

## A SIMPLE METHOD FOR THE PRESERVATION OF SERA BY DESICCATION IN THE FROZEN STATE WITHOUT THE USE OF REFRIGERANTS

BY R. I. N. GREAVES (*Fellow of Gonville and Caius College*)  
AND MURIEL E. ADAIR (*John Lucas Walker Student*)

*From the Department of Pathology, University of Cambridge*

A METHOD for the preservation of antisera by desiccation has been described by Hartley *et al.* (1923). This method has been extensively modified by Hartley (1936), who has described a technique whereby sterile serum is dried in a vacuum over  $P_2O_5$ . He has obtained by his process dry stable preparations of antitoxins, anti-pneumococcus serum, agglutinating and precipitating antisera, the toxins of diphtheria, scarlet fever and *B. dysenteriae* (Shiga), and complement. According to this method, 80–100 ampoules of serum are put into a desiccator which contains a large dish of  $P_2O_5$ , the pressure in the desiccator is reduced slowly until small bubbles appear in the fluid, when the desiccator is closed to permit dissolved gases to escape without boiling. The next day the pressure is further reduced, a high vacuum pump being used in the final stages of evacuation. A scaly stage of dryness is reached in 1–5 days, according to the volume of fluid; the  $P_2O_5$  is then renewed and desiccation is continued until a selected number of ampoules show no further loss of weight. The ampoules are then sealed and preserved at a low temperature. The dried material is readily soluble, solution being facilitated by warming to  $37^\circ C$ . If small quantities only of serum are to be dried, the contents of the ampoules are frozen before introduction into the desiccator, and the pressure is reduced rapidly, the serum remaining in the solid state throughout the drying process. In the case of complement, 3.5 g. of NaCl are added to each 100 c.c. of fresh guinea-pig serum, which is frozen before desiccation. Comparative tests of the original solutions and solutions of the dried products have shown that very little or no loss of specific activity occurs as a result of desiccation.

Elser *et al.* (1935) have published a method for the desiccation of serum in the frozen state. By its application they have been able to obtain “a product which shows no material reduction in volume; is porous in texture; breaks up readily into fine powder; is readily soluble in water and yields, when restored to the fluid state, a clear solution”. They have shown that antisera and antigens, yeasts and bacteria, including such micro-organisms as *N. gonorrhoeae* and *N. meningitidis*, when dried in this way, may be preserved so that, even after many years, no change is observed on restoration to the fluid state. Their method consists of preliminary freezing of the material and subsequent rapid evaporation, drying being accomplished by the condensation of water vapour

on refrigerating coils. This interesting method unfortunately demands the use of apparatus which is not available in most laboratories.

We were confronted with the problem of providing precipitating antisera for the use of a class of more than a hundred students. In order to obviate the inoculation of a very large number of rabbits, it was necessary to preserve the antisera obtained throughout the year after successive injections of smaller batches of animals. We have adopted a simple and rapid method of drying serum in the frozen state which, so far, seems to be satisfactory for the preservation of antisera for class purposes and also for research work. The process needs no apparatus not usually available in a laboratory, except an efficient high vacuum pump.

Lancefield & Quastel (1929) and other workers have shown that if proteins be dried rapidly over  $P_2O_5$  at ice-box temperature, no denaturation occurs. Adair & Robinson (1931) dried solutions of haemoglobin and crystalline egg albumin in platinum basins over  $P_2O_5$  in a high vacuum, and found that under these conditions the protein solution supercooled and then froze rapidly. The weight of protein became constant after 2 or 3 days at  $0^\circ C.$ , the dried protein so obtained having a high ratio of surface to volume. Proteins dried in this way were apparently undenatured as they were readily soluble and could be recrystallized. The dry weights were practically equal to those obtained after dehydration at  $105^\circ C.$ , whereas haemoglobin and egg albumin dried at room temperature by ordinary methods were found to contain 4–12 per cent of water. It was considered that when proteins are dried slowly at room temperature, the hard skin which forms over the surface prevents the interior from reaching a state of equilibrium. The above experiments indicate the advantages of the desiccation of proteins in the frozen state.

