later reported that 14/20 vaccinations were successful with similar material after 3 years' storage (79).

Dried lymph, therefore, yielded variable results in different hands. It was generally noticed that reconstitution of this material was difficult, and that grinding with pestle and mortar was necessary to get a good suspension. An explanation of the irregularity of results was advanced by Voigt (88) who suggested that different operators might well vary in the thoroughness with which they ground the material. He recognized clearly that the necessity for grinding the lymph was a serious drawback and drew attention to the danger of respiratory infection as a result of this process. As protection for the operator, he advocated the wearing of a mask.

During the short time remaining before the First World War, further accounts, mostly favorable, of the use of dried lymph in the tropics were rendered by Sorel and Arlo (86), Manteufel (64), Kersten (56), and Ringenbach (75).

The drying of lymph with the aid of refrigerants. After the war, during which little further work on this subject was reported, the problem was again tackled, but now with the aid of new techniques. D'Arsonval and Bordas (25), 1919, contended that it was dangerous to expose lymph to sulfuric acid vapor during the drying process. They used a moisture condenser, cooled by liquid air, or solid CO_2 and acetone and

claimed that vaccine dried in this way gave good results under tropical conditions.

At this time, Wurtz and Camus (90) described a new technique whereby the lymph was first frozen and then dried over H_2SO_4 or P_2O_5 while thawing. By this means, they hoped to bring the virus from "l'état d'activité" to "l'état de sommeil". Subsequent manipulations were done in a dry atmosphere, and the dried lymph was stored *in vacuo*. Although stated to preserve its activity after several weeks' exposure to a temperature of 37 C, the authors enjoined strict precautions to be observed in its use. Thus it was to be kept as cool as possible in transit and ground in a chilled mortar with cold glycerin-water when reconstituted. They also stipulated that the actual vaccination should be performed in a place sheltered from the sun.

Otten's method. By far the most detailed study of the desiccation of lymph from the liquid state *in vacuo* was made by Otten. Although he used the early technique of drying over sulfuric acid, his investigations are reviewed separately from those of others who experimented with this method, both because they were carried out at a later date, and in view of the remarkable results which he achieved.

His first paper on the subject was published in both Dutch and German (69, 70) and dealt with preliminary experiments in which buffalo lymph was either dried at room temperature over sulfuric acid or was frozen and dried at −15 C. As a result of storage experiments he concluded that nothing was gained by freezing, and henceforward most of the work was done with lymph dried by the first method.

It is perhaps unfortunate that Otten relied on the percentage success rate of vaccinations with his dried lymph as an index of its potency, so that no clear quantitative estimations of deterioration rates were given. His results are also rather difficult to assess because individual samples were stored for varying periods at different temperatures, which makes comparison difficult. Nevertheless, it is obvious that his dried lymph retained its potency much better than glycerinated control suspensions. He concluded that the life of dried lymph held at 37 C could be measured in months, while that kept at 41–45 C remained efficacious for some weeks, provided always that the glass containers remained vacuum-tight. Lymph which had been exposed to the atmosphere for any length of time gave poor results. Like Hager (43), Otten attributed the resistance of the virus to drying to its being enclosed in epidermal cells.

Later, a paper was published in both English and German (71, 72) confirming his earlier work, and laying stress on the optimum time for harvesting the crude pulp. This was, generally speaking, 3 days after inoculation. Lymph thus obtained lost more weight on drying than did that removed on the second or fourth days, and it tended to give better results when stored. It was thought that on the third day, the inflammatory reaction had reached its height, with more water in the tissues, and a greater concentration of virus. The technique now adopted was to dry the lymph in thin layers over H_2SO_4 for 24 hours *in vacuo*. The dried material was then removed from the desiccator and ground to a fine powder, which could be transferred to glass tubes in measured amounts. The tubes were then evacuated to a pressure of 0.25 mm Hg and sealed. Lymph dried in this way lost about 80% of its weight as moisture. It produced 86–92% successful vaccinations after storage at the prevailing temperature for 1¼–2¼ years, and it was further observed that the dried lymph was more effective after storage at 37 C for one year than glycerinated lymph kept at room temperature for 1½ months.

