THE FREEZING OF HUMAN SERUM AND PLASMA IN MEDICAL RESEARCH COUNCIL TRANSFUSION BOTTLES, BEFORE DRYING BY SUBLIMATION FROM THE FROZEN STATE

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(With Plates V–VII and 1 Figure in the Text)

Introduction

Human serum was dried in this department at the outbreak of war with a view to its possible use as a stable substitute for whole blood for transfusion purposes. In the absence of any definite knowledge of dosage, the arbitrary unit of 200 c.c. was chosen, because this was approximately the amount of serum which could be obtained from a pint of clotted blood, and also because this amount of serum could conveniently be frozen before drying in 12 oz. medical-flat bottles. The technique of freezing was to place the bottles of serum on racks in a cold-room maintained at $-20°$ C., the racks being slightly inclined from the horizontal so that the serum froze in a wedge, thus giving a reasonably large surface from which evaporation could occur during the drying process. Large amounts of human serum have been dried by sublimation from the frozen state on apparatus based on the experimental model described by Greaves & Adair (1939), after prefreezing by this simple technique.

There are, however, certain disadvantages to this technique. First, experience has shown that 200 c c. are too small a unit; the ideal unit would appear to be 400 c.c., since this amount of serum contains approximately the same amount of protein as a full Medical Research Council transfusion bottle of plasma. Secondly, none of the standard transfusion equipment will fit the 12 oz. medical-flat bottle. The Medical Research Council Blood Transfusion Outfit is described in the *M.R.C. War Memorandum* No. 1, Appendix B, 1940. Thirdly, stationary freezing in a wedge carried out in a cold-room is a slow process resulting in the formation of large crystals, some concentration of protein and an expression of fat on to the surface, all factors tending to prolong the time of re-solution after drying. The first two of these difficulties could be removed by wedge-freezing 400 c.c. of serum in the standard transfusion bottle, but this produces so thick a wedge that $5\frac{1}{2}$ days are required for drying; in consequence the output of the drying plant is greatly reduced. If serum could be frozen rapidly on the inside periphery of the standard M.R.C. transfusion bottles, the resulting large surface and small depth of the frozen material

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would greatly reduce the drying time, and the small crystal produced by rapid freezing should result in improved solubility after drying.

Flosdorf & Mudd (1935) introduced a method of freezing serum on the inside periphery of ampoules by rotating them slowly at an angle slightly inclined from the horizontal in a bath of solid CO_2 dissolved in methyl-cellosolve which gives a temperature in the region of $-80°$ C.; with small quantities of serum in ampoules this gives rise to moderately rapid freezing. This method was tried with 400 c.c of serum in a transfusion bottle, and gave a very satisfactory product. Freezing was complete in 20 min., giving a fairly small crystal size, and after drying the solubility of the material was found to be very satisfactory From a practical point of view the cost and handling of the large quantities of dry-ice, which would have been necessary for the scale of drying contemplated, render this method unsatisfactory.

Lanyon (1941) has shown that it is the speed of freezing rather than the temperature of the refrigerant which is the critical factor in determining crystal size, and describes a method of obtaining a product similar to that obtained with dry-ice by slowly rotating the bottles at an angle of 15° to the horizontal in an air stream of about 100 ft /min at a temperature of $-20°$ C. The elimination of large crystals is to some extent aided by the continuous rotation and consequent stirring of the freezing material which occurs with this technique.

On a small scale this method has been found to be entirely satisfactory, but on a large scale certain difficulties were encountered. Lanyon (1941) pointed out that to get the best results it was necessary to bring the bottles more nearly to the horizontal position after freezing had commenced. On a large scale great difficulty was encountered in getting an even draught on each bottle, with the result that freezing was unequal from bottle to bottle, and it was found to be impossible to alter the angle of inclination of the rack during freezing. Alterations in the temperature of the room, the plugging of bottles which was essential for maintaining sterility but which prevented cold air from entering the bottles, and the freezing of really clean serum shortly after filtration presented even greater difficulties It was found that if the temperature rose to the region of $-15°$ C. violent frothing occurred, whereas in the region of -17 to $-19°$ C., with clean serum and plugged bottles, the serum was apt to supercool and then suddenly to freeze into a solid lump. This lack of uniformity in freezing, particularly since it was usually impossible to detect the incorrectly frozen bottles without removing the plugs, rendered this method of freezing unsuitable for routine work

Recently Lanyon (personal communication) has got greatly improved results by keeping the temperature as low as possible, producing a maximum draught and reducing the speed of rotation to approximately four per minute.