As stated above, Hartley (1936) has recommended that if small quantities only of serum are to be dried, the ampoules of serum should be frozen before introduction into a desiccator containing  $P_2O_5$ , which may then be brought rapidly into a high vacuum when the serum remains solid during the whole drying process. Unfortunately only a very small amount of serum can be dried in one desiccator in this way. We found that if the total volume was as much as 20 c.c., the contents of some of the ampoules thawed, owing to the heat generated by the deliquescent  $P_2O_5$ , and on thawing the serum boiled and frothed so that losses were incurred. Elser *et al.* (1935), who encountered the same difficulty, suggested that it might be overcome by placing the sera in ampoules submerged in a freezing mixture and connected to a pyrex manifold containing  $P_2O_5$ . We found, however, considerable difficulty in effecting joints between ampoules and manifold that would retain a high vacuum for the period necessary for the completion of drying.

The disadvantages of drying serum in evaporating basins are obvious, as transference of the dried product to vessels suitable for storage would occasion losses of material and risk of contamination. If serum contained in bottles or ampoules be placed in a desiccator charged with  $P_2O_5$  which is then brought

rapidly into a high vacuum, the violent boiling and frothing which precede freezing cause losses unless disproportionately large vessels are used for a given volume of fluid. As stated by Hartley (1936), such frothing may be avoided if the pressure in the desiccator be reduced gradually over a period of hours or days, but such a delay is undesirable for our purposes.

We find that this difficulty can be avoided by first placing the bottles of serum in a desiccator which contains no  $P_2O_5$  or other drying agent, and reducing the pressure to about 2 cm. of Hg, when the serum begins to froth. Closing the tap, however, causes the bubbles to subside. Reopening and reclosing several times produces, after about 15 min., a quiescent fluid in a high vacuum. If air be then readmitted and the bottles of sera quickly transferred to a second desiccator charged with  $P_2O_5$ , which is then brought into a high vacuum, the serum evaporates rapidly and freezing takes place with scarcely any frothing. It should be noted that, with this method of freezing, the change from the liquid to the solid state is practically instantaneous. This rapid solidification minimizes the chance of deleterious changes which, as suggested by Elser *et al.* (1935), may occur during slow freezing owing to concentration of salts and other agents. The desiccator may then be kept in a cool cupboard for 3 or 4 days. The serum is but little reduced in volume by drying and is very readily soluble in distilled water. This method seemed to us to provide a simple and satisfactory drying process.

We had then to consider the choice of containers in which to dry the serum. A vessel with a short and wide exit is the most satisfactory, as rapid evaporation is facilitated. Consequently ordinary ampoules with long narrow necks are not entirely suitable, although we have used them with some success. We have found that vaccine bottles make very satisfactory containers, and their use is economical as, unlike ampoules, they can be used many times. It is essential for the successful application of the method that the correct size of bottle be used for a given volume of serum, because, on the one hand, if the bottle contain too little fluid partial drying may take place before freezing occurs, so that a scaly product, difficult to dissolve, is obtained, and, on the other hand, if too great a volume be present the turbulence which is sometimes manifested at the moment of freezing may cause ejection of some of the contents of the bottle. Using amber vaccine bottles supplied by R. B. Turner, we have found experimentally that 1 c.c. of serum is the most suitable quantity for the 3 c.c. size of bottle, 1.5 c.c. for the 6 c.c. bottle, 3 c.c. for the 12 c.c. bottle and 5 c.c. for the 25 c.c. bottle.

We have found that it is essential to use an adequate amount of  $P_2O_5$  for the drying of a given quantity of serum. The  $P_2O_5$  in the desiccators has been placed in small dishes, of the type usually employed for section staining, with an internal diameter of about 5 cm. and a depth of about 2.5 cm. One of these dishes filled with  $P_2O_5$  will dry 3 c.c. of serum. Accurate weighing of  $P_2O_5$  for use in desiccators would be difficult, and we have found this rough method of measurement to be adequate.