Samples of lymph which had been dried by Otten no less than 18 years previously were examined in 1950 (20). This material had been stored throughout at an average temperature of 22 C, and different ampules yielded from 30 to 85% successful vaccinations. The titer of different batches varied from 1/250 to 1/8,000 as tested on the guinea pig cornea. By this time, most of the lymph used in Indonesia was being dried by Otten's method, and the Pasteur Institute at Bandoeng could produce 10.8 million doses *per annum* (20).

Although this method of drying lymph has been highly successful in Indonesia, it may be stated here that attempts by the writer to reproduce these results using sheep lymph met with little success (19). In one experiment, Otten's strain of virus in the form of buffalo pulp (kindly sent by Dr. R. Green from the Institute for Medical Research, Kuala Lumpur) did not survive better than a batch of sheep lymph dried under the same conditions. In addition there is a decided risk of airborne scatter of finely powdered vaccine when handling the end product, while

reconstitution is difficult, due probably to denaturation of the slowly dried protein.

The large scale use of dried lymph. By the late 1920's the use of dried lymph was becoming general in many tropical countries, notably in French Equatorial Africa (11, 12), French East Africa (59), Indochina (35) and in the Belgian Congo (60). Boulnois (9) described the successful results obtained in India with dried vaccine although he did not state its source. He concluded that dried lymph afforded a high degree of protection against variola, both in India (9) and in Africa (10).

Untoward reactions to dried lymph. Since most of the dried lymph from the Continental laboratories had undergone no preliminary treatment to remove contaminating bacteria, it is perhaps surprising that there were few reports of untoward reactions following its use. It is possible that the drying process itself helped to reduce secondary contaminants. This was noted by several authors, including Voigt (88), who made use of this phenomenon in the preparation of vaselinated lymph, which was notoriously liable to gross contamination. Voigt dried the crude pulp and then added the powder to the vaseline, hoping in this way to minimize the number of extraneous organisms.

Boulnois (9) mentioned that in 14,000 vaccinations performed in India with dried vaccine there were no deaths. One case each of tetanus and post-vaccinal encephalitis was recorded, and there were 16 cases of abscess, one being severe. Boulnois remarks that this record is much better than would be expected from a consideration of the standard of care after vaccination.

Gauducheau (38) thought that dried lymph which had been stored for long periods tended to give a generalized eruption similar to variola, but I can find no other references to the occurrence of this type of reaction.

Table 1 summarizes the methods used for drying lymph from the liquid state.

The use of vaseline and lanolin. Reference has been made in the preceding paragraphs to the use of glycerinated, lanolinated and vaselinated lymph. Glycerol was used to reduce the number of contaminating bacteria; when mixed with the ground pulp, it acts over a period, the process being known as "ripening". It was generally agreed, however, that glycerol also damaged the virus (1, 24, 57, 68, 88). Attempts were made, therefore, to find other suitable vehicles for the crude pulp. Vaseline was mentioned by Harvey (46), 1905, who also referred to the danger of growth of secondary contaminants in this medium. He considered that vaselinated lymph did not keep better than glycerinated material. In a later paper (47) he stated that the use of vaseline was introduced by Colonel Bamber, I.M.S., but gave no reference; the method, however, was known in 1890 (4).

King (57), 1920, was the chief advocate of lanolinated lymph. He stipulated that the lanolin should be neutral in reaction, anhydrous, and free of chlorine and glycerine fats. He said that lymph prepared in this manner kept better in India than vaselinated material and condemned dried lymph as being too variable in its behavior on storage.

Carefully controlled large scale trials in India of glycerinated and lanolinated lymph were reported by Cunningham (23) and Cunningham and Cruickshank (24). They came to the unequivocal conclusion that glycerinated lymph was superior to the lanolinated variety, both in its keeping properties and the type of vesicle produced.

