HET MECHANISM OF THE PROTECTIVE ACTION OF GLYCEROL AGAINST HAEMOLYSIS BY FREEZING AND THAWING

by

J. E. LOVELOCK

National Institute for Medical Research, Mill Hill, London (England)

It was discovered by POLGE, SMITH AND PARKES[1] that spermatozoa could be frozen without loss of motility if glycerol was included in their suspending medium. SMITH found that the protective action of glycerol could be applied also to red blood-cells. This was followed by a report of the successful preservation of red blood-cells in bulk at —79° C (SLOVITER[3]). The successful transfusion of human cells preserved by glycerol at low temperatures has also been described (MOLLISON AND SLOVITER[4]).

The first systematic investigation of the nature of the protection provided by glycerol was made by SMITH, POLGE AND SMILES[5]. By means of a specially constructed freezing stage they were able to observe directly the freezing and thawing of cell suspensions. In the discussion of their observations they comment upon the modification of the shape of the ice crystals by glycerol, and upon the greater volume of fluid left unfrozen in its presence. They do not ascribe the protective action of glycerol directly to these effects but suggest that the mechanical stress of freezing must be greatly reduced by its presence. Other potentially adverse effects of freezing are discussed by PARKES[6]. Among these he drew attention to the concentration of the electrolytes of the suspending medium as ice forms. LOVELOCK[7] suggested that with the red blood-cell the greater part of the destructive action of freezing could be attributed to the concentration of the electrolytes of the cell and of its suspending medium. The critical region of temperature in which damage occurs rapidly, —3° to —40° C, was shown to coincide with that region in which the cell is exposed to concentrated salt solution. The nature of the destructive action is complex. In NaCl solutions between 0.8 *M* and 2.0 *M* the cells are sensitive to mechanical shock, sudden chilling, and to transfer back to isotonic media. In stronger solutions the structural integrity of the cell is destroyed.

These suggestions are confirmed by the results reported in this paper, and it is shown that glycerol functions by preventing the excessive increase in salt concentration which otherwise occurs on freezing. This protective action is exerted in full only when glycerol is present both within and without the cell. On the basis of this proposed mechanism of the action of glycerol it has been possible to suggest an improved technique for the bulk preservation of red blood-cells. This has been tried on a practical scale (CHAPLIN AND MOLLISON[8]) and found successful.

References p. 36.

METHODS

Red blood-cells

Human blood to which "acid-citrate-dextrose" anticoagulant had been added was stored at +4° C until required for use. Blood older than 10 days was not used. The cells were separated by centrifuging and then washed three times in lightly buffered NaCl solution (NaCl 0.15 *M*, Na_2HPO_4 0.005 *M*, and NaH_2PO_4 0.005 *M*). After the final washing the cells were packed by centrifuging and stored at 0° C. The washed cells were always used within 5 hours of their separation from the plasma.

Glycerol

The glycerol used was of analytical reagent quality. Because of the viscosity and hygroscopicity of the pure glycerol it was in most experiments dispensed as a 50% by weight solution in water. Care was taken to ensure that neither the glycerol nor the other reagents and apparatus used were contaminated with copper. Traces of this element have been shown (JACOBS[9]) to alter profoundly the permeability of the red blood-cell to glycerol.

Measurement and maintenance of low temperatures

In all experiments temperatures were measured in terms of the E.M.F. generated by a calibrated copper constantan thermocouple. Temperatures between 0° and —30° C were obtained by means of a thermostatically controlled alcohol bath. This was capable of maintaining the temperature to which it was set to within ±0.1°. Temperatures below —30 C° were obtained by means of a bath consisting of an open-necked Dewar flask containing 10 litres of alcohol. The temperature of this bath was adjusted to the required temperature by the addition of solid carbon dioxide. Its heat capacity was sufficiently great, and heat losses sufficiently small for the temperature to remain constant during the 10 minutes required for most experiments.

Measurement of haemolysis

In most experiments the damage which occurred to the red blood-cells was assessed by measuring the quantity of haemoglobin released into the suspending solution. After exposure to experimental conditions the red blood-cells remaining undamaged were removed by centrifugation. The supernatant solution of haemoglobin was diluted with 0.1% Na_2CO_3, shaken to convert the haemoglobin to oxyhaemoglobin, and the concentration of the latter measured colorimetrically.