The pump we have used is a Cenco Megavac, and it is important that it should be in such a condition that a high vacuum can be obtained quickly. The internal and external apertures of the desiccator and the tap connected with the pump should be of moderately large bore, if a high vacuum is to be obtained quickly, and the line of closure between the lid and the base of the desiccator should, moreover, be broad and well ground.

The volume of serum that can be dried in a desiccator is limited, for if the quantity of heat liberated by the reaction between water and  $P_2O_5$  is too large, in proportion to the size of the desiccator, the serum will not freeze. It has been found that 21 c.c. of serum can be dried in a desiccator of 8 in. internal diameter, using seven dishes of  $P_2O_5$ , placed on a perforated zinc platform at the bottom of the desiccator, with the bottles of serum on a second zinc platform supported by a glass tripod about 2 in. above the level of the  $P_2O_5$ .

The process may be summarized as follows:

(1) The appropriate amounts of serum are measured into sterile bottles.  
(2) The bottles are put into a desiccator, containing no drying agent, which is gradually brought into a high vacuum, so that a quiescent fluid is obtained in the way previously described.

(3) The vacuum is quickly released and the bottles are transferred to a second desiccator charged with dishes filled with fresh  $P_2O_5$ . A high vacuum is then rapidly attained. If a mercury gauge be connected in series with the pump, and the outflow of air be regulated by a tap in the early stages of evacuation, the desiccator is not subjected to a sudden strain. After the contents of the bottles have frozen the desiccator tap is turned off.

(4) The desiccator is kept in a cool place for 3 or 4 days. At the end of the period allowed for drying, air is slowly admitted through a chain of scrubbing towers containing granular  $CaCl_2$ . The bottles are closed with sterile rubber stoppers, through which hypodermic needles are inserted. The bottles are then evacuated on the high vacuum pump, the needles are withdrawn and the stoppers finally sealed in a bath of molten high vacuum wax (Everett No. 2). We have stored the bottles of dried sera at  $0^\circ C.$ , but this precaution does not seem to be essential.

(5) Serum dried by this process is readily soluble without heat or shaking. The serum can be reconstituted by adding a volume of distilled water calculated from a comparison of dry and wet weights or from the refractive index, but if it is to be used in high dilution, it is sufficiently accurate to add a volume of distilled water equal to the original volume of the serum and to make up to the necessary dilution with saline.

Tests of the activity of antisera before and after drying have been carried out by the optimal proportions method of Dean & Webb (1926), in which a wide range of dilutions of antigen is arranged, each in a volume of 1 c.c., so that the amount of antigen is halved in each successive tube; to each tube is added 1 c.c. of an appropriate dilution of antiserum. Such an experiment is known as a rough test, and indicates approximately the proportions of antigen

and antibody necessary for the most rapid particulation. A fine test is then set up in which the amount of antigen differs but little from tube to tube, and the proportions are more accurately determined. The result is expressed as the antigen-antibody ratio, e.g. a ratio of 1 in 10 indicates that 1 part by volume of antigen is in optimal proportions with 10 parts of antiserum.

The following examples may be cited. In the case of No. 1722, anti-crystalline egg albumin serum, fine tests performed on 26 January 1936 gave an optimal ratio of 1 to 33 expressed in terms of 1 per cent. crystalline egg albumin to antiserum. The serum was dried the same day and on 26 April 1936 it was redissolved and again tested; the ratio was found to be unchanged. No. 1807 anti-horse serum was tested on 18 January 1936, and gave a ratio of 1 to 32. It was dried on the same day and retested on 28 April 1936, when the ratio was unchanged. No. 2588 anti-crystalline human albumin serum was tested on 18 January 1936, and gave a ratio of 1 to 77.4. It was dried on the same day and a subsequent test carried out on 30 April 1936 gave a ratio of 1 to 81.2.

Tests on guinea-pig complement dried by this process were made as follows. On 15 January 1936, 1 c.c. samples of pooled complement were measured with an Ostwald pipette into 3 c.c. vaccine bottles and dried. On 9 May 1936, random samples were taken and titrated against sheep cells and four minimal haemolytic doses of anti-sheep cell haemolysin prepared in the rabbit. There was no detectable difference between individual samples, which were actually superior to a sample of fresh pooled guinea-pig serum used as the control. Since even pooled samples show variation, this can in no way be interpreted as a quantitative experiment, but shows that the drying process can have produced no great deterioration in the specimen.

Table I records the results of experiments made to determine the rate of desiccation of different quantities of sera dried by this method. The volumes of the sera were measured with standard pipettes and the weighings were carried out on an Oertling air-damped balance. The weights are recorded to four places of decimals, but in view of the hygroscopic nature of dried serum, the fourth place is probably not exact, in spite of the speed with which weights can be determined with this type of balance. It will be seen that for quantities greater than 4 c.c., 4 days must be allowed for drying. For smaller quantities, 3 days would appear to be sufficient.

Preliminary experiments have been carried out as to the application of this process to the drying of bacteria. The cultures were suspended in sterile inactivated guinea-pig serum and distributed in 0.25 c.c. quantities into pyrex test-tubes of approximately 0.5 cm. internal diameter and 15 cm. length, plugged with cotton-wool. 25 to 30 of these tubes were placed in a beaker containing about 15 c.c. of water, which was then placed on the stage of a CO<sub>2</sub> microtome, and the water and the contents of the tubes were frozen. The beaker was then put into a desiccator, containing seven dishes of P<sub>2</sub>O<sub>5</sub>, sufficient to dry 21 c.c. fluid, which was evacuated rapidly. The evaporation of

Table I

Size of bottle	c.c.	c.c.	Volume of anti-serum	Wet weight of anti-serum	Weight of anti-serum in g. after												I.c.c. anti-serum wet weight	Dry weight	Loss of weight of anti-serum in g. after								
					1 day			2 days			3 days			4 days					5 days			3 months					
					day	days	months	day	days	months	day	days	months	day	days	months			day	days	months	day	days	months			
Anti-horse serum 1597	25	10	10-3178	1-1076	1-1007	0-9981	0-9975	0-9972	0-9945	0-9918	0-9995	9-2102	0-0069	0-0026	0-0006	0-0003	0-0027										
Anti-horse serum 1807	25	7	7-1796	0-6094	0-6047	0-6030	0-6032	—	0-5993	1-0254	0-0856	6-5702	0-0047	0-0017	0-0002	—	0-0039										
Anti-horse serum 1597	25	4	4-1451	0-4590	0-4571	0-4558	0-4558	—	0-4532	1-0362	0-1133	3-6861	0-0019	0-0013	0-0000	—	0-0026										
Anti-egg albumin serum 1722	12	4	4-1152	0-3412	0-3401	0-3394	0-3396	—	0-3386	1-0288	0-0847	3-7740	0-0011	0-0007	0-0002	—	0-0016										
Anti-horse serum 1807	12	3	3-0671	0-2603	0-2588	0-2584	0-2589	—	0-2573	1-0224	0-0858	2-8068	0-0015	0-0004	+0-0005	—	0-0016										
Anti-horse serum 1807	12	3	3-0706	0-2606	0-2587	0-2584	0-2586	—	0-2564	1-0235	0-0854	2-8100	0-0019	0-0003	+0-0002	—	0-0022										
Anti-horse serum 1606	25	3	3-0810	0-2958	0-2956	0-2950	0-2950	—	0-2949	1-0270	0-0983	2-7852	0-0002	0-0006	0-0000	—	0-0001										
Anti-egg albumin serum 1722	25	2	2-0445	0-1704	0-1699	0-1693	0-1697	—	0-1694	1-0223	0-0847	1-8741	0-0005	0-0006	+0-0004	—	0-0003										
Anti-egg albumin serum 1722	12	2	2-0616	0-1705	0-1699	0-1697	0-1696	—	0-1690	1-0308	0-0845	1-8911	0-0006	0-0002	0-0001	—	0-0006										
Anti-egg albumin serum 1722	12	1-5	1-5293	0-1258	0-1257	0-1252	0-1256	—	0-1255	1-0195	0-0837	1-4035	0-0001	0-0005	+0-0004	—	0-0001										
Anti-horse serum 1807	6	1-5	1-5290	0-1327	0-1311	0-1306	0-1306	—	0-1286	1-0193	0-0857	1-3963	0-0016	0-0005	0-0000	—	0-0020										
Anti-horse serum 1807	6	1-5	1-5311	0-1290	0-1281	0-1281	0-1289	—	0-1282	1-0207	0-0855	1-4021	0-0009	0-0000	+0-0008	—	0-0007										
Anti-horse serum 1807	6	1-5	1-5310	0-1288	0-1279	0-1279	0-1281	—	0-1278	1-0207	0-0852	1-4022	0-0009	0-0000	+0-0002	—	0-0003										
Anti-human globulin serum 2636	3	1	1-0241	0-1040	0-1027	0-1021	0-1021	—	0-1007	1-0241	0-1021	0-9201	0-0013	0-0006	0-0000	—	0-0014										
Anti-human globulin serum 2636	3	1	1-0243	0-1032	0-1018	0-1012	0-1012	—	0-1002	1-0243	0-1002	0-9211	0-0014	0-0006	0-0000	—	0-0010										
Anti-human albumin serum 2588	3	1	1-0228	0-0945	0-0936	0-0936	0-0938	—	0-0938	1-0228	0-0938	0-9283	0-0009	0-0000	+0-0002	—	0-0000										
Anti-human albumin serum 2588	3	1	1-0222	0-0952	0-0942	0-0940	0-0945	—	0-0944	1-0222	0-0945	0-9270	0-0010	0-0002	+0-0005	—	0-0001										

the ice in the beaker ensures that the serum containing the organisms is retained in the solid state throughout the drying process. As soon as the ice in the beaker was seen to have evaporated, the culture tubes were removed, constrictions were made with an oxygen blowpipe at the open ends and the tubes were evacuated and sealed off. We have dried cultures of *C. diphtheriae* and *S. aertrycke* by this method and have reclaimed living pure cultures after the bacteria had been in the dried state for a few days, but the cultures have not been investigated further nor has the condition of the organisms during the period of desiccation been ascertained, but we suggest that they may be protected by an enveloping layer of dried serum as by a capsule, rather than desiccated.

#### SUMMARY

A simple process is described for the desiccation of antisera after freezing by rapid evaporation. Freezing has been brought about by the rapid evaporation which takes place in a high vacuum in the presence of  $P_2O_5$ . The violent frothing which takes place when serum is exposed to a high vacuum has been controlled by a preliminary evacuation of the serum in the absence of the drying agent.

No special apparatus except an efficient high vacuum pump is necessary for this process.

This process has been applied for the preservation of (a) anti-horse serum, anti-ox serum, anti-human serum, anti-human globulin and anti-crystalline human albumin sera; (b) anti-sheep cell haemolysin; (c) guinea-pig complement; (d) various *Salmonella* antisera.

Preliminary experiments are described of the application of this method to the drying of bacteria.

We desire to thank Dr P. Hartley for his valuable advice and for showing us his paper prior to publication.

#### REFERENCES

- ADAIR, G. S. & ROBINSON, MURIEL E. (1931). A direct method for the measurement of the hydration of proteins. *J. Physiol.* **72**, 2P.
- DEAN, H. R. & WEBB, R. A. (1926). The influence of optimal proportions of antigen and antibody in the serum precipitation reaction. *J. Path. Bact.* **29**, 473.
- ELSER, W. J., THOMAS, R. A. & STEFFEN, G. I. (1935). The desiccation of sera and other biological products (including micro-organisms) in the frozen state with the preservation of the original qualities of the products so treated. *J. Immunol.* **28**, 433.
- HARTLEY, P., EAGLETON, A. J. & OKELL, C. C. (1923). The desiccation of serum and other protein solutions, with special reference to the reagents used in the Wasserman reaction. *J. Path. Bact.* **26**, 53.
- HARTLEY, P. (1936). (Personal communication.)
- LANCEFIELD, R. C. & QUASTEL, J. H. (1929). A note on the antigenicity of crystalline egg albumin. *J. Path. Bact.* **32**, 771.

(MS. received for publication 18. vi. 1936.—Ed.)