THE THIRD PHASE

Addition of "protective" substances to liquid suspensions of vaccinia virus. The beginning of the next decade saw a new approach to the problem. Hitherto, lanolin and vaseline had been added to liquid lymph only because they were considered to be suitable inert vehicles. During the 1930's, however, a more positive attitude was adopted in that various substances with definite physical or chemical activities were added to fluid suspensions, and, it was maintained, protected the virus against high temperatures.

Morosow (67) contended that glycerinated lymph became acid in reaction on storage, and that this harmed the virus. As a buffering agent, he added egg white in concentrations of approximately 26% or 40% and claimed that glycerinated lymph thus treated showed improved keeping qualities. He suggested that the lysozyme present in egg white exerted a bactericidal influence on contaminating organisms.

Some workers added sugars of various kinds to calf lymph. Silber and Wostrouchowa (85) asserted that saccharose preserved virus held at 37 C. Akasawa (2) tested many sugars in this way, including glucose, mannose, galactose, fructose, maltose, lactose, salicin, arabinose

TABLE 1

Summary of methods used for drying vaccine lymph from the liquid state

DATE	ORIGINATOR AND METHOD	USED BY	OPINION
?	? Jenner Drying on threads		
?	Unknown Drying on ivory "points"	Warlomont, 1883	Good
?	Unknown Drying on glass slips		
1881	Reissner Drying in bulk over H_2SO_4 at atmospheric pressure	Hager, 1883 Schulz, 1889 Bauer, 1890	Good Good Doubtful
1902	Blaxall Drying over H_2SO_4 *in vacuo*		
1906	Carini Blaxall's method, but dried lymph stored *in vacuo*	Tomarkin and Serebrenikoff, 1910	Good
1909	Achalme and Phisalix Carini's method, but dried lymph stored in large pieces	Leger, 1911 Ross, 1911, 1913	Poor Good
1909	Camus Carini's method; gum arabic added	Joyeux, 1909	Indifferent
1919	D'Arsonval and Bordas Drying over refrigerated condenser		
1919	Wurtz and Camus Lymph frozen, and dried while thawing		
1926; 1932	Otten Essentially Carini's method	Collier, W. A., 1950 Collier, L. H., 1952	Excellent Poor

and xylose. None was effective, but he reported good results with a preparation termed "Japanischer Kandizucker" in 30% concentration.

Long and Olitsky (63) tried the addition of cysteine hydrochloride in 1/2,000 concentration to suspensions of testicle-passed vaccinia. They hoped that this reducing agent would prevent oxidation and inactivation of virus stored in the liquid state and reported that the addition of cysteine to virus suspended in broth, Ringer solution or distilled water prolonged its life at 37 C. Anaerobiosis was increased by storing the suspensions under layers of petrolatum.

The preservative influence exerted by milk, lecithin, and egg yolk at 25–30 C was noted by Hirano (48), and among other substances tested Yaoi and Arakawa (91) found that 5% glue prolonged the life of vaccinia virus, but that the addition of gum acacia or agar to lymph resulted in rapid deterioration.

The use of culture virus. At about this time, virus propagated by tissue culture or in embryonated eggs became available. Eagles and McClean (29), 1929, found that virus cultivated in chick embryo tissue deteriorated rapidly even in the cold room. They were particularly interested to discover a way of preserving such suspensions at low temperatures, and to this end

investigated the effects of adding cysteine hydrochloride, glycerol, agar, gelatin, borax buffer solution, fresh minced tissue, Hartley's broth and "hormone" broth (65). The influence of storage under anaerobic conditions and of 1% phenol as a bactericide was also investigated. They concluded that anaerobic storage in the presence of 0.25% agar at ±1 C gave the best results for virus grown in chick embryo tissue and plasma. Phenol did not impair the infectivity of the virus unless the suspension was frozen. Virus grown in Maitland and Laing's kidney medium was also well preserved at ±1 C under anaerobic conditions.