Procedure for freezing red blood-cells

All experiments were made in flat bottomed test tubes, the dimensions of which were — length 10.0 cm, diameter 0.5 cm, and wall thickness 0.1 cm. The usual experimental procedure was to place 0.1 ml of packed cells in one of these tubes, followed by 0.9 ml of the suspending medium. When glycerol was present the mixed suspension was kept for 10 minutes at 40° C to ensure that the cells were fully permeated by the glycerol. The suspension was cooled by immersion in a bath set at the required temperature. It was left there for 10 minutes after freezing had occurred either spontaneously, or by seeding with an ice crystal.

When it was required to freeze suspensions to temperatures between —35° and —50° C it was found necessary to modify the procedure just described. This was to avoid the suspension spending too long at temperatures where rapid destruction occurs as it proceeded towards the required temperature. The modification consisted of freezing the suspension first by immersion in a bath at —80° C, and then placing in a bath at the required temperature. By this means only a very short time, 5 to 10 seconds, was spent in the critical region. The additional haemolysis due to the rapid freezing to —80° C was almost always less than 3%.

In experiments where it was required to prevent the entry of glycerol into the cells before freezing the following procedure was adopted. Before use the red blood-cells were washed in 0.9% NaCl containing 10^{-5} *M* $CuSO_4$. This concentration of copper was also added to the glycerol solution used to suspend the cells. The cells were suspended as usual in the glycerol solution, but the suspension was divided into two portions. The first was frozen immediately and the second kept at +40° C for 20 minutes to allow the entry of glycerol in spite of the copper ions, and then frozen. The second operation was made to ensure that the damage which occurred when the cells were frozen without equilibration was not due to some directly damaging effect of the copper ions.

Measurement of the melting points of glycerol, NaCl and water mixtures

The melting points of solutions of glycerol and NaCl in water between 0° and —40° C were measured as follows: 50 ml of a solution of known composition was partially frozen by immersion in an alcohol bath at —80° C. The slush of ice and solution was then transferred to a double jacketed vessel and stirred continuously. The temperature of the mixture was followed by means of a copper constantan thermocouple. The melting point was taken as that temperature at which the last traces of ice were disappearing.

References p. 36.

Measurement of the permeability of red blood-cells to strong glycerol solutions

0.05 ml of carefully washed packed red blood-cells were added to 10 ml of 1.5 *M* glycerol, and the time taken for haemolysis to occur measured. Measurements were made at temperatures between 45° and —10° C. The measurements made at —10° C were with supercooled solution. No difficulty was experienced in maintaining this temperature for long periods provided that the solution and apparatus was kept free of dust. Measurements were made at pH 7.0. This was maintained by the addition of 0.01 *M* phosphate buffer to the glycerol solution. The effect of copper ions on the permeability of the red blood-cells to glycerol was determined using glycerol solutions containing 10^{-5} *M* copper.

RESULTS

The haemolysis which occurs when red blood-cells are frozen in solutions of NaCl and glycerol

Fig. 1 shows the haemolysis which occurs when suspensions of red blood-cells containing various concentrations of glycerol are frozen. In this experiment the cells were suspended in 0.16 *M* NaCl and were frozen for 10 minutes. The immediate effect of the glycerol is a reduction in the extent of the critical range of temperatures in which haemolysis occurs. If sufficient glycerol is added—more than 2.5 *M*—haemolysis is prevented completely. The most unfavourable temperature for the survival of red blood-cells frozen in the presence of glycerol is between —32° and —35° C.