It was noted during these experiments that the serum that had supercooled and 'snap-frozen' had no crystals visible to the naked eye, the amorphous appearance of the material being similar to that obtained by the rapid-freeze

method of Adair & Robinson (1931), or to the appearance of small quantities of serum frozen in liquid air. The material certainly appeared more amorphous than the 'degassed-snap-frozen' material produced by the method of Greaves & Adair (1936). If a method could be devised of obtaining this 'snap-freeze' with the serum distributed round the inside periphery of the bottle, it would be reasonable to assume that an extremely good and very soluble dried product would result.

Previous experiments had shown that if an M.R.C. transfusion bottle containing 400 c.c. of serum was spun on its vertical axis at a speed of 750 rev./min. a hollow cone was formed in the fluid which elongated till eventually it reached the bottom, the serum thus being distributed round the inside periphery of the bottle. This method of spinning was abandoned at the time because it was thought that the mechanical difficulties involved in running a number of spinners at this rate would be very great and also because it was thought that spinning would have to be carried out in a liquid refrigerant bath, causing even greater difficulties.

Lanyon's demonstration (1941) that an air stream could be as effective as a liquid refrigerant bath rendered vertical spinning a more attractive proposition. If one spinner could be made to accommodate several bottles the mechanical difficulties would be still further reduced. A vertical high-speed spinner was, therefore, constructed which has given consistently good results over several months' routine use. By using an induction motor to drive the apparatus, all bottles spin at the same speed, causing the distribution of the serum in every bottle to be identical, and therefore the drying time to be uniform. Consequently a drying routine can be maintained.

It has been found possible to insure that the serum shall 'supercool' and 'snap-freeze' so that on drying a very rapidly soluble product is produced.

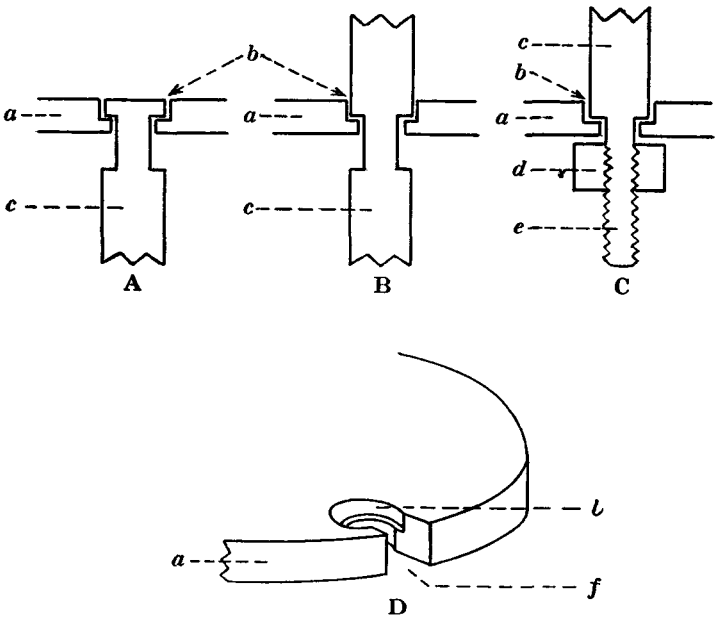
Description of apparatus

The apparatus has been constructed on the simplest possible lines, as can be seen from the photograph (Pl. V). By making each spinner to take five bottles the number of sets of bearings is reduced to a fifth, and the drive mechanism is greatly simplified. The framework is constructed from 2 by 2 in. wood, and the top and bottom cross pieces of two lengths of $1\frac{1}{2}$ in. angle iron to take the bearing cases. There is so little vibration of the unit during operation that it is unnecessary to bolt it to the floor. Four spinners are in use, but provision has been made to add two further spinners if required.