Goodpasture and Buddingh (39) working with egg cultured vaccinia found that although virus suspended in 3% mucin, 30% gum arabic, glycerol-saline or egg yolk deteriorated rapidly at room temperature, it survived with undiminished titer after 6 weeks' storage at 25 C if suspended in normal inactivated rabbit serum; living virus was still present after storage at 37 C for 4 weeks. Further similar experiments were later recorded, in which both rabbit and ox serum were found to be effective preservative agents (13).

Gallardo and Sanz (36) found that virus grown in chick embryo tissue culture or on the intact chorioallantoic membrane, and suspended in Tyrode's solution or in physiological saline respectively, would maintain unimpaired potency for 14 months at −10 C. By contrast, Gastinel and Fasquelle (37) reported that glycerinated suspensions of egg-cultured vaccinia deteriorated seriously during storage for 6 months at −20 C although dried material survived well at this temperature. At room temperature, however, both glycerinated and dried suspensions showed marked falls in titer after one month. Neither the method of drying nor concentration of glycerol was stated.

Experiments with purified virus. After the pioneer studies of Eagles and Ledingham (28) and Craigie (22), 1932, on the purification of vaccinia virus, Amies (3) reported that elementary bodies suspended in nutrient broth retained some activity for several weeks at 37 C. Even so, the infectivity showed a steady decrease at this temperature as judged by intracutaneous titration in rabbits. Control suspensions in physiological saline deteriorated even more rapidly.

Behrens and Ferguson (5) investigated the influence of a number of so-called protective substances on vaccinia purified by iso-electric precipitation (6). Virus suspended in 0.1% gelatin preserved its activity unimpaired for many months at 5–10 C. Good results also attended the use of 1% peptone, but glucose, glycerol, serum and cysteine hydrochloride (1/1,000 or 1/2,000) were found to have little or no preservative influence. No mention is made of storage under anaerobic conditions with cysteine, as recommended by Long and Olitsky (63), and no storage temperature above 10 C was used.

Eagles (27) described a number of long term storage experiments using elementary bodies suspended in phosphate buffer (pH 7.3), 0.25% agar-saline, broth, and saline alone. Most suspensions in broth or in 0.25% agar survived well at 4 C for many months, sometimes for more than a year. Again, the influence of higher temperatures was not investigated.

THE FOURTH PHASE

Drying of vaccinia virus from the frozen state. The history of this method of preserving protein and other solutions is reviewed by Greaves (41) and will not be fully considered here. The method was first described by Shackell (84), 1909, and had his discovery not been neglected for many years, it is probable that far more would be known today about the preservation of biological substances. Briefly, the method consists in freezing the solution, either in a cold bath or by using the rapid extraction of heat occurring during evaporation of moisture under high vacuum. Drying then takes place under high vacuum from the frozen state, the continuous evaporation of water vapor in the presence of a suitable condenser keeping the product frozen until the process is complete. By this means, untoward effects due to concentration of potentially deleterious agents such as salts are completely avoided (30, 84). The frothing which is liable to occur when protein solutions are exposed to a high vacuum can be eliminated by freezing the liquid before reducing the pressure (30, 84) or by centrifuging the material during the evacuation process (40). The method was shown to yield a product of high solubility, and denaturation of proteins was minimal. From about 1935 onwards the technique was used increasingly for the preservation of a wide range of more or less

labile biological agents such as complement, antisera, blood plasma, bacteria and viruses.

Rivers and Ward (76), 1933, first used this method for preserving vaccinia; they dried tissue cultured virus from the frozen state. A preparation with an original intracutaneous titer of 10^{-5} still produced a reaction at a dilution of 10^{-3} after storage for 4 weeks at 37 C. Later, egg albumin or 2.5% gum acacia was added before drying, and material treated in this way was successfully used to immunize humans by the intracutaneous route (77). Experiments in which culture virus was mixed with an equal volume of horse serum before freeze drying were described by Lloyd and Mahaffy (62). Their preparations would retain undiminished titers for up to 14 days at 37 C.