It has been shown that the critical range of temperature corresponds to that region in which the cell is exposed to concentrated salt solution during freezing (LOVELOCK[7]). One effect of adding glycerol to the suspending medium is to reduce the concentration of salt in equilibrium with ice as freezing occurs. In view of the fact that the "salt buffering" action of glycerol might explain its protective properties, it was of some interest to try other means of lessening the concentration of salt which occurs on freezing. The simplest of these is a reduction of the initial NaCl concentration before freezing. In Fig. 2 is shown the haemolysis which occurred when red blood-cells were

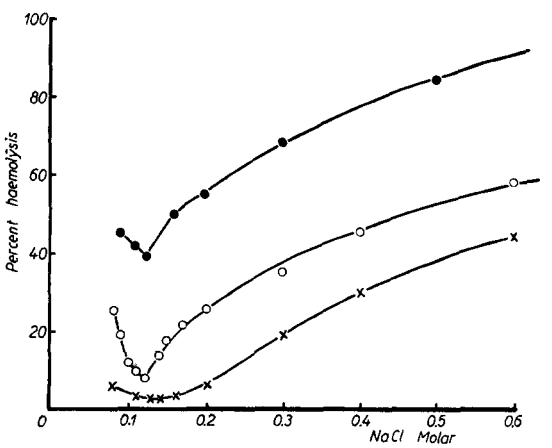

Fig. 2. The effect of the initial NaCl concentration of the suspending medium on the haemolysis of red blood-cells. The glycerol concentrations were (×) 1.0, (o) 1.5, and (●) 2.0 *M*, respectively. The cells were frozen to —30° C for 10 minutes.

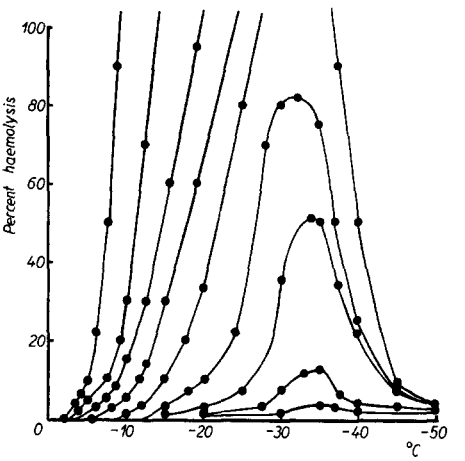

Fig. 1. The haemolysis when red blood-cells in suspension in 0.16 *M* NaCl containing various concentrations of glycerol are frozen. The curves shown are from left to right those corresponding to glycerol concentrations of 0.0, 0.15, 0.3, 0.5, 0.75, 1.0, 1.5, 2.0 and 2.5 *M*. In all experiments the cells were frozen for 10 minutes at the temperatures indicated.

References p. 36.

frozen while suspended in various concentrations of NaCl and glycerol. The results indicate that at NaCl concentrations above 0.11 *M* there is a close connection between the NaCl content of the suspending medium and haemolysis on freezing. The survival at the optimum concentration, 0.11 *M*, is so much better than at the normal value, 0.16 *M*, that a reduction in the initial NaCl content of suspending media may be of practical value.

The effect of strong NaCl solutions upon red blood-cells with and without the presence of glycerol

Red blood-cells suspended in solutions of NaCl stronger than 0.8 *M* are haemolysed if suddenly cooled or if resuspended in 0.16 *M* NaCl. These effects, particularly the latter, are responsible for much of the damage which occurs when red blood-cells are frozen. The possibility that some of the protective action of glycerol might be due to an inhibition of these destructive effects was therefore examined. Table I shows the haemolysis which occurred when cells suspended in strong NaCl solutions containing various concentrations of glycerol were resuspended in 0.16 *M* NaCl containing the same glycerol concentrations. This experiment shows that the destructive effect of sudden transfer from strong to weak NaCl is but little affected by glycerol, even in high concentration. A similar experiment showed that the haemolysis caused by sudden cooling in 1.0 NaCl was also largely unaffected by the presence of glycerol.

TABLE I

THE EFFECT OF GLYCEROL UPON THE HAEMOLYSIS WHEN RED BLOOD-CELLS ARE TRANSFERRED FROM STRONG NaCl TO 0.16 *M* NaCl

(Cells were suspended in the strong NaCl solutions containing various concentrations of glycerol at 0° C. After 10 minutes they were transferred to 20 times their volume of a solution the composition of which gave a final NaCl concentration of 0.16 *M*)

Glycerol concentration Molar	*Per cent. haemolysis after transfