

HIGH-VACUUM CONDENSATION DRYING OF PROTEINS FROM THE FROZEN STATE

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(With Plates IV and V and 12 Text-figures)

INTRODUCTION

IF proteins are dried *in vacuo* from the liquid state, they tend to form hard scales, which, if weighed daily, show a slow, continuous loss of weight for several months. Presumably, a pellicle, which reduces the rate of evaporation, is formed on the surface of the scales. Proteins dried in this way are perhaps partly denatured, for they dissolve slowly. Nevertheless, this method of desiccation has been used successfully for many years in the preparation of international standards of antisera.

As early as 1909, Shackell demonstrated that if protein solutions were dried *in vacuo* from the frozen state, solubility seemed to be unimpaired. By this method of desiccation he preserved guinea-pig complement, the immune serum of hog cholera, and rabbit brain containing the fixed virus of rabies, and furthermore applied the method to the drying of meat, fruit and vegetables. Recent work has entirely substantiated Shackell's claims as to the great value of his products.

If protein solutions are dried from the frozen state, little reduction in volume takes place, so that, on evaporation of the ice, a porous mass is left, exposing a high ratio of surface to volume, which greatly facilitates desiccation and subsequent solution. The dry mass dissolves rapidly and completely in water, yielding a fluid which apparently does not differ in physical properties from the original solution (Adair & Robinson, 1931; Greaves & Adair, 1936; Elser, Thomas & Steffen, 1935; Flosdorf & Mudd, 1935, 1938). Proteins dried in this way may be preserved for long periods of time without undergoing deterioration.

Adair & Robinson (1931) dried solutions of haemoglobin and crystalline egg albumin in platinum basins over phosphorus pentoxide in a high vacuum. They found that under these conditions the protein solutions first supercooled, then rapidly froze throughout and remained in the solid state during the process

of desiccation. The weight of the dried material became constant after 2–3 days, and, if the basins were subsequently placed in an oven at 105° C., remained unchanged. They therefore considered the proteins to be in an anhydrous state.

If a protein solution is brought rapidly into a high vacuum it tends to froth violently before it freezes, so that if it is contained in bottles or ampoules suitable for storage, losses will ensue unless they are disproportionately large for the volume of fluid in them. Hartley (1936) showed that this frothing was not due to boiling of the solution, but to the liberation of dissolved gases. He found that by reducing the pressure slowly with a water pump, frothing could be controlled, till eventually a quiescent fluid resulted. Greaves & Adair (1936) made use of this observation for drying, from the frozen state, material in its final containers. They found that if vessels containing the protein solution were placed in a desiccator without any drying agent, and the pressure was reduced to about 12 mm. Hg, frothing commenced, which subsided on closing the desiccator tap. By reopening and reclosing the tap several times they obtained a quiescent fluid after 15–30 min. Air was then admitted to the desiccator and the bottles transferred to a second desiccator charged with phosphorus pentoxide, which was then quickly brought to a high vacuum. The protein solutions then evaporated so rapidly that they “snap” froze, and dried from the frozen state.

Flosdorf & Mudd (1938) have redescribed this process, which they term “a degassing self-freezing procedure”, but they were in error when they stated that prior to their communication “self-freezing in the final container has not been feasible”.

As continuous evaporation entails continuous abstraction of heat, it is clear that an evaporating system gradually undergoes a reduction of temperature, and, unless heat is supplied, the frozen mass may have so low a vapour pressure that the process of drying becomes extremely slow. The considerable heat of dilution of phosphorus pentoxide is probably an important factor in the efficiency of the processes of Adair & Robinson (1931) and Greaves & Adair (1936). Their techniques are applicable to small volumes of solution only, because a large amount of phosphorus pentoxide is necessary to dry a small volume of protein from the frozen state, so that only about 21 c.c. can be accommodated in an 11-in. desiccator. Consequently, for drying quantities up to 5 l. under sterile conditions it became necessary to devise a new technique.

The recent publications of Elser *et al.* (1935) and of Flosdorf & Mudd (1935) record methods of drying proteins from the frozen state which may be applied to large volumes. Their technique is one of condensation drying *in vacuo*, in which the water which distils from the protein solution is collected on a cold surface. More recently, Flosdorf & Mudd (1938) have adapted their apparatus for the use of a chemical desiccant, Drierite.

In the procedure of Flosdorf & Mudd (1935) vessels containing the protein solution are attached to manifolds connected with a receptacle cooled to

–80° C. by immersion in a bath of solid CO₂ dissolved in methyl cellosolve, which serves as the condenser. The protein solutions are frozen before desiccation in specially designed ampoules, which are immersed and rotated in the same refrigerant mixture so that the solutions freeze round the internal periphery of the ampoules, ensuring a large surface for evaporation. The ampoules are then individually connected to a manifold which in its turn is connected, with the interposition of the condenser, to a Cenco Megavac pump which serves to evacuate the system. After evaporation has started, the loss of heat of the protein is to some extent compensated by exposure of the ampoules to room temperature. It is stated that a small depth of frozen protein is dried in 18 hr.

Elser *et al.* (1935) criticized the use of a manifold because of the considerable obstruction presented to the passage of water vapour by tubes of narrow bore, and the difficulties of maintaining high vacua in an apparatus in which there are many vacuum joints, all of which are prone to leak.

As sterility is often of paramount importance in biological work, it is convenient to be able to work with plugged containers. The introduction of plugs into containers connected to a condenser or absorbent by means of a manifold adds appreciably to the obstruction of the passage of water vapour, and may lead to thawing of the frozen protein solution. For this reason alone, an apparatus of this type is undesirable.

On the other hand, it has been argued that ampoules containing dried protein can be sealed *in vacuo* without detachment from the manifold and the danger of admitting air thus avoided, and, moreover, that the use of a manifold is advantageous because the ampoules can be exposed to room temperature during the process of drying, and sufficient heat thus automatically supplied to regulate the temperature of the frozen serum. Exposure of the containers during desiccation of the protein to room temperature is undoubtedly a labour-saving device for temperature regulation, though our work has shown that a thermostatically controlled water bath would be more efficient. Methods of temperature regulation will be discussed more fully later.

APPARATUS AND EXPERIMENTAL METHODS

We began our experiments with the object of producing an apparatus in which the use of large amounts of solid CO₂ were unnecessary on account of its deleterious effects on buffer solutions and possibly on globulin solutions, and because the large quantities required would, at the current price in England, entail high running costs. We therefore based our preliminary experiments on another suggestion made by Elser *et al.* (1935), who had used a large insulated steel box as a desiccator and employed refrigerator coils for condensing the water vapour evolved during the evaporation of the protein solution. These authors, however, abandoned the method for several reasons; first, they found the desiccator was apt to leak; secondly, they were unable to install automatic control of either the refrigerator or of the temperature of the frozen protein;

thirdly, as the solutions were frozen in a layer on the bottom of the desiccator, it was difficult to maintain sterile conditions. The advantages of this method seemed to us, however, to be considerable, provided the difficulties enumerated could be overcome.

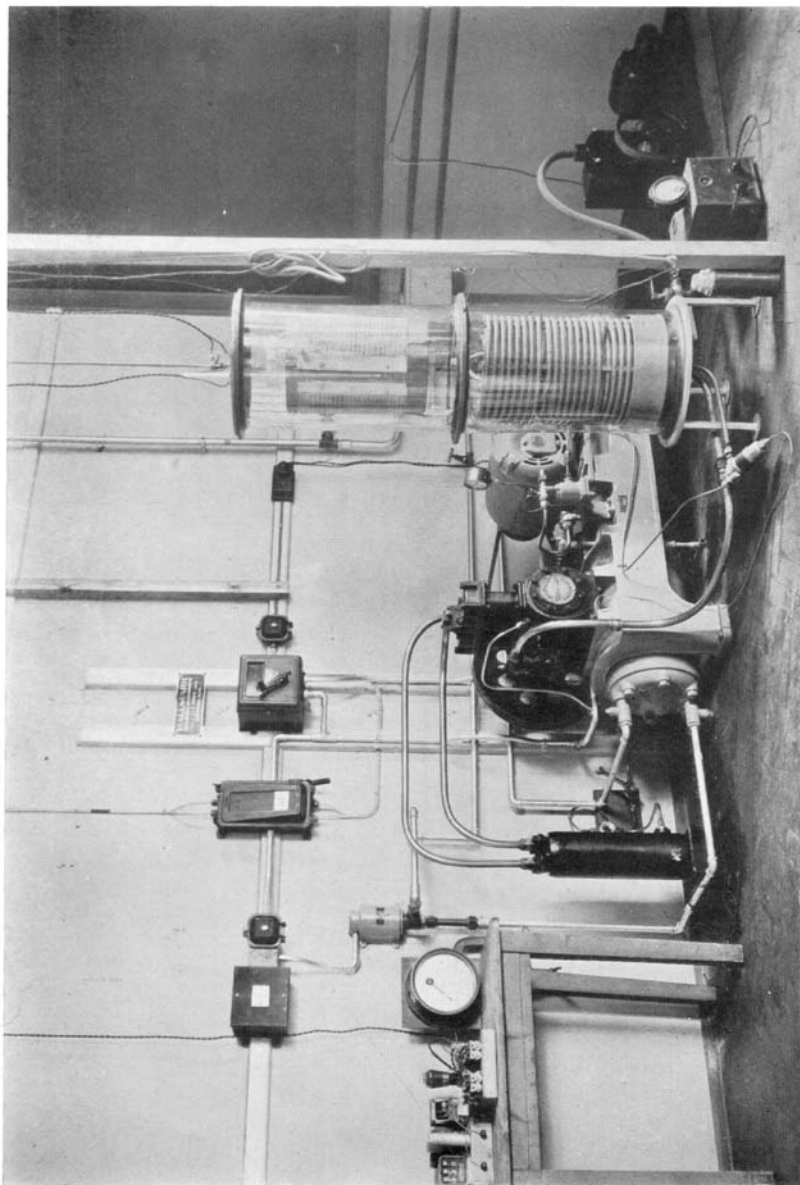
The necessity of an efficiently regulated supply of heat to accelerate evaporation of the frozen protein was recognized by Elser *et al.* (1935). The importance of this factor in the desiccation of proteins from the frozen state, if dryness is to be achieved within a reasonable time, has been fully confirmed by our experimental work.

A. *Desiccating chambers*

As desiccators we have employed throughout the vessels designed in the Cavendish Laboratory, Cambridge, for experiments in high vacua (Cockcroft & Walton, 1932). These are cylinders constructed of resistance glass 14 in. in diameter and 24 in. long, which are sealed to steel plates of 18 in. diameter and $\frac{3}{8}$ in. in thickness by means of a low vapour pressure plasticine, Apiezon Q. Such vessels, if supplied with a suitable tap, will maintain a vacuum of the order of 0.0001 mm. Hg or less for very long periods.

In preliminary work on condensation drying, a tube of liquid air was used as the condenser. In such experiments, the desiccator was constructed as shown in Text-fig. 1. Through the bottom steel plate of 18 in. diameter, which is supported on three metal legs, a $\frac{3}{4}$ in. hole was drilled. Into this hole the vacuum junction box was fixed as shown in Text-fig. 2. Into the junction box a B14 glass joint is fixed and used for connecting a Pirani vacuum gauge for the measurement of pressure. In our early experiments a discharge tube provided with two aluminium electrodes was used, the character of the discharge given by this tube serving as a rough guide as to the degree of vacuum attained. Apiezon Q was used to seal the lower rim of the glass cylinder to the bottom plate and the upper rim to a steel ring of 18 in. external and 12 in. internal diameter. The lid of the vessel consisted of a steel plate of 17 in. diameter provided with two handles. The difference in diameter between the steel ring and the lid left a convenient $\frac{1}{2}$ in. groove for the final seal with Apiezon Q. This is the only seal to be broken and remade when the apparatus is in use. If the Apiezon Q is kept at a suitable temperature (about 25° C.) it is very easily applied and may be readily removed with an ordinary "putty knife" at the end of an experiment. The sealing of the glass cylinders and the lids to the metal plates has on no occasion in our experience been the source of a leak.

A 2 $\frac{1}{2}$ in. hole was drilled in the centre of the steel lid, and through this was passed the closed end of a pyrex tube 2 in. in diameter and 32 in. in length which served as the condenser. The flange at the open end of the tube was reflected back for 11 in. to form an outer tube of 3 in. diameter; the rim of the flange rested on the steel lid, to which it was sealed with Apiezon Q. In this way, 21 in. of the tube was inside the desiccator, giving the surface for condensation, while the upper part was provided with a vacuum jacket between the



General view of apparatus. The nickel-plated reflector screen has been removed to show the copper heating cylinder which holds the 20-litre bottle with its heating mats and thermostatic thermometer. To the right of the chamber is seen the Pirani vacuum gauge and the Conco-Megavac pump, to the left the compressor unit with the oil trap. On the table on the left is seen the automatic temperature control apparatus.

inner and outer tube when the desiccator was evacuated. During the process of desiccation the tube was filled with liquid air approximately up to the level of the steel lid.

The vessels containing the protein solution to be dried were placed around the condenser on tiers of circular metal trays, beneath which heating elements were fixed.

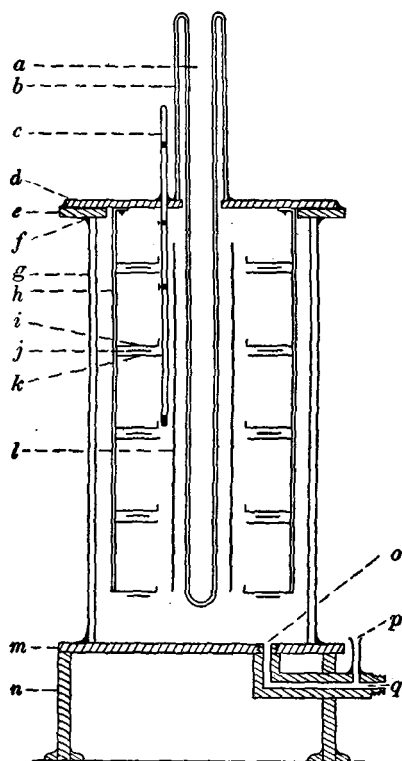


Fig. 1.

Fig. 1. Section of a desiccator chamber, with a tube containing liquid air as the condenser. *a*, central pyrex tube of the condenser. *b*, reflected pyrex tube of the condenser. *c*, thermo-static thermometer. *d*, steel lid. *e*, steel ring. *f*, Apiezon Q seals. *g*, glass cylinder. *h*, support arms for heating trays. *i*, heating trays. *j*, heating mats. *k*, polished reflector screens. *l*, polished reflector screen. *m*, Lower steel end plate. *n*, steel supporting legs screwed to floor. *o*, entrance to vacuum junction box. *p*, B 14 glass joint for vacuum gauge. *q*, exit from vacuum junction box, threaded for $\frac{3}{8}$ in. metal vacuum tap.

Fig. 2. *A*, bottom plate of desiccator. *B*, B 14 glass joint for Pirani gauge. *C*, Apiezon sealing wax. *D*, exit, with threaded end for $\frac{3}{8}$ in. vacuum tap.

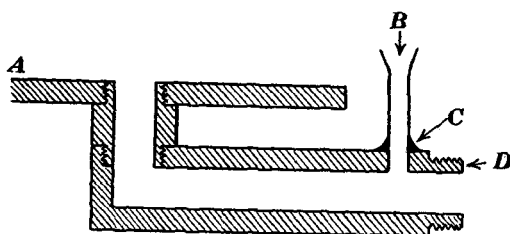


Fig. 2.

After a preliminary "degassing" of the protein solutions by evacuation of the desiccator with a water pump, the Megavac pump was connected through a liquid air trap to the desiccator and a high vacuum rapidly attained, whereupon the protein solutions supercooled and then "snap" froze. The desiccator tap was then turned off and liquid air was poured into the central condenser, when there was a further improvement in vacuum and desiccation proceeded

rapidly. The method has several advantages, among which may here be mentioned the possibility of quantitatively collecting the condensate, which may be of importance if the material to be dried contains volatile substances. It should be pointed out that in a vacuum-tight apparatus in which "degassed" material is being dried, there is no need to keep the vacuum pump running once a good vacuum has been obtained. With prefrozen material, however, dissolved gases are slowly liberated as desiccation proceeds, and only in the terminal stages should the pump be turned off. As constructed, the desiccator accommodated about 250 c.c. of material, but the surface for condensation was limited. In designing a large model, capable of drying up to 5 l. of material, it was decided, therefore, to utilize refrigerator coils as the condenser.

In this apparatus the desiccator consists of two glass cylinders (Pl. IV and Text-fig. 3). The lower cylinder houses refrigerator coils, and the upper contains the material to be dried.

Three holes are drilled through the bottom steel plate of the lower cylinder; two are placed near the centre, and into these are soldered the entrance and exit of the refrigerator "evaporator coils", while the third, which is about 5 in. from the centre of the plate, communicates with a vacuum junction box, by which the desiccator is connected to a Pirani gauge, for measurement of the vapour pressure, and to the Megavac pump.

The vacuum junction box (Text-fig. 2) is constructed from square brass rod, all the joints being screw-fitted and soldered. The Pirani gauge is inserted into a B 14 glass joint which is sealed into the junction box with Apiezon sealing wax. The outlet to the pump is fitted with a metal vacuum tap having a bore of $\frac{3}{4}$ in.

The two glass cylinders are separated by a steel ring of 18 in. external and 10 in. internal diameter, to which they are sealed with Apiezon Q. A second steel ring of 18 in. external and $12\frac{1}{2}$ in. internal diameter is sealed to the top of the upper cylinder. The metal lid and the method of sealing the desiccator have been described previously. With this form of apparatus it has been found to be convenient to attach the stands used to house the vessels containing protein to the metal lid. We have employed three lids for the desiccator, one without any fittings, used to close the apparatus between experiments and for vacuum testing purposes, one fitted with a stand to contain a 20 l. bottle (see Pl. IV, and Text-fig. 3), and one fitted with tiers of trays, to carry a large number of ampoules (see Pl. V).

All the vacuum seals between the glass cylinders and the steel plates have been made with Apiezon Q, but alternative seals could be made by cutting a channel in the steel plate and sinking the glass cylinder in this in a bed of Apiezon sealing wax, or Bank of England sealing wax. We have found it impossible to make a perfect glass-to-steel seal on the first application of Apiezon Q, due perhaps to a slight contraction of the glass cylinder under vacuum. Our technique for overcoming this has been as follows. After the first application of the Apiezon Q, the chamber is evacuated, until a vacuum of

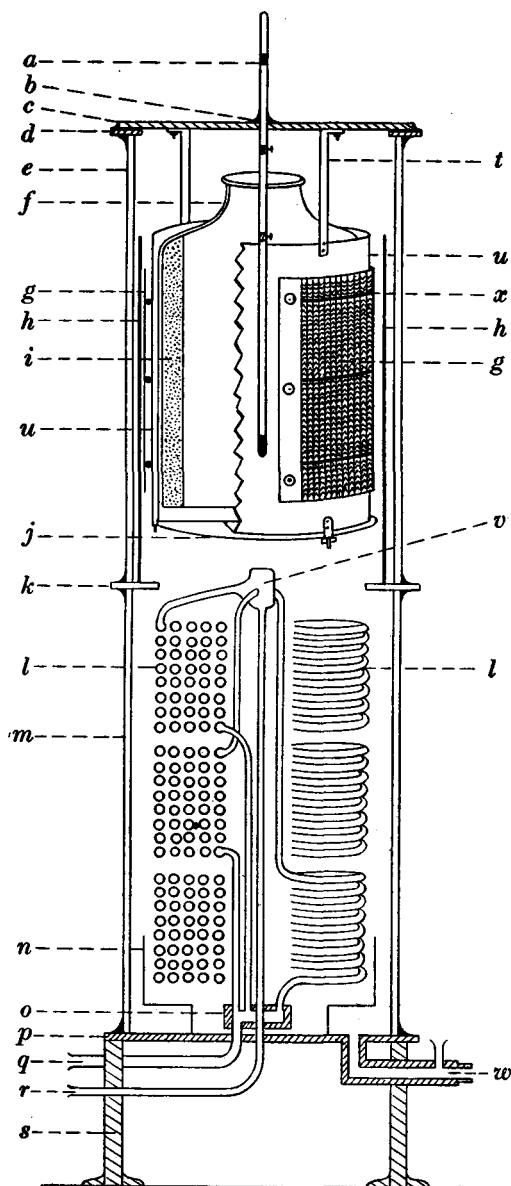


Fig. 3. Explanatory diagram showing details of construction of the desiccator chamber shown in Plate IV. *a*, thermostatic thermometer. *b*, Apiezon Q seal. *c*, steel top plate. *d*, upper steel ring. *e*, upper glass cylinder. *f*, 20 l. bottle. *g*, heating mat. *h*, polished reflector screen. *i*, layer of frozen serum. *j*, detachable copper bottom plate of heating cylinder. *k*, middle steel ring. *l*, refrigerator evaporator coils condenser. *m*, lower glass cylinder. *n*, drip tray for collecting condensate after defrosting. *o*, junction box for the return pipes from the evaporator coils. *p*, bottom steel end plate. *q*, return pipe to the compressor. *r*, entrance pipe to evaporator coils. *s*, steel legs screwed to floor. *t*, supporting arms for heating cylinder. *u*, copper heating cylinder. *v*, junction box to distribute gas to three sets of evaporator coils. *w*, vacuum junction box. *x*, three lines of porcelain bead insulators; also represented by three dots between *g* and *u*.

say 0.5 mm. Hg has been obtained; if the Apiezon Q is now worked in firmly, the Pirani gauge will show a progressive improvement in vacuum up to the maximum of the pump, in our case 0.0001 mm. Hg. Now on closing the tap and disconnecting the pump this vacuum should be maintained for at least a fortnight.

As the Pirani gauge used does not start to record before a pressure of 1 mm. Hg has been attained, it is convenient to fit a small mercury gauge to the middle steel ring so that the preliminary stages of evacuation may be observed.

B. Refrigeration

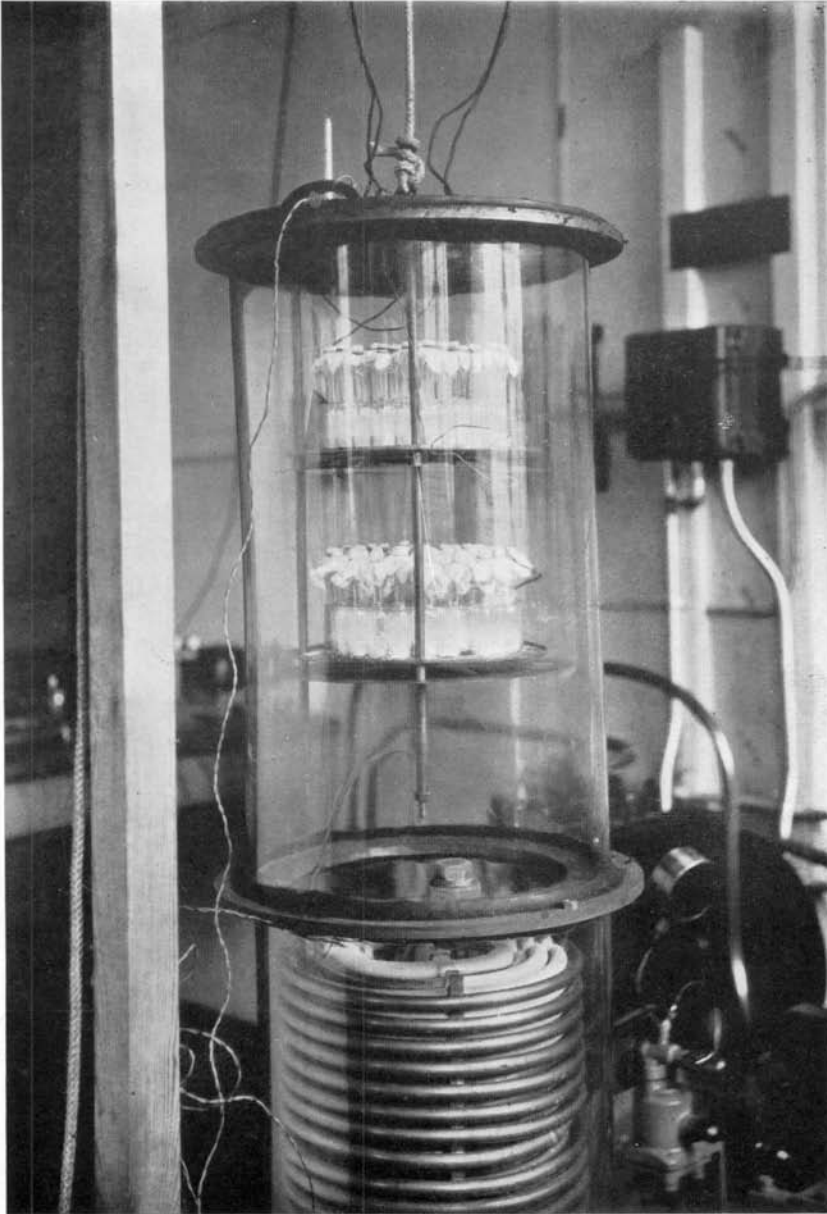
Preliminary experiments were carried out with a compressor unit from a discarded "Isadore" SO₂ refrigerator. With this equipment, the temperature of the coils rarely sank below -20° C., but the data obtained were sufficient to indicate that the form of apparatus was efficient, and, furthermore, that it was possible to devise an automatically controlled system for supplying heat to the frozen protein during the process of drying.