Each spinner is constructed from six $\frac{1}{4}$ in. thick circular steel plates supported by four $\frac{3}{8}$ in. vertical steel rods. Three of these are fixed by being passed through holes in the steel plates and welded in position, but the fourth can be removed by unscrewing a nut below the bottom plate and removing the rod from a key-way made by countersinking the upper surfaces of the plates (see Text-fig. 1). The bearings at the top and bottom are 12 mm. 'self-aligning

ball-races', and the thrust is taken on a steel ball in the bottom bearing case. It is advisable that the spinners should be roughly dynamically balanced, but fine balancing has not been found necessary.

The drive is taken from a $\frac{1}{3}$ h.p. vertical mounting repulsion induction electric motor, running at a speed of 1425 rev./min. On the spindle of the motor is a ten-tooth magneto sprocket and this is coupled to a sixteen-tooth sprocket on the shaft of the first spinner by means of a short length of cycle chain. In this way a speed of $890\frac{5}{8}$ rev./min is obtained for this spinner. The remaining spinners are coupled through short lengths of cycle chain and double



Text-fig. 1. A. Fitting of removable rod into key-way of top disk. B. Fitting of removable rod into key-way of middle disks. C. Method of tightening removable rod by screwing up nut under bottom disk. D. View of countersunk key-way in a disk. *a*, section of circular steel disk; *b*, countersunk groove in key-way; *c*, section of the removable rod, *d*, nut for tightening up rod, *e*, screwed end of removable rod to take tightening nut; *f*, opening into countersunk key-way.

eight-toothed sprockets. Adjustment of the tension of the chains is obtained by making the positioning of the bearing cases adjustable over a short range. The chains are lubricated by a preliminary heating in a bath of grease and graphite. Recently greatly improved smoothness of running has been achieved by replacing the chains with small V-belts.

Since the transfusion bottles vary slightly in diameter, the rods cannot be made to fit them very closely, but by binding the rods with insulating tape in places corresponding with the bulges of the bottles a perfect fit is obtained, since there is enough elasticity in the tape cushions to cancel out the inequalities of the bottles.

Experiments

The series of photographs reproduced in Pls. VI and VII show stages in the formation of the cone when 400 c.c. of serum are spun vertically at a speed of 890⅝ rev./min. It will be seen that after its apex reaches the bottom of the bottle the cone opens out till its sides are nearly vertical. The effect of increasing the speed of rotation would be to open the cone out still further. Theoretically it would be best to run at such a speed that the sides of the cone became actually vertical, but as the increased speed of rotation would create greater mechanical strains and wear in the apparatus, it did not seem justifiable. If 400 c.c. are placed in the bottle the cone just reaches the bottom at a speed of rotation of 712 rev./min. In determining the optimal speed at which to run it must be remembered that there is an increase in volume on freezing and also that from a practical point of view it is convenient to have a window at the bottom when the serum is frozen so that complete freezing can be observed by direct inspection. With 400 c.c. of serum and a speed of 890⅝ rev / min., a window ¾ in. in diameter is formed at the bottom of each bottle.

Serum may be spun-frozen satisfactorily on this apparatus under widely differing conditions of temperature and draught, but if the amorphous structure of 'snap-frozen' material is required the conditions are somewhat more stringent.

Supercooling will only occur if the rate of cooling is not too fast, and therefore both temperature and draught must be taken into account. The rotation of the rods of the spinners creates an appreciable draught and therefore it is best to carry out the freezing in still cold air rather than in a cold air stream, as the rate of cooling will be more uniform. When spinning at a rate of 890⅝ rev./min. the optimal temperature is −17° C., though satisfactory results have been obtained up to −11° C. and down to −20° C.; the region above −11° C. has not been explored. Below −20° C. cooling is too fast at this speed of rotation and large crystals start to appear. With faster rotation of the spinners the lower limit of temperature would rise, and vice versa. Supercooling only occurs with very clean material. The separated serum is therefore first clarified with paper pulp and then filtered through a Seitz bacterial filter. Before spin-freezing the filtered serum is precooled overnight in a cold room at 2° C.