In 1937 Kaiser published the first of a series of papers on the freeze drying of vaccinia (52). Originally, approximately 1.5% glucose was added to partially purified virus to protect the virus against protein denaturation. The material was treated with 0.3% "zephirol" before drying to reduce bacterial contamination. This dried vaccine retained a high titer after storage for 20 months (53). Further investigations into methods of preservation were later described (55). Lymph was ground in a cooled ball mill and centrifuged to get rid of extraneous matter; gelatin which had been partially broken down by autoclaving was added in 10% concentration to the supernatant fluid containing the virus, which was then dried from the frozen state. "Zephirol" was used as before. Eagles (unpublished material) stated that dried vaccine prepared in this manner was used extensively by the German Army during the 1939–1945 war.

After reviewing different methods in current use for preserving viruses, Hoffstadt and Tripi (49), 1946, described storage experiments with the freeze dried infective agents of Shope's fibroma, vaccinia and infective myxomatosis of rabbits. Of the three, vaccinia survived best.

A review of the preservation of microorganisms and of biological substances in general by freeze drying was published by Fasquelle and Barbier (31), 1950. They described their own work on the preservation of vaccinia and stated that virus propagated in different animals varied in its resistance to drying and storage. They thought that bovine virus was more readily preserved than that derived from the rabbit, whereas egg cultured virus was the most labile. It was stated, without quoting actual figures, that a series of cultures of bovine virus dried 15 years previously had conserved "all their virulence". On the other hand, a dried chorioallantoic culture lost all activity after only 6 months, although kept at 4 C.

Hahn (45) described the production of a combined yellow fever-smallpox vaccine, consisting of 5 parts of embryo mince infected with yellow fever virus and one part of a ⅛ suspension of sheep lymph. The mixture (which also contained 15% gum arabic) was shell frozen and dried in 0.5 or 1.0 ml quantities, being then sealed under dry nitrogen and stored at 0 C. The titers of both viruses were found to be satisfactory after storage for 16 months at this temperature. Unfortunately, the initial titers of the preparations were not mentioned.

All the work with virus dried from the frozen state described above had been done by the prefreezing technique using methods similar to the so-called "lyophile" (33) or "cryochem" (34) processes. In 1951, however, a brief account of the application of Greaves' centrifugal freeze drying method (40) to the preservation of vaccinia virus was given by the author (18). By contrast with the findings of earlier workers (3, 5, 27), peptone and broth could not be shown to protect purified virus in the liquid state; freeze drying, however, exerted a marked preservative influence on such preparations and on crude vaccine pulp. Better results were obtained when the dried suspensions were sealed under high vacuum than when nitrogen packing was used.

This communication was a preliminary account of experiments described in a doctoral thesis (19) to be published elsewhere, in which the development of a stable smallpox vaccine is fully recorded. Using purified virus freeze dried in 5% peptone, a vaccine has been produced which has yielded 100% successful primary vaccinations after storage for at least 4 months at 37 C, or for at least 12 months at 22 C. Such material also offers the advantage of easy reconstitution.

A summary of methods used for drying vaccinia virus from the frozen state is given in table 2.

It is hoped that this review of attempts to solve the important problem of preserving vaccinia virus has conveyed some idea of the complexity of the task and of the variable results obtained with different techniques. It may be

TABLE 2

Summary of reports on the drying of vaccinia virus from the frozen state

DATE	MATERIAL	AUTHOR
1933	Tissue culture virus	Rivers and Ward
1935	Egg culture virus + egg albumin or gum acacia	Rivers and Ward
1935	Tissue culture virus + horse serum	Lloyd and Mahaffy
1937	Partially purified virus + glucose	Kaiser
1942	Partially purified virus + gelatin	Kaiser
1951	Lymph and chick embryo yellow fever vaccine + gum arabic	Hahn
1951 1952	Lymph: purified virus + peptone, broth, etc.	Collier, L. H.

wondered, with some justification, how much real progress has been made since Jenner was successful in sending to Vienna virus dried on threads. It is rather chastening to reflect that quite recently, along with a description of a modern technique of freeze drying smallpox vaccine, Hornibrook and Gebhard (50) also advocated its preservation in the form of impregnated threads wound on steel needles.

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