The refrigerator plant finally selected for the purpose of drying 5 l. quantities of material is a type 9 W.H. Hallmark three-cylinder methyl chloride compressor. The evaporator consists of tinned copper tubing, arranged in the form of closely nested coils, and divided into three equal circuits to ensure as equal a distribution of the refrigerant as possible. A tray, capable of holding 5 l. of water, surrounds the base of the coils, so that when they are defrosted at the end of an experiment, water can be removed easily by suction from a water pump through a length of rubber tubing. This method is preferable to providing a tap for emptying the tray, as an additional opening into the desiccator chamber is avoided.

The compressor unit is fitted with condenser and evaporator gauges so that the constant-temperature expansion valve can be exactly regulated, and any moisture in the system, which might collect and freeze the bellows of the expansion valve, is removed with a metal drying tube containing calcium chloride, which is provided with the necessary shut-off valves so that it may be removed and recharged.

An oil separator removes oil which may come over with liquid methyl chloride and collect in the region of the expansion valve, which is the lowest part of the system. At the end of each experiment the oil is returned to the crank case through a by-pass. A water condenser is fitted to the compressor unit, so that the efficiency of the system does not depend on the room temperature.

The temperature of the evaporator has been recorded by means of a thermocouple, wound round one of the coils near its entrance and tied firmly with tape. The thermocouple leads leave the desiccator through $\frac{1}{16}$ in. holes drilled horizontally through the steel ring which separates the two glass cylinders. Glass tubing is inserted through the length of these holes, and the two leads, from



Showing arrangement for drying ampoules. The ampoules are standing in glass dishes, placed on the metal trays beneath which the heater mats are situated. The reflector screens beneath the trays can be seen. The photograph was taken during the desiccation of 500 c.c. of serum distributed in 5 c.c. quantities in plugged ampoules. Some condensed ice can be seen on the evaporator coils.

which the insulation is removed for a similar length, are passed through the glass and sealed in with Apiezon sealing wax.

Records of the coil temperature have shown that this compressor unit can maintain a temperature between -43 and -45°C ., if 250 W. are supplied for the purpose of controlling the evaporation of the frozen serum. In the final stages of the experiment when less work is done, a temperature of -52°C . may be attained. A greater quantity of heat can of course be neutralized by this compressor if a coil temperature higher than -40°C . be permitted; but in the experiments to be recorded such higher temperatures have been avoided. Experiments have also been carried out with a four-cylinder compressor which could maintain a temperature of -40°C . if 350 W. were supplied, but, as will be shown later, such a quantity of heat cannot be utilized under the experimental conditions which have been considered advisable.

In the method for the desiccation of small volumes of protein solutions from the frozen state, "snap" freezing was brought about by the rapid evaporation of the solution in a high vacuum in the presence of phosphorus pentoxide. It is clearly desirable to expose the maximum surface area of frozen material during the drying process, and this is most easily obtained by having the material distributed over the entire inside periphery of the bottle. Since this would be impossible to obtain by "snap" freezing methods, it is necessary to freeze the 5 l. volumes of solution before introducing them into the desiccating chamber.

The bottles used for the drying of 5 l. batches of serum were 23 cm. in diameter by 35 cm. in length and had a capacity of 20 l. Before use they were plugged and sterilized. As an accurate knowledge of temperature conditions is of paramount importance in all the experiments, a thermocouple is arranged to record the temperature of the frozen protein throughout the process of drying. Preliminary experiments had shown, that with a 2 cm. layer of frozen protein, less than 0.5°C . difference could be recorded between the upper and lower surfaces of the layer; the thermocouple is therefore placed in the region of the system which is the warmest at the end of the experiment, namely, at the region of contact of the protein with the wall of the bottle.

The thermocouple is prepared by removing the insulation from 18 in. of the thermocouple wire, and a "looped couple" is made and threaded through a narrow glass tube fitting as exactly as possible the shape of the inside of the 20 l. bottle and finally bent so that the ends are reflected back over the outside of the neck of the bottle where they may be fixed firmly with rubber bands. The thermocouple is sterilized before introduction into the bottle, and it can be readily and cleanly removed at the end of an experiment and may be used repeatedly.

By the courtesy of the Superintendent of the Low Temperature Research Station, Cambridge, we have been allowed to use a room maintained at -20°C . for preliminary freezing of protein solutions. A 5 l. batch of solution is divided into 16 parts of 312.5 c.c. and cooled to 0°C . A cardboard clock face, marked with 16 divisions, is attached to the neck of a 20 l. bottle, which is then laid on

its side on rollers in the -20° C. room and thoroughly cooled. The 16 portions are then poured in, with sterile precautions, at about 90 min. intervals, the bottle being turned through 180° or less, as indicated by the numbers on the clock face, before the addition of each successive amount of 312.5 c.c. By this means the solution may be frozen in a fairly uniform layer around the internal wall of the bottle. When the whole amount is frozen, the cotton-wool plug is replaced by a sterile cap made by inserting 0.5 in. thickness of cotton-wool between two pieces of gauze. Such a cap, which is firmly fixed by rubber bands, has been found to be entirely adequate to maintain sterility, although it permits of much more rapid evaporation than does an ordinary cotton-wool plug.

We are fully aware of the fact that a temperature of -20° C. only brings about slow freezing and may well be above the eutectic point of solutions of proteins in the presence of salts. The concentration of salts that takes place under such conditions has been considered to provide a possible cause of denaturation of proteins (Elser *et al.* 1935). Actually, we have experienced no such effects, e.g. antisera have been found to be rapidly and completely soluble after desiccation following such a method of preliminary freezing, and have retained their original potency. It is possible that the temperature of -20° C. may inhibit denaturation from such causes.

The material which has been frozen slowly presents after drying a somewhat crystalline appearance due presumably to the fact that large ice crystals have formed and evaporated. The dried product which has been instantaneously frozen forms an amorphous mass.

If the protein is to be dried in ampoules, rather than in one large vessel, the requisite number of ampoules are filled to a depth of not more than 2 cm. and plugged, and the contents are allowed to freeze overnight at -20° C. Alternatively, they may be "degassed" and "snap" frozen, thus avoiding preliminary freezing.

C. Methods of heating, and the construction of holders for the 20 l. bottle, and for ampoules

As previously stated, three steel lids have been used for the desiccator. One carries the stand for supporting a 20 l. bottle and another a series of trays for holding ampoules. With this arrangement the electrical connexions for the thermoregulators and the thermocouple wires need not be disturbed, and the rapid introduction of the frozen and removal of the dried product is facilitated, as the stands can be raised and lowered without delay into the desiccator by means of a rope and pulley.

For the 20 l. bottle, the stand is a cylinder, of 12 in. depth and 10 in. diameter, made from 18-gauge copper, the seam being slotted so that an adjustment can be made for slight variations in the diameters of the bottles. The cylinder is attached to the steel lid by three brass arms. The base of the cylinder is a circle of 18-gauge copper, reinforced at the edges, which can be

readily attached and detached from the cylinder for insertion and removal of the bottle. The base is kept brightly polished, to deter radiation of heat downwards on to the evaporator coils.

The heating element consists of two Creda resistance mats each of 18 ohms resistance, connected in series, which encircle the copper cylinder except for a space of 1 in. on either side. They are insulated from the cylinder by three rows of porcelain beads. In one of the spaces mentioned a thermostatic mercury thermometer is fitted into a copper sleeve. The upper end of the thermometer passes through a hole drilled in the steel lid, and is sealed in with a brass washer and Apiezon Q. The thermostat is set externally at the required temperature by means of a magnet and may be adjusted during the course of an experiment.

Four electrical connexions, two to the heating element and two to the thermostat, are inserted through the top plate as follows. In each case, about $1\frac{1}{2}$ in. of platinum wire is inserted into a short piece of thermometer tubing, the walls of which are slightly distended at one end to prevent its being sucked in, and at the distension the wire is sealed. The glass tube is then passed through a hole drilled in the steel lid and sealed in with Apiezon sealing wax. External and internal connexions are then soldered to the appropriate ends of the platinum wire. As thermocouple leads cannot be treated in this way, owing to the formation of secondary couples, the copper and constantan wires, after removing their insulation coverings, have been sealed into suitable lengths of glass tubing with Apiezon sealing wax, and the glass tubes similarly sealed into holes drilled in the steel lid. Rigorous tests have shown that the Apiezon wax seals are efficient in retaining a high vacuum.

The copper-constantan thermocouples are conveniently made from Lewcos insulated twin-twisted thermocouple wire. No attempt was made to weld the couple junction since it was found that simple twisting followed by soft-soldering using pure resin as the flux was efficient. By decreasing the length of the thermocouple wire its resistance is lowered, and the sensitivity increased; a few experiments will therefore determine a suitable length for use over the desired temperature range with the galvanometer available.

In order to shield the upper glass cylinder from heat radiated from the resistance mats, a brightly polished, nickel-plated 18-gauge copper cylinder, of 12 in. diameter, is arranged so that it rests on the upper surface of the central steel ring which separates the two glass cylinders.

If the apparatus is to be used for drying proteins in ampoules, a tiered copper stand is connected to the steel lid by three brass arms (Pl. V). Each stage is heated by means of a resistance mat of 7 ohms fixed to the under side, and insulated from the stage by a mica sheet. Beneath each mat a disk of polished copper is fixed to protect the stage below from radiant heat. The thermostatic thermometer is fitted into a metal sleeve on one of the stages. To facilitate loading and unloading of the desiccator, the ampoules are placed in glass dishes, one of which stands on each stage. Since only one stage is thermo-

statically controlled, it is necessary to have a similar distribution of ampoules on each stage.

The thermostatic thermometer used is a sensitive mercury thermometer with a very fine column, which operates in conjunction with a thermionic-valve relay. When the temperature at which the thermostat is set has been attained, the mercury column makes contact with an adjustable steel wire, thus short-circuiting the grid of the valve, thereby altering its plate current, which in turn operates the relay. The diagram of the circuit is given in Text-fig. 4. With this well-recognized method, no current passes along the mercury column, so that there is no tendency for the column to break, even if it is very fine. This thermostat is very sensitive, and will operate with variations of temperature of the order of 0.001°C . Recently we have substituted a Sun-Vic hot-wire vacuum switch for the thermionic valve relay.