The time for freezing to occur depends on the rate of cooling. Since the cold-room used for spinning is also used by other people it is impossible to maintain an even temperature, and therefore for routine work a longer time has to be given than would be necessary under better regulated conditions. The routine spin is for 2½ hr., enabling five spins to be carried out each day, the last run being turned off automatically by a time-switch. Thus with four spinners a hundred bottles can be spun-frozen each day.

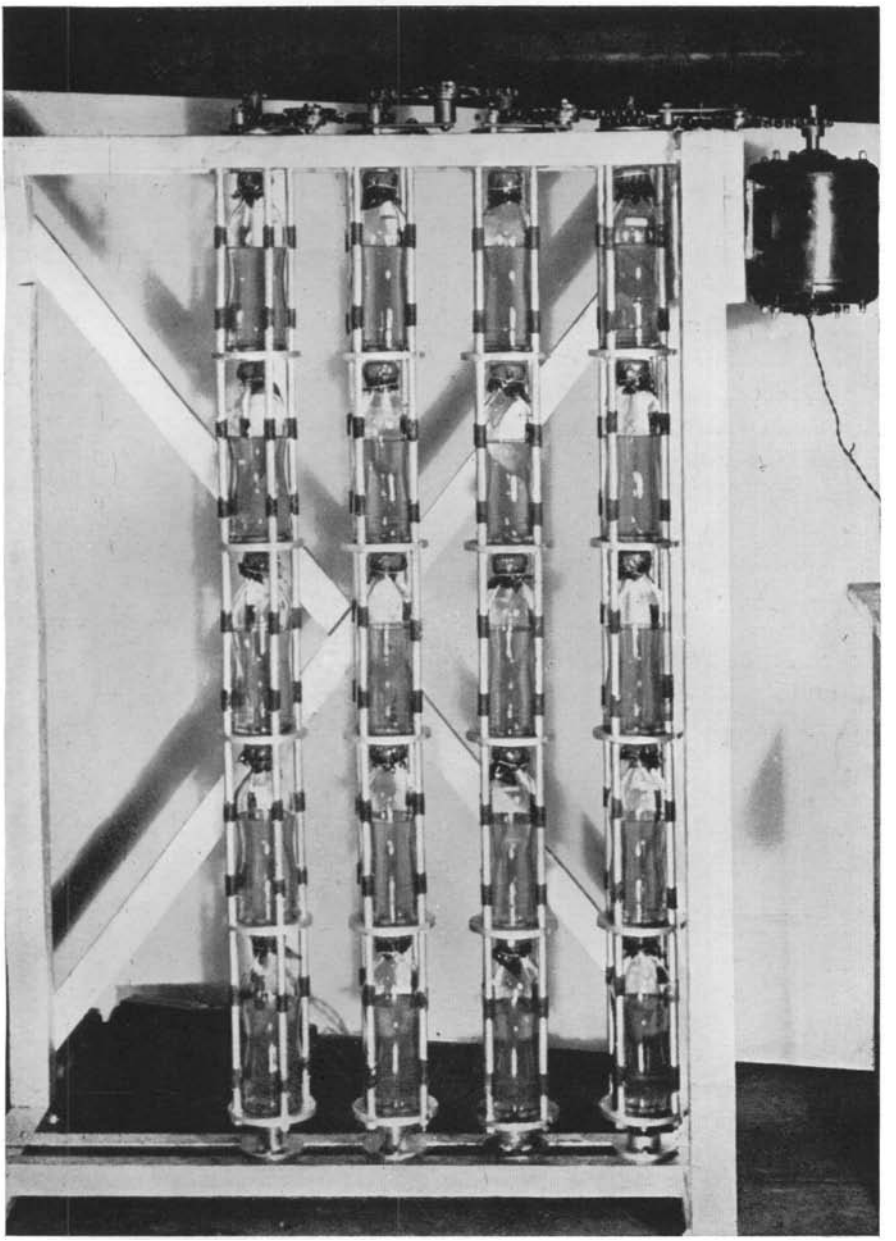
Discussion

The importance of rapid freezing when protein solutions are to be dried from the frozen state has long been recognized. Adair & Robinson (1931) found that the very rapid freezing which occurs when a protein solution is rapidly exposed to a high vacuum in the presence of a desiccant without previous 'degassing' gave a satisfactory product for accurate protein work. Elser *et al.* (1935) considered that slow freezing might lead to a partial denaturation of the proteins in serum on account of the concentration of the salts which occurs, even though this concentration is taking place at a low temperature. Flosdorf & Mudd (1935) stressed the importance of rapid freezing and used dry-ice in methyl-cellosolve as the refrigerant. Freezing in this mixture is not, however, as rapid as the freezing by evaporation of Adair & Robinson (1931), Greaves & Adair (1936), and Flosdorf & Mudd (1938). The authors of the last paper are of the opinion that the 'snap-frozen' material is slightly more rapidly soluble after drying than material prefrozen in dry-ice.

On theoretical grounds there are several reasons why serum which has been rapidly frozen should after drying be more rapidly soluble than slowly frozen material. The physical condition of slowly frozen serum may be visualized as consisting of a relatively small number of large ice crystals with thick layers of hydrated protein and salt in between. With rapidly frozen material very many more ice crystals of smaller size will be formed, and being more closely packed the films of hydrated protein and salt will be much thinner and per unit volume will present a larger surface area. The initial rate of solution should be directly proportional to the surface area of the interface between the dry protein and the water used for solution, thus the greater surface area of the rapidly frozen material should lead to more rapid solution of the dried product. If a film of water is placed between two crystals of a sparingly soluble salt, the time required for saturation by diffusion should be proportional to the square of the thickness, thus if the thickness is halved the time for saturation is decreased four times, the greater dispersion of the rapidly frozen material should therefore greatly increase the speed of re-solution after drying. The surface energy of finely divided protein particles is greater than that of coarsely divided particles and thus the increased surface energy of the protein particles which result after drying serum that has been rapidly frozen should aid the speed of re-solution.

The method of freezing described, which gives very favourable conditions for rapid supercooling, yields small crystals and consequently there is great dispersion of the particles of dried protein, a large surface area, increased surface energy and small thickness of these particles, all factors tending to hasten the re-solution of the dried product.

The 200 c.c. quantities of serum frozen slowly in a wedge and then dried took from 5–10 min. to dissolve to a normal concentration at room temperature,



General view of the apparatus, fully loaded There are four spinners, each of which accommodates five bottles The black marks on the rods are the cushions of insulating tape which insure a good fit for the bottles