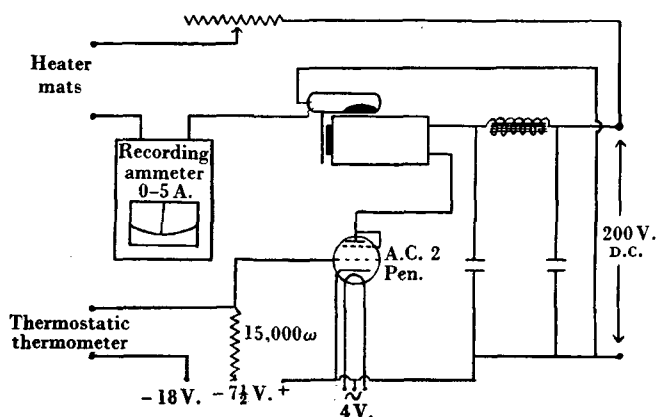


Fig. 4. Circuit diagram of thermionic valve relay used to maintain the heating cylinder or trays at a constant temperature.

The amount of heat supplied has been determined in most of the experiments by recording the time during which the heating current is passing by means of tracings on a smoked drum, and measuring the lengths of the periods during which the current is on and off. More convenient determinations of the duration and strength of the current have also been made by including a recording ammeter in the heating circuit.

When the protein which has been undergoing desiccation is dry, the continuous reduction of temperature due to the latent heat of evaporation will, of course, cease. If the thermostat connected with the heating circuit is set below room temperature, conduction of heat from the steel lid of the desiccator to the copper cylinder will raise the temperature above that of the thermostat setting and the heat will be permanently cut off. If, on the other hand, the thermostat is set above room temperature, there will be a tendency for heat to be conducted away from the cylinder, but the rate of cooling will be constant, and the heating current will be turned on and off at regular intervals. From observations of the

record of heat supplied, it can therefore be ascertained when the process of desiccation is complete. This information is of great value, as, when protein solutions are dried from the frozen state, the appearance of the product gives little or no information as to whether dryness has been attained.

D. *Methods of controlling and recording temperatures*

The simple thermionic-valve relay thermostat previously described and used to maintain the stands or the cylinder at a constant temperature has been found to be an extremely effective form of temperature control. Moreover, if the movements of the relay are recorded a visual indication of the "dry point" can be obtained.

For experimental work it has been highly important to observe the temperatures of the serum and the coils, and the observations have been made throughout with copper-constantan thermocouples. By connecting these to a recording galvanometer continuous records of temperatures have been obtained. For our recent work we have used a Cambridge Scientific Co. six-point recording twin galvanometer. One galvanometer records up to three temperatures from +50 to -50° C., the other two temperatures from 0 to -100° C., and pressures from 1 to 0.0001 mm. Hg.

Though for work with ampoules a stage temperature of +37° C. has been found entirely satisfactory, our work with 5 l. quantities in single bottles soon showed that by maintaining the heating cylinder at +37° C. we were not able to utilize all the heat the refrigerator was capable of neutralizing, so that the drying time was unnecessarily long. By applying 250 W., the maximum load of our refrigerator, the cylinder temperature rose to +90° C., and if the thermostat was set at this temperature drying proceeded very rapidly, but it was necessary to stand by to readjust the thermostat to +37° C. when the temperature of the serum reached +37° C.

To avoid the necessity of this manual change, the relay system shown in Text-fig. 5 was constructed from second-hand G.P.O. telephone relays. Two contacts were fitted on the drop arms of a recording galvanometer, separated by a distance equivalent to a change of 2° C. in temperature. These contacts could be moved along the drop arm and set at any predetermined temperatures.

When the serum temperature reached the point at which the break contact was set, a momentary impulse of 31 V. was placed across the field coil of relay B.K., thus the operation of this relay broke the field circuit of relay M.K. which in its turn broke the field circuit M.B.K. On relay M.B.K. is situated a mercury tip switch, connected in series with a similar switch on relay R.K.; these switches control the heater current, and their position is indicated visually by the two neon "pilot lights". Only when both lights are on is the heater current passing. Thus the momentary impact on the break contact has caused the heater current to be switched off.

Since the serum is not yet dry it will continue to cool and soon an impulse will come on the make contact; this will apply a momentary impulse of 31 V.

to the field coil of relay M.K. which will switch on its own holding voltage of 6 V. and will apply a holding voltage of 6 V. to the relay M.B.K. with the result that the heating current is again switched on.

In this way the serum temperature may be maintained within narrow limits till the thermionic-valve relay thermostat is readjusted.

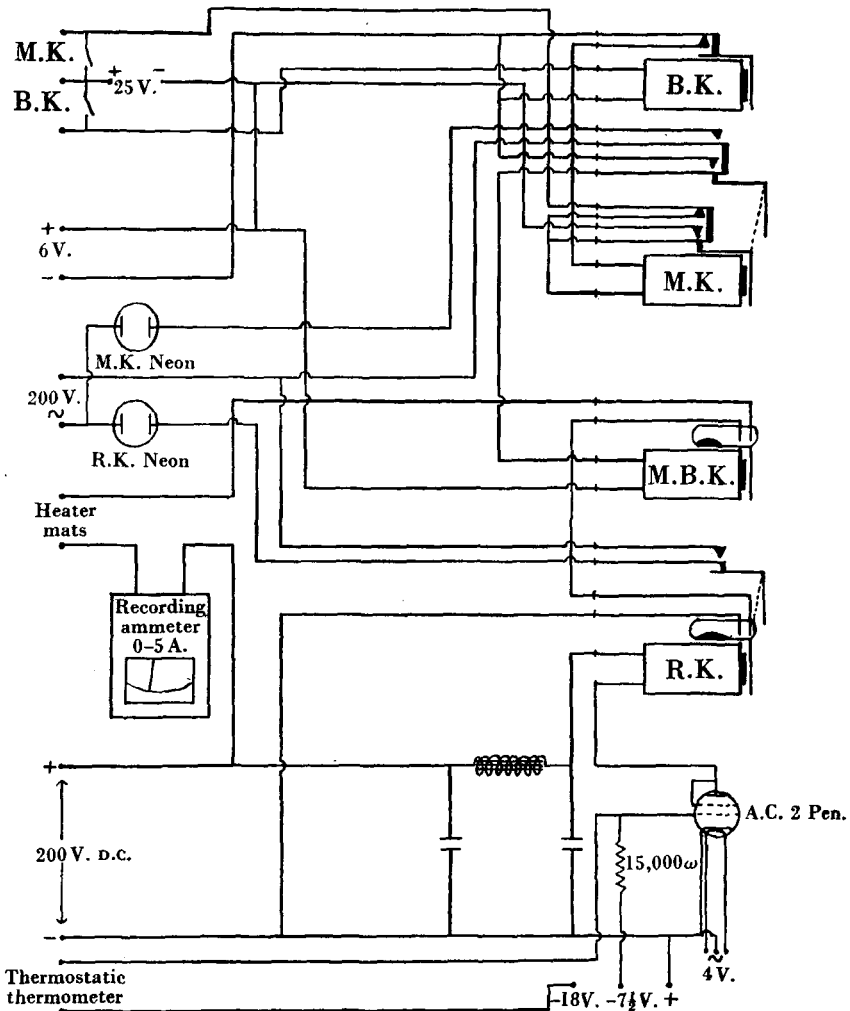


Fig. 5. Circuit diagram of method for operating a secondary thermostatic relay direct from a thermocouple recording the serum temperature.

Another somewhat simpler method of obtaining the same result is shown in Text-fig. 6. By utilizing two previously set stage thermostats, it is possible to arrange to switch over from one to the other at any predetermined serum temperature by means of a single galvanometer contact. In the arrangement as shown in the diagram the momentary closure of the galvanometer contact

causes a momentary high striking potential to be applied to the relay, which throws over the three switch mechanisms causing the application of a low holding potential to its own field coil, the insertion of extra resistance in the heater circuit, and the change over from the high- to the low-temperature thermostat. The advantage of this circuit is that if a recording galvanometer is not available, a Weston micro-relay, suitably shunted, could be made to operate this relay mechanism at any predetermined serum temperature.

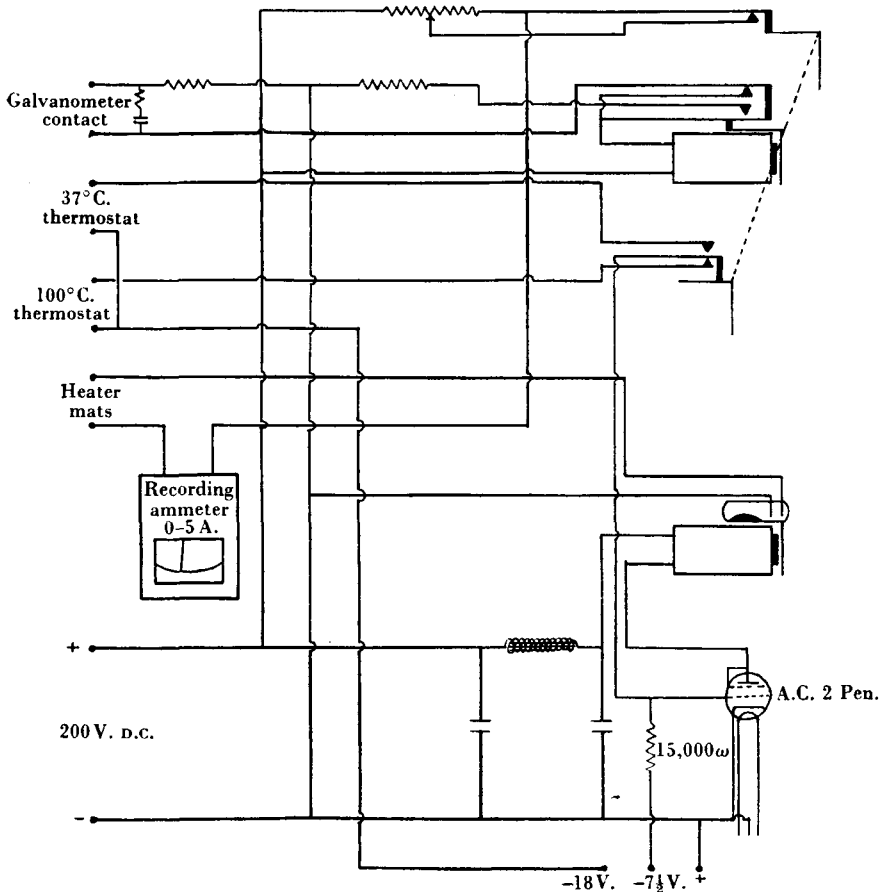


Fig. 6. Circuit diagram of method for the switching over from one thermostat to another pre-set thermostat, at any pre-determined serum temperature.

It should perhaps be emphasized that the thermionic valve relay thermostat is the essential temperature control, and additional relays are only required when a speeding up of desiccation of large volumes is desired.

EXPERIMENTS

All preliminary experiments for the calibration of this apparatus were carried out using 5 l. quantities of a low-potency tetanus antitoxin prepared in the horse, because it was readily available in large bulk, and it also enabled potency tests to be carried out on the dried product at the same time as solubility and sterility tests. In all cases these 5 l. quantities were prefrozen on the inside periphery of 20 l. bottles at -20°C . as already described. The frozen serum was brought from the -20°C . chamber and placed in the apparatus, which was then rapidly evacuated, a temperature of -45°C . having previously been attained on the evaporator coils.

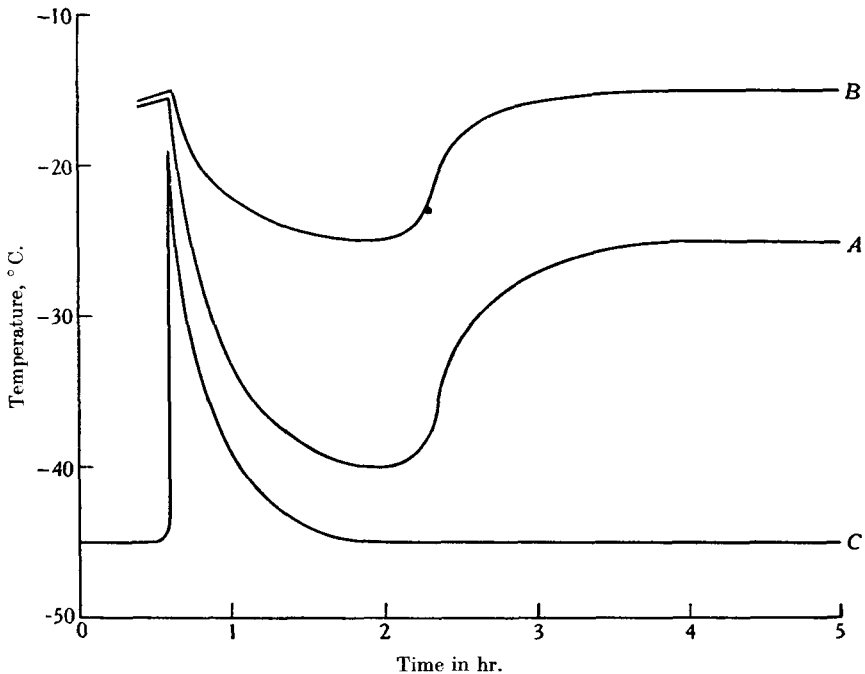


Fig. 7. Temperature curves obtained during the drying of 5 l. quantities of serum with and without plugs. Curve *A* shows the temperature of the serum in the unplugged bottle, curve *B* that of the serum in the plugged bottle. Curve *C* shows the temperature of the refrigerator evaporator coils.

As it is frequently necessary to work under sterile conditions, we determined at the outset to ascertain whether the drying process could be carried out efficiently if the vessels were suitably plugged. Two experiments were therefore carried out, one with the bottle unplugged, the other with the bottle plugged.

Text-fig. 7 shows a portion of the records of the serum temperatures under these two conditions. Inspection of curve *A*, which refers to the unplugged bottle, shows that the initial temperature of the serum on introduction into the apparatus was -16°C ., rising to -15°C . while the desiccator was being

evacuated. The sudden rise of temperature of the refrigerator coils (curve *C*) until it almost equals that of the serum corresponds to a pressure of approximately 1.5 mm. Hg, and it may be assumed that at this pressure evaporation becomes exceedingly rapid and a considerable load is therefore thrown on to the refrigerator. This sudden rise is followed by a rapid fall in temperature of the evaporator coils accompanied by a simultaneous fall of the serum temperature, which will be seen to be always lagging a few degrees behind that of the condenser. This fall in serum temperature is due to the abstraction of heat caused by evaporation, and the serum which had previously been at a temperature above its eutectic point probably now becomes completely frozen.

These records also show that 90 min. after the beginning of the experiment the serum temperature was -40°C ., and the condenser temperature was again -45°C .

Under these conditions evaporation is slow, owing to the low vapour-pressure difference between the serum and condenser. Therefore 250 W. were applied to the heating mats and the thermostat on the copper heating cylinder set at $+100^{\circ}\text{C}$. The temperature of the serum then rose during the course of 2 hr. to -25°C ., which was maintained over a further period of several hours, giving a temperature difference of 20°C . between the serum and the refrigerator coils, and a vapour-pressure difference of approximately 0.423 mm. Hg.

Curve *B* records the temperature of the serum while being dried in a plugged bottle. The same rise in temperature of the condenser was observed, corresponding to a pressure of approximately 1.5 mm. Hg, but it will be noted that in this case the subsequent fall in temperature of the serum did not run parallel to that of the coils, but was less rapid, only attaining -27°C ., instead of -40°C . as in the case of the unplugged bottle. After 90 min. 250 W. were again applied, the thermostat on the copper cylinder being set as before at $+100^{\circ}\text{C}$. The serum temperature now rose to -15°C ., giving a temperature difference of 30°C ., and a vapour-pressure difference of 1.196 mm. Hg.

It would seem therefore that the inhibitory effect of the plug on the rate of evaporation is automatically compensated by an increased temperature and vapour-pressure difference between the coils and the condenser. Under these conditions the same amount of heat is utilized so that the drying time is not influenced.

Our next experiment was made in order to discover the heat absorption at different temperatures of the heating cylinder. The result is shown in Text-fig. 8; this curve may be used, as will be shown later, for the calculation of the maximum refrigerator load for any set of circumstances.

Text-fig. 9 records the complete analysis for the drying of 5 l. of serum, batch A, in a plugged bottle with the heating cylinder maintained at $+37^{\circ}\text{C}$. throughout. Curve *A* is a record of the actual serum temperature throughout. The initial temperature of the serum was -10°C ., falling to -30°C . during the preliminary stage of evaporation. When this temperature had been attained, 120 W. were applied and the thermostat on the heating cylinder set

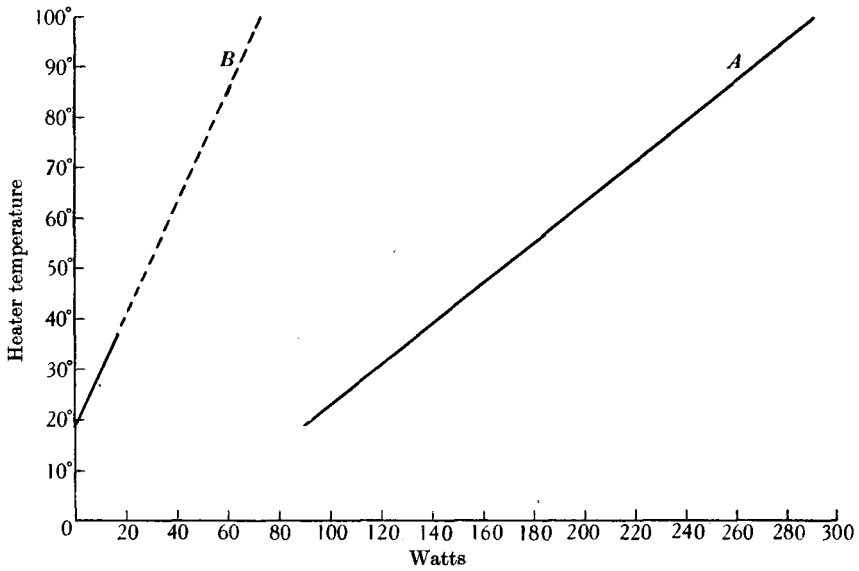


Fig. 8. *A* represents the heat absorbed by 5 l. of serum, distributed so that it had a surface area of 2500 sq. cm., at different temperatures of the heating cylinder. *B* represents the heat loss of the system at different temperatures of the heating cylinder. Continuous line represents values actually plotted and the dotted line assumed values.

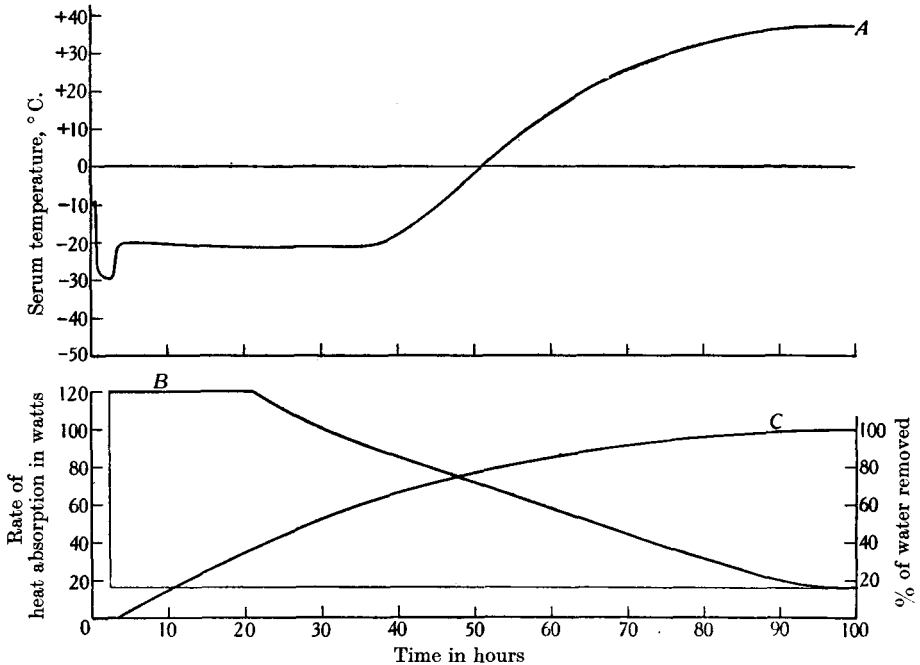


Fig. 9. Records obtained during the drying of 5 l. of tetanus antitoxin with the heating cylinder set at $+37^{\circ}\text{C}$. Curve *A* records the actual serum temperature throughout the experiment. Curve *B* and the thin lines enclose an area which represents the heat utilized to dry the serum. Curve *C* records the percentage dryness attained at different periods of the experiment, and is calculated from the heat utilization area.

at $+37^{\circ}\text{C}$. After the heating current had been turned on, the serum temperature rose to -20°C . and fell again very gradually to -21°C . during the course of about 30 hr., after which it rose, reaching 0°C . in $55\frac{1}{2}$ hr. and $+37^{\circ}\text{C}$. 95 hr. after the beginning of the experiment.

The heating energy of 120 W. passed continuously for the first $22\frac{1}{2}$ hr., at which time the heating cylinder reached $+37^{\circ}\text{C}$., and the thermostat therefore came into operation. The graphic record of the periods during which the current was and was not passing showed that for the next 37 hr. the "on" periods became progressively shorter and the "off" periods longer, till from the 94th to the 100th hour the relationship between these periods was constant. The serum was assumed therefore to have reached the equilibrium dry point.

By accurate measurement of these "off" and "on" periods it was therefore possible to ascertain the mean rate of heating at any period of the experiment, and by plotting these values, curve *B*, representing the rate of heat absorption, was obtained.

It may be noted that at the end of the experiment, when equilibrium had been attained, 17 W. were utilized to maintain the temperature of the system at $+37^{\circ}\text{C}$. This represents the heat loss of the system at this temperature. A base-line must therefore be drawn at 17 W., when an area will be demarcated between it and the heat utilization curve, which represents the heat in joules absorbed by the serum during evaporation. Since 4.182 joules = 1 cal., this area represents 4,507,636 cal., or 901.5 cal. were required to dry 1 c.c. of serum from the frozen state. From the record of the amount of heat absorbed at any given time throughout the experiment, it is possible to calculate the amount of water removed from the serum at each stage of the process of desiccation. These values, plotted as percentages of the total water, are recorded by curve *C* in Text-fig. 9, which shows that 59% of the water had been removed when the temperature of the serum started to rise from -21°C ., and that 78% had been removed when 0°C . was reached.

The latent heat of evaporation of ice at different temperatures can be calculated from Clapeyron's equation $\frac{dP}{dT} = \frac{\lambda}{T(v_2 - v_1)}$, which gives a value of 665 cal. between 0 and 5°C ., and 672 cal. between -25 and -30°C . Within the accuracy of this analysis it is fair to assume an average value of 670 cal./g. of ice over the whole range.

Our figure of 901 cal./g. is considerably above this figure, but part of the discrepancy can be accounted for by the fact that our figure also includes the heat utilized for raising the temperature of (a) the bottle from -35 to $+37^{\circ}\text{C}$., (b) the copper cylinder, (c) the dried solid, and (d) in warming the water vapour as it passes between the heating elements and the radiant heat screen on its way to the condenser, but most of the discrepancy is probably accounted for by the energy required to drive off hydrated water from the protein. Though we do not consider the figure of 901 cal./g. to be strictly accurate, it is justifiable to utilize this figure for computing the percentage dryness curve.

The antitetanus serum, desiccated as described in this experiment, was found to yield a satisfactory product as far as could be determined by tests of solubility, sterility and potency. Further experiments were therefore carried out (batches B and C) in order to investigate the possibilities of accelerating the early stages of the process. The records of the serum temperatures in these experiments are given in Text-fig. 10, and for purposes of comparison curve *A* of the previous experiment.

In the second experiment (Text-fig. 10, curve *B*, batch B), as soon as the temperatures of the condenser and the serum had fallen to the minimum values the thermostat on the heating cylinder was set at $+100^{\circ}\text{C}$. and the heating current adjusted so that 250 W. were applied. A secondary thermostatic control was arranged to operate from the recording galvanometer, on which the thermocouple from the serum was recording serum temperature; as previously described the "break" contact was set at 0°C . and the "make"

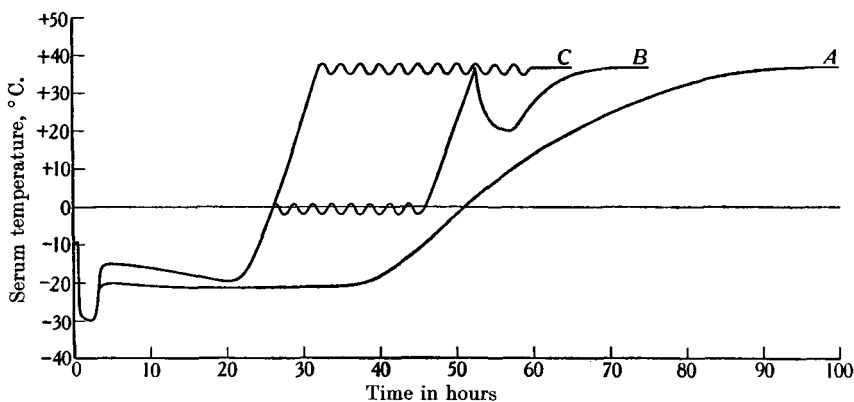


Fig. 10. Records of the serum temperature observed under different experimental conditions as described in text.

contact at -2°C ., so that while this secondary thermostat was in operation the temperature of the serum could not rise above 0°C .

Curve *B*, Text-fig. 10, shows that the effect of raising the temperature of the heating cylinder from $+37$ to $+100^{\circ}\text{C}$. was to raise the temperature of the serum from -20 to -15°C ., which is presumably above its eutectic point.

The serum temperature rose to 0°C . in 27 hr. as opposed to 51 hr. in the previous experiment; at this temperature it was held by the secondary thermostat for a further 21 hr. On release from this secondary thermostat the serum temperature rose in about 5 hr. to $+37^{\circ}\text{C}$., when the thermostat on the copper cylinder was readjusted from $+100$ to $+37^{\circ}\text{C}$., which automatically cut off the supply of heat until the process of evaporation of the serum had abstracted sufficient heat to bring about the reduction of temperature of the cylinder to $+37^{\circ}\text{C}$., when the thermostatic valve relay again came into operation. The temperature of the serum meanwhile started to fall and was reduced from $+37$ to $+20^{\circ}\text{C}$. in the course of about 5 hr.; it then rose slowly again to $+37^{\circ}\text{C}$.,

and inspection of the record of the heating circuit showed that the equilibrium dry point was reached 76 hr. after the beginning of the experiment.

Curve *B*, which records the serum temperature, shows that the reduction in time of 24 hr. is to be attributed to the early part of the experiment, and indicates that the process would have been still more rapid if the thermostat on the copper heating cylinder had been adjusted to $+37^{\circ}\text{C}$. as soon as the temperature of the serum rose to 0°C . An automatic mechanism which could be used to perform this adjustment has been suggested (Text-fig. 6).

As solubility, sterility and potency tests were again satisfactory, it was decided still further to accelerate the drying process and to repeat the experiment (batch *C*), arranging the serum temperature thermostatic control so that the "make" contact was at $+35^{\circ}\text{C}$. and the "break" contact at $+37^{\circ}\text{C}$. The temperature of the serum during the progress of this experiment is recorded by curve *C*, Text-fig. 10. The process of drying was completed in 60 hr. The tests

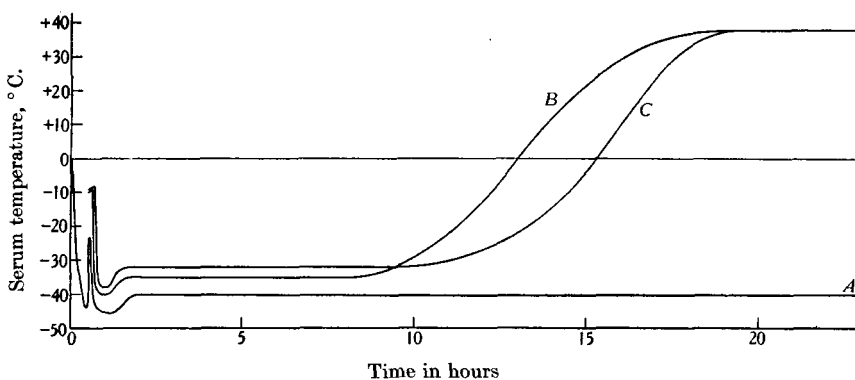


Fig. 11. Temperature records of 2.5 c.c. quantities of serum dried in ampoules, *B* without, *C* with plugs. Curve *A* represents the temperature of the refrigerator evaporator coils throughout the experiment.

for solubility, sterility and potency again indicated that the serum had not undergone denaturation by being dried under these conditions.

Text-fig. 11 records an experiment in which 2.5 c.c. quantities of anti-tetanus horse serum were dried in ampoules with and without plugs. In this case the recording galvanometer was adjusted so that three temperature records were obtained. Curve *A* shows the temperature of the condenser coils, curve *B* the temperature recorded from a thermocouple fixed into an unplugged ampoule, and curve *C* into a plugged ampoule.

The necks of the ampoules were 5.5 cm. long, with an orifice area of 0.133 sq. cm.; their internal diameter was 2 cm., giving a depth of fluid of 0.8 cm. when containing 2.5 c.c. of serum. The serum was frozen at -20°C . before introduction into the desiccator. After the temperatures of the condenser coils and the serum had fallen to the minimum values, the heating stage thermostat was set at $+37^{\circ}\text{C}$. and the heating current turned on.

Text-fig. 11 shows a temperature difference of 5° C. between the serum in the unplugged ampoules and the condenser, and of 8–9° C. between the serum in the plugged ampoules and the condenser. Even in the latter case, therefore, the difference is less than between the coils and the plugged 20 l. bottle. This is probably due to the more favourable relationship between the area of the orifice and the surface area of the serum in the case of the ampoules. The ratio

$$\frac{\text{area of aperture}}{\text{surface area of serum}} = 1/7\cdot38 \text{ for ampoules and for the 20 l. bottle} = 1/35\cdot3.$$

As in the case of the experiments previously discussed, the presence of a plug is shown to make no difference to the total time taken for attaining the equilibrium dry point. Experiments in which 5 c.c. of serum were placed in similar ampoules, showed that doubling the depth of fluid slightly more than doubled the total time taken to attain the equilibrium dry point.

If different quantities of serum in ampoules are required to be dried at the same time, it is inadvisable to set the stage thermostat above 37° C., since the smaller quantities would dry before the larger and the dry powder would then be exposed to a high temperature.

Percentage dryness of final product

In experiments in which 5 l. quantities were dried in bulk, only indirect methods could be adopted for estimating the residual water content of the dried serum.

If at the close of an experiment the 20 l. bottle was immediately transferred to a second desiccator, containing a small amount of phosphorus pentoxide, and the system was evacuated with the Megavac pump, a slight alteration only was observed in the appearance of the desiccant. Previous experience had shown that the quantity of phosphorus pentoxide used would have become practically liquefied by 5 c.c. of water and appreciably wetted by 3 c.c.

10 c.c. of the original serum when dried over phosphorus pentoxide weighed 0.7347 g. 5 l. would therefore have a dry weight of 367.35 g., and 1 c.c. of residual water would be less than 0.3% of the dry weight.

Table I, column 1, records the dry weights of 10 c.c. quantities of serum after desiccation at a stage temperature of +37° C. with the evaporator coils at –45° C., until the heat absorption curve and the thermocouple readings indicated that the equilibrium dry point had been attained. The weighing bottles containing the serum were subsequently transferred to a small desiccator charged with phosphorus pentoxide and were weighed daily on four subsequent days, the desiccator being evacuated between each weighing.

At a temperature of +37° C., the vapour-pressure of pure water is 46.65 mm. In the large desiccating chamber, the tension of aqueous vapour in the region of the evaporator coils at –45° C. should be approximately 0.061 mm. Assuming that the tension of aqueous vapour over the dried serum at +37° C. is 0.061 mm., the “relative humidity” of the dried serum should be equal to the ratio $0.061/46.65 = 0.001308$. The relationship between the water bound by colloids

Table I

Species	Net wt. of 10 c.c. serum g.	Dry wt. after condensation over drying g.	Dry wt. after 24 hr. P ₂ O ₅ g.	Dry wt. after 48 hr. P ₂ O ₅ g.	Dry wt. after 72 hr. P ₂ O ₅ g.	Dry wt. after 96 hr. P ₂ O ₅ g.	Net loss of wt. over P ₂ O ₅ g.	Loss of wt. over P ₂ O ₅ per g. dry solid g.
Rabbit	10-2607	0-8608	0-8583	0-8577	0-8573	0-8572	0-0036	0-0042
Rabbit	10-2632	0-8608	0-8579	0-8572	0-8574	0-8565	0-0043	0-0050
Rabbit	10-2609	0-8608	0-8582	0-8571	0-8574	0-8567	0-0041	0-0048
Guinea-pig	10-1951	0-6870	0-6848	0-6835	0-6838	0-6835	0-0035	0-0051
Guinea-pig	10-2155	0-6879	0-6844	0-6838	0-6845	0-6838	0-0031	0-0048
Horse	10-1671	0-7743	0-7716	0-7711	0-7712	0-7709	0-0034	0-0044
Horse	10-1673	0-7741	0-7717	0-7707	0-7696	0-7696	0-0043	0-0056

and the relative humidity has been investigated by Katz (1917). He did not record any data for serum, but in the case of serum albumin he found that 1 g. protein bound 40 mg. water at a relative humidity of 0.02. From his data it is not possible to make an exact estimate of hydration at a relative humidity of 0.0013, but it seems almost certain that it exceeds the value 2.6 mg. calculated on the assumption that hydration is directly proportional to the relative humidity, and it is unlikely that it exceeds 10 mg., the value calculated on the assumption that hydration is proportional to the square root of the relative humidity.

It may be noted from Table I that the observed values for the loss of weight over phosphorus pentoxide, which range from 4.2 to 5.6 mg./g. of dry solid, lie within the limits deduced from the measurements of Katz on serum albumin. These observations indicate that the efficiency of the drying process approaches that of an ideal system, where the protein has reached equilibrium at the extremely low relative humidity of 0.0013.

Sealing of vessels containing dried protein

In the preparation of biological standards, Hartley (1936), after the stage of complete dryness has been attained, constricts the neck of the ampoule and continues the exposure of the dried product to the dehydrating action of phosphorus pentoxide *in vacuo* for a further 7-10 days. The ampoules are then filled with pure dry nitrogen gas and either sealed, or evacuated and then sealed. In the majority of cases we have found it satisfactory to seal the vessels after admission of dry air, although theoretically nitrogen is preferable.

The procedure has been as follows. At the end of an experiment the vacuum is slowly replaced by air or nitrogen, and since the gas must pass over the refrigerator coils it is dried while being admitted to the desiccator. The bottle is then transferred to a desiccator constructed from one glass cylinder which contains a small quantity of phosphorus pentoxide, and the desiccator is evacuated. After 3 days, dry air or nitrogen is slowly readmitted, and the bottle is removed from the desiccator and the cotton-wool plug is immediately replaced by a sterile glass plate, which is sealed on with Apiezon Q, or, if a more permanent joint is required, with Apiezon sealing wax.

In the case of plugged ampoules, the glass dishes containing the ampoules are placed over phosphorus pentoxide in a desiccator, constructed from one glass cylinder, from which they are more readily available than from the large desiccator. About a dozen ampoules at a time are then transferred to a small desiccator also containing phosphorus pentoxide and from this they are taken one at a time, and sealed. The rate of diffusion of gases through the narrow plugged necks of the ampoules is sufficiently slow for these manipulations to be carried out with safety.

If very rapid re-solution is essential it is advisable to seal the ampoules in a high vacuum and to dissolve the dried material in water from which the air has been driven out by boiling. In this way air pockets, which inhibit the contact of water with the solid, can be eliminated.

Biological assay of the dried antitoxins

(1) Tests on 5 l. quantities, dried in one container.

The 20 l. bottles, containing the total solids (about 375 g.) from 5 l. of tetanus antitoxin, were transferred from Cambridge to a sterile room at the National Institute for Medical Research, Hampstead.

The sterile glass plate was removed with aseptic precautions and about 5 g. of the dried serum were removed by means of a sterile scoop. This sample was used for potency tests and for other examinations. 5 l. of sterile distilled water were added to the main bulk and the neck of the bottle closed with a previously sterilized cotton-wool plug. The dried serum dissolves rapidly, but it is an advantage to rotate the vessel gently in order to wet the large amount of dried solid quickly, and so accelerate solution. Samples of the main bulk of dissolved serum were withdrawn and used for potency and sterility tests.

Sterility tests. 0.5 c.c. quantities were tested on fluid and solid media at 37° C. and at room temperature, for the presence of aerobic and anaerobic organisms. No growth occurred in any of the tests applied to any of the samples examined.

Potency tests. In order to obtain strictly comparable solutions for the tests for potency the following procedure was adopted:

Four solutions of antitoxin were used for each of these tests.

Solution (a). The parent antitoxin, diluted 1 in 10 with saline.

Solution (b). Calculating from a preliminary estimation of the total solids of the parent antitoxin, a weighed portion of the 5 g., removed as described, was dissolved so that it contained the same percentage of solids as the parent antitoxin, diluted 1 in 10 (i.e. solution (a)).

Solution (c). The rest of the dried solid (main bulk) was dissolved in 5 l. of distilled water. 5 c.c. were removed and made up to 50 c.c. with saline.

Solution (d). The dissolved main bulk was passed through a Seitz filter. 5 c.c. of the filtrate were made up to 50 c.c. with saline.

By comparing solutions (a) and (b), any loss of potency due to the drying

process could be estimated, while a comparison of solutions (c) and (d) should reveal any loss of potency subsequent to Seitz filtration of a dissolved dried antitoxin.

The tests of the original serum and the dissolved dried antitoxin were carried out at the same time and with the same solution of test toxin, a control series of tests of the toxin solution with standard antitoxin always being included. Exactly similar series of antitoxin dilutions, and mixtures of these with the toxin solution, were made and injected into groups of mice. By observation of the symptoms produced, survival time and mortality, a very close comparison of the relative potency of the original serum and the solutions of the dried antitoxin could be made. The results of the tests of three batches of tetanus antitoxin, desiccated as described in the foregoing text, are summarized below.

Batch A. 5 l. of tetanus antitoxin, natural serum,* of low potency (see p. 429 and curve *A* of Text-fig. 10).

Sample	Potency, units per c.c.	Comment
(a) Parent antitoxin (control)	67 to 72	Nearer 67 than 72
(b) Dissolved antitoxin (separate sample)	67 to 72	Nearer 72 than 67
(c) Dissolved antitoxin (main bulk)	67 to 72	Nearer 72 than 67
(d) Main bulk after Seitz filtration	67 to 72	Nearer 72 than 67

Batch B. 5 l. of tetanus antitoxin, natural serum, of low potency (see p. 432 and curve *B* of Text-fig. 10).

Sample	Potency, units per c.c.	Comment
(a) Parent antitoxin	67 to 72	Nearer 67 than 72
(b) Dissolved antitoxin (separate sample)	67 to 72	Nearer 72 than 67
(c) Dissolved antitoxin (main bulk)	67 to 72	Nearer 72 than 67
(d) Main bulk after Seitz filtration	67 to 72	Nearer 67 than 72

Batch C. The parent material for this experiment was the dried antitoxin Batch A dissolved and Seitz-filtered, of which 4.8 l. were used for the experiment. That is, it was a tetanus antitoxin, natural serum, of low potency which had been dried, dissolved, dried a second time and then redissolved in 4.5 l. of sterile distilled water (see p. 433, and curve *C* of Text-fig. 10).

Sample	Potency, units per c.c.	Comment
(a) Parent antitoxin	67 to 72	Nearer 67 than 72
(c) Dissolved antitoxin (main bulk)	67 to 72	Nearer 72 than 67
(d) Main bulk after Seitz filtration	67 to 72	Nearer 72 than 67

The results of these tests show that 5 l. quantities of antitoxin can be reduced to the dry condition and sterility maintained; the whole of the protein dissolves quite readily and there is no loss of potency. Actually, as regards batches A and B, the samples of dissolved antitoxin proved to be slightly more potent than the original parent, due most probably to slight deterioration of the parent material during the interval between beginning the desiccation and the performance of the potency tests. The three main bulks of dissolved antitoxin

* Whole serum, unheated and without antiseptic.

were Seitz-filtered, not because of cloudiness or non-sterility, but in order to see whether any loss of potency occurred as a result of this procedure; in two cases no loss occurred and in one case it was very small.

The results of batch C are of particular interest as they relate to an antitoxin which had been dried twice, the second desiccation being conducted under conditions which might be expected to cause damage to the protein and consequent loss of potency (see Text-fig. 10). There was no evidence whatever either of denaturation, impaired solubility or loss of potency.

(2) *Tests on 5 c.c. quantities of antitoxin dried in ampoules.*

As it was desired to determine quantitatively the loss of potency which might occur on desiccation and at the same time to obtain a sterile end product, 5 c.c. quantities of the antitoxin were delivered into a series of ampoules by means of a previously sterilized precision distribution apparatus (Hartley, 1936) and subsequently dried as described in the text.

For the purpose of the sterility tests, 5 c.c. of sterile distilled water were added to the contents of an ampoule containing the dried solids from 5 c.c. of antitoxin. The solutions were tested in the way previously described, and all the samples tested proved to be sterile.

For the purpose of the potency tests, the contents of an ampoule were dissolved in 5 c.c. of sterile distilled water, the solution transferred to a 50 c.c. graduated flask, the ampoule washed out with successive 5 c.c. quantities of salt solution and the volume made up to 50 c.c. 5 c.c. of the parent antitoxin were diluted to 50 c.c., and the subsequent dilutions required for testing were prepared from these first dilutions.

Batch D. The parent material for this experiment was the dried and dissolved batch B, i.e. a tetanus antitoxin, natural serum, of low potency.

Sample	Potency, units per c.c.	Comment
Parent antitoxin	62 to 67	Nearer 67 than 62
Redissolved antitoxin (ampoule)	67 to 72	Nearer 67 than 72

Batch E. The parent material for this experiment was a concentrated globulin solution of tetanus antitoxin, prepared from batch B, after drying and re-solution, by separating and concentrating the globulin fraction.

Sample	Potency, units per c.c.	Comment
Parent concentrated antitoxin	200 to 220	No detectable difference in potency
Dissolved concentrated antitoxin (ampoule)	200 to 220	

Batch F. The parent material was a concentrated diphtheria antitoxin, globulin solution, which had been prepared from a diphtheria antitoxin of low potency, the final globulin solution being diluted with four volumes of normal horse serum to provide an adequate volume of material for a large-scale experiment.

Sample	Potency, units per c.c.	Comment
Parent concentrated antitoxin	235	Potency determined by the intracutaneous method of testing
Dissolved concentrated antitoxin (ampoule)	More than 220, less than 235	

The results of these tests show that 5 c.c. quantities of antitoxin can be dried in ampoules, sterility maintained and soluble products obtained. In two experiments no detectable loss of potency occurred on desiccation, and in one case the loss was trifling.

DISCUSSION

An analysis of these experiments reveals certain factors which are helpful in interpreting some of the fundamental principles underlying this method of drying.

At a definite pressure evaporation suddenly becomes exceedingly rapid and in consequence "snap" freezing occurs in a liquid and a sudden fall in temperature in a frozen material (see Text-figs. 7, 9, 10, 11, 12).

This pressure has not been measured exactly, but is reached when the Pirani gauge situated as in our apparatus indicates about 1.5 mm. Hg. Presumably in the neighbourhood of the serum the pressure is higher, standing at about 4 mm. Hg. To produce desiccation from the frozen state it is essential to work below this pressure.

So long as other conditions remain constant, the actual temperature of the serum depends on the degree of obstruction to the flow of water vapour to the condenser. The greater this obstruction, the higher the serum temperature. Excessive obstruction is most commonly caused by a poor vacuum due to leakage, an inefficient vacuum pump, tubes of narrow bore, such as long and narrow necks of ampoules, or manifolds constructed from long and narrow tubes, or to unsuitable cotton-wool plugs for the ampoules. Any of these causes, if excessive, may lead to so serious a rise in serum temperature that thawing results.

Text-fig. 12 records an experiment designed to show the effect of variations of pressure, stage temperature and condenser temperature on the temperature of material undergoing desiccation from the frozen state.

Text-fig. 8 shows that there is a definite relationship between the heater temperature and the amount of heat absorbed for a given surface area of serum. An increase in heater temperature results in a rise in serum temperature, and the extent of this rise depends largely on the degree of obstruction which exists; if obstruction is low, large increases in heater temperature cause slight rises only in serum temperature. We would go so far as to say that it is impossible to melt frozen serum so long as it is in a good vacuum with little obstruction to hinder the flow of water vapour to the condenser; in fact we have seen ice showing no tendency to thaw when in contact with a white-hot wire.

It will be seen, therefore, that the form of apparatus described, in which obstruction is reduced to a minimum and where heating is under complete control, offers considerable advantages over other types when really low serum temperatures are required during desiccation. With the condenser at -45°C . it is possible to dry proteins at -38°C .; slight alterations to the heating stands,

to prevent heat being conducted down the supporting rods, would enable even lower temperatures to be maintained, but since the speed of drying would then become very slow, this alteration does not seem to be justified.

As shown below, lowering of the condenser temperature would lower the serum temperature to a very slight extent only. The serum temperature at -35°C . and the condenser at -45°C . (Text-fig. 11) gives a vapour-pressure difference of $0.173 - 0.061 = 0.111$ mm. Hg under conditions in which obstruction is at a minimum. Assuming the vapour-pressure of ice at -184°C . to be nil, liquid air in the condenser would give the same vapour-pressure difference

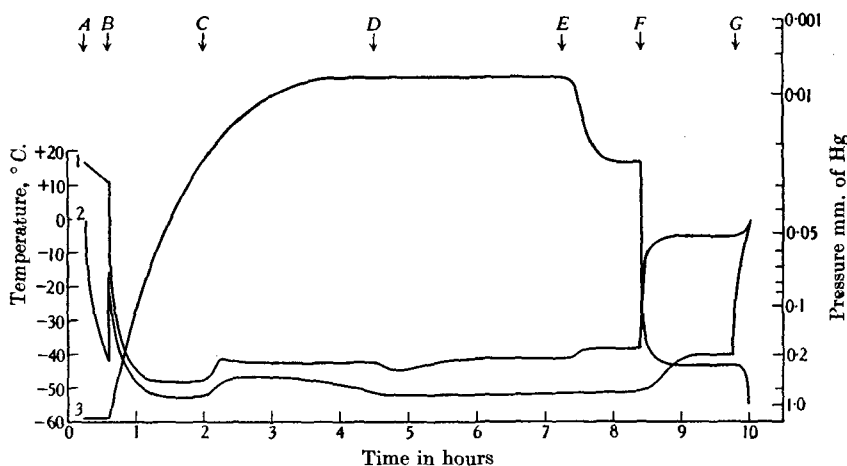


Fig. 12. Experiment designed to show the effects of variations of pressure, stage temperature and condenser temperature on the temperature of material undergoing desiccation from the frozen state. 0.5 l. of distilled water were placed in each of two evaporating dishes on the two stages (see Plate V) and left in a "water-pump vacuum" overnight to de-gas. Curve 1 records the temperature of the distilled water during desiccation. Curve 2 records the temperature of the refrigerator evaporator coils. Curve 3 the pressure of the system as recorded by the Pirani gauge. At A the refrigerator was started. At B the vacuum pump was turned on; this was followed almost immediately by a "snap" freezing of the distilled water. At C, heat was applied, the stage temperature being set at $+50^{\circ}\text{C}$. At D, the stage temperature was lowered to $+25^{\circ}\text{C}$. At E, the vacuum pump was turned off. At F, air was admitted to the system to increase the pressure. At G, the refrigerator was turned off.

of 0.111 mm. Hg when the serum is at a temperature of -39.5°C . Thus a drop of condenser temperature of 139°C . would cause a drop of 4.5°C . only in serum temperature.

Since a definite number of calories is required to evaporate 1 g. of ice at a definite temperature, the speed of evaporation must depend directly on the rate of absorption of heat; and since at a given heater temperature the greater the surface area of serum the more heat is absorbed, a volume of serum will dry more quickly the more it is spread out. On the other hand, within certain limits, the same speed of desiccation could be obtained by decreasing the surface area of the serum and at the same time increasing the temperature of the heater.

The influence of the degree of vacuum on the system has already been discussed when considering the effects of obstruction to the passage of water vapour (p. 439).

Since it is the total pressure which affects the system, namely, the partial pressures of indifferent gases and of water vapour, it is desirable to use a Pirani electrical gauge which records the total pressure rather than the McLeod type gauge which only records the partial pressure of indifferent gases other than water vapour.

The pressure recorded by the Pirani gauge depends upon its situation; in the apparatus described this is between the condenser and the vacuum pump. If preliminary vacuum tests are made by evacuating the *dry* chamber, the Pirani gauge gives a true reading of the pressure of the residual air. It has been our criterion in these preliminary tests that a vacuum at least as good as that for which the pump was designed should be obtained and maintained over two weeks. If everything is operating satisfactorily during the process of desiccation, the Pirani gauge, on account of its position, shows a pressure rather lower than that corresponding to the vapour pressure of ice at the temperature of the coils.

We have shown experimentally that the effect of placing an obstruction to the passage of water vapour from the evaporating substance to the condenser is to cause an increased vapour pressure over the evaporating substance which results in a rise in its temperature. Since plugging of the ampoules is essential to ensure sterility, all other sources of obstruction should be eliminated where possible. Desiccators undoubtedly reduce obstruction to a minimum, but in the past reliable desiccators of large enough capacity were difficult to obtain.

The desiccator chambers evolved in the Cavendish Laboratory, however, overcome this difficulty; glass cylinders can be bought in almost any size, and steel end-plates sealed with Apiezon Q are entirely satisfactory. Alternatively, weldless steel cylinders, though more expensive, could be substituted for the glass cylinders. The consequent lack of visibility would be no disadvantage, since the whole desiccating process is recorded on instruments and adjusted and controlled externally. Small desiccators constructed on these lines from glass cylinders 7 in. in diameter by 12 in. long, with steel end-plates and vacuum junction boxes, are excellent substitutes for ordinary laboratory vacuum desiccators.

Probably the most important practical advantage of the apparatus described is the ease of determining when the equilibrium "dry point" has been reached. With the Flosdorf & Mudd apparatuses dryness is calculated on a time basis coupled with the weighing of sample ampoules, and though they claim a product which should only contain 0.5% of its dry weight of water, Flosdorf & Webster (1937) show that on analysis some of their samples, taken at random, reveal wide differences in the residual water. This variation is completely abolished by estimating the "dry-point" from the heat utilization chart, a method which is applicable even when different quantities and thicknesses of material are being dried at the same time.

The thermocouple recording the serum temperature also gives some indication as to when the dry point is reached, for the serum temperature will then be equal to that at which the stage thermostat is set. This observation shows the dry point to be reached an hour or two before the heat absorption curve shows the process to be completed, but presumably this is due to the extreme sensitivity of the thermostatic thermometer we have used.

In some cases it may be desirable to dry at a temperature below the eutectic, though with antibody solutions we have not been able to detect any difference between material dried below or above the eutectic temperature. Again, there may be cases where it is undesirable to raise the temperature above 0° C. until a definite proportion of water has been removed. This can readily be accomplished by a simple calculation and adjustment of the apparatus as described, though again we have been unable to detect any difference in the final product if the temperature has been raised above 0° C. when the antisera have had quite widely differing water contents. It is possible, however, that certain substances may require careful control, as Flosdorf & Mudd (1938) have shown that some viruses do not survive desiccation by their process.

Flosdorf & Mudd (1935) stress the point that to avoid thawing the frozen serum during desiccation it is necessary to ensure a very favourable surface-to-depth ratio of the serum in the ampoule. We have not observed this critical ratio; in fact it has been possible to dry a thickness of 1 in. of serum perfectly satisfactorily.

The maximum heat which can be applied in practice depends, first, on the maximum heat which the refrigerator can neutralize without undue rise in temperature, and secondly, on the maximum temperature to which it is considered advisable to adjust the heater. In practice we have never raised the heater temperature above 100° C., for this is the maximum temperature on our thermostatic thermometer, but with higher reading thermometers there is no reason why much higher temperatures should not be used, providing always that there is a secondary relay operating on the serum temperature, which will prevent it ever getting above +37° C. or whatever temperature is considered to be the highest safe maximum towards the end of the experiment.

With the condenser at -45° C. and the stage at +37° C. our dry-weight experiments show 0.42-0.56% of the dry weight to be residual water. This is possibly of no importance (Flosdorf & Webster, 1937), but if it is desired to remove the last traces of water this can be done simply by placing in a single-cylinder desiccator in a high vacuum over a very small quantity of phosphorus pentoxide. This has been our usual practice, and it will be seen from Table I that a period of 3 days over phosphorus pentoxide is desirable to attain complete dryness.

The apparatus described is probably too large for many purposes, but by utilizing the experimental data quoted it should be possible to design an apparatus to meet any requirements by deciding, first, the maximum surface area of protein which is to be exposed to evaporation and, secondly, the maxi-

imum temperature at which it is desired to run the "heater". Once these two variables are fixed it is possible to estimate the maximum heat neutralization which is required of the refrigerator, and hence its size.

Perhaps this is best explained by an example. Suppose 500 c.c. is the maximum volume it is desired to dry at one time. If this is distributed in 5 c.c. quantities in ampoules of such a size that 5 c.c. gives a depth of fluid of 1 cm., the surface area exposed to evaporation will be 500 sq. cm. By referring to Text-fig. 8 it is seen that a surface area of 2500 sq. cm. would require 134 W., and therefore 500 sq. cm. would require 27 W. to maintain the heater temperature at $+37^{\circ}$ C. In our case at this temperature 17 W. was the loss of the system, but in a smaller apparatus this would probably be less, say 10 W., in other words only 17 W. could be utilized in evaporation of the serum. Assuming 50% loss on the refrigerator side, 40 W. would be the maximum heat neutralization required of the refrigerator. On the other hand, if the 500 c.c. were to be dried in 2.5 c.c. quantities in similar ampoules the surface area would be 1000 sq. cm. and the heat requirements, assuming the same heat loss as before, would be 44 W., so that 88 W. heat neutralization will be the maximum required of the refrigerator.

Our refrigerator was designed to neutralize 250 W. at -45° C. when drying 5 l. quantities of protein, for smaller quantities a much smaller refrigerator could be used. Moreover, since the maximum heat is only applied to the serum for a short period, it is possible to operate with smaller refrigerators, never making use of the maximum possible heat input, though the drying time will be slightly longer.

The amount of heat which a refrigerator can neutralize falls rapidly with the temperature, so that, if economy is taken into consideration, it may not always be possible to run the condenser at -45° C. Our original experiments were carried out with the condenser at -20° C., but undoubtedly lower temperatures give more latitude for the provision of plugs, and alterations in the conditions of desiccation, and, moreover, the percentage of water remaining at the end of desiccation is much less.

The apparatus as shown in Pl. V has 124 ampoules, each containing 5 c.c. arranged in equal numbers on each of two stages. More than two stages can, of course, be fitted, and by having variable spacing pieces the number of stages required for containers of different sizes can be assembled; the heating mats are then connected in series, and the thermostat fixed to the most convenient stage.

If ampoules of one size only are to be used, an ideal stand could be constructed from a copper cylinder with honeycomb-like compartments arranged on the inside periphery in such a way that the ampoules were held at an angle of 10° from the horizontal with their necks pointing centrally. In this way a large number of ampoules could be accommodated and a very favourable ratio of surface area to volume of the protein would be provided.

The design of the apparatus shown in Pl. IV could be varied in many ways.

If a large supply of cold brine was available, alcohol cooled by the brine could be circulated through the condenser, and it would be possible to run a number of chambers simultaneously using only one refrigerator to cool the brine. The apparatus could be so constructed that the preliminary freezing could be carried out *in situ* if the refrigerator coils were arranged around the internal periphery of the desiccator while the heating trays were situated in the middle, separated from the coils by a polished reflector screen. Since under these conditions maximum cooling would be required when the desiccator was not evacuated and therefore not vacuum "lagged", the outer walls of the chamber would require to be suitably insulated.

SUMMARY

1. Methods are described for the preservation by desiccation from the frozen state of large volumes of protein, both in bulk and in ampoules.

2. The principle is one of high-vacuum condensation drying, utilizing liquid air for quantities up to 250 c.c. or refrigerator evaporator coils for quantities up to 5 l. Desiccators, not manifolds, are used throughout.

3. Use is made of an accurate automatic control of heat to the frozen material. By recording this heat input a visual record is obtained indicating when the end-point of desiccation has been reached, and, furthermore, it is possible to calculate from this record the approximate percentage dryness of the material at any period of desiccation.

4. The control of the apparatus is such that within stated limits the conditions of desiccation can be altered at will.

5. In this form of apparatus obstruction to the flow of water vapour is so small that plugged containers may be used, thus preventing contamination of the material while it is being dried.

6. In its final form the apparatus is entirely automatic and the running costs are extremely low.

7. Suggestions are made for designing forms of apparatus to meet many requirements.

ACKNOWLEDGEMENTS. We would like to thank Dr W. B. Lewis of the Cavendish Laboratory, Dr R. Gane of the Low Temperature Research Station, and Mr G. S. Adair of the Department of Physiology, Cambridge, for much valuable advice, and especially Dr P. Hartley of the Department of Biological Standards at the National Institute for Medical Research, Hampstead, for his co-operation in this research and for testing the dried products.

We are also grateful to Messrs J. and E. Hall of Dartford, for the loan of the refrigeration equipment.

REFERENCES

- ADAIR, G. S. & ROBINSON, MURIEL E. (1931). A direct method for the measurement of the hydration of proteins. *J. Physiol.* **72**, 2P.
- COCKCROFT, J. D. & WALTON, E. T. S. (1932). Experiments with high-velocity positive ions. *Proc. Roy. Soc. A*, **136**, 619.
- 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.
- FLOSDORF, E. W. & MUDD, S. (1935). Procedure and apparatus for preservation in "lyophile" form of serum and other biological substances. *J. Immunol.* **29**, 389.
- (1938). An improved procedure and apparatus for preservation of sera, micro-organisms and other substances—the cryochem process. *J. Immunol.* **34**, 469.
- FLOSDORF, E. W. & WEBSTER, G. W. (1937). The determination of residual moisture in dry biological substances. *J. biol. Chem.* **121**, 353.
- GREAVES, R. I. N. & ADAIR, MURIEL E. (1936). A simple method for the preservation of sera by desiccation in the frozen state without the use of refrigerants. *J. Hyg., Camb.*, **36**, 507.
- HARTLEY, P. (1936). A simple laboratory method for the desiccation of serum and other protein solutions. *Quart. Bull. Hlth Org. L.o.N.* **5**, 735.
- KATZ, J. K. (1917). Die Gesetze der Quellung. *Kolloidchem. Beih.* **9**, 1.
- SHACKELL, L. F. (1909). An improved method of desiccation, with some applications to biological problems. *Amer. J. Physiol.* **24**, 325.

(MS. received for publication 26. iv. 39.—Ed.)