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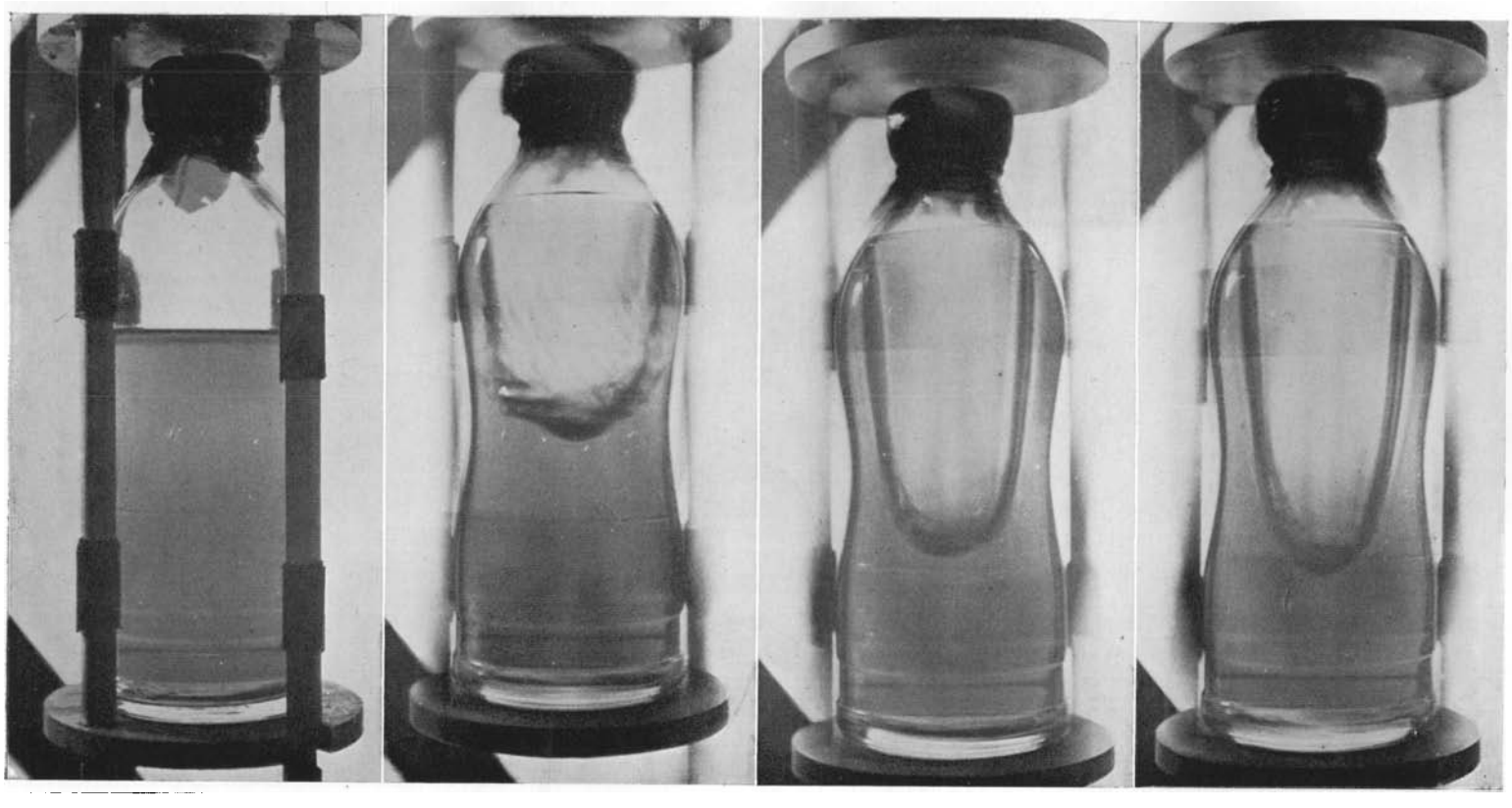
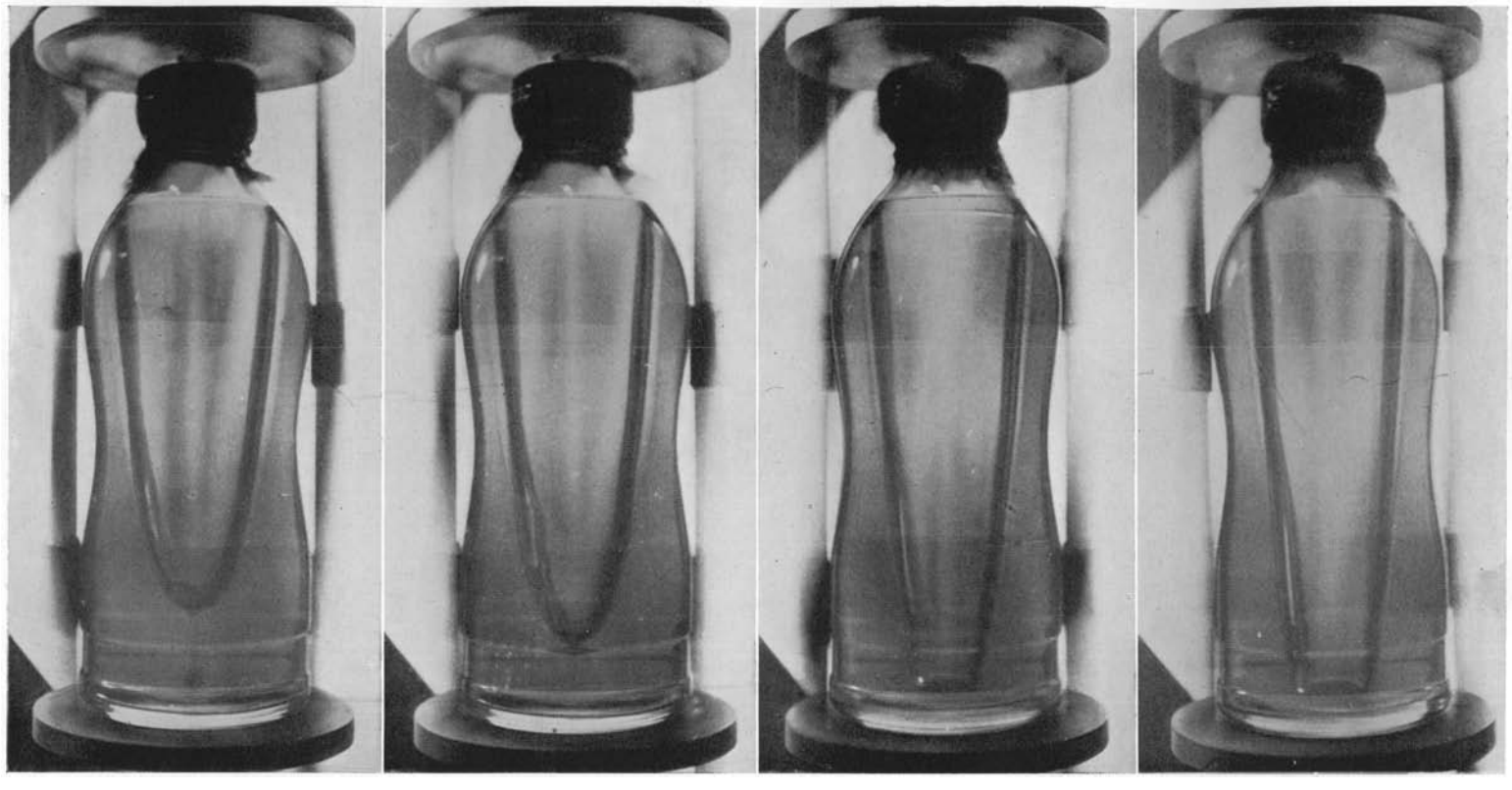


1 2 3 4

Fig 1 A bottle containing 400 c c of serum before spinning has begun Note the height of the fluid Figs 2–8 A series of photographs taken during spinning at 4 sec intervals showing stages in the formation of the hollow cone The speed of rotation was 890$\frac{3}{4}$ rev /min



5 6 7 8

For description of Figs 5–8 see under Pl VI

whereas 400 c.c. quantities of 'spun-snap-frozen' serum took less than 30 sec. under similar conditions.

Small quantities of human plasma have also been frozen by this method and have behaved in exactly the same way as human serum.

SUMMARY

1. A method is described for 'spin-freezing' human serum or plasma in the standard Medical Research Council transfusion bottles.

2. The method consists in spinning the bottles at a high speed on their vertical axes which causes a hollow cone to be formed down the centre of each bottle, and the serum to be distributed evenly round the inside periphery. A maximum surface with a minimum depth of frozen material results, so that large quantities may be dried rapidly.

3. The advantages of rapid freezing are discussed and the conditions necessary to cause the material to supercool and then 'snap-freeze' are given. This rapid freezing gives rise to very small crystals and consequently a great dispersion of the particles of dried protein. If serum frozen in this way is dried by sublimation from the frozen state, the re-solution of the resulting dried product is extremely rapid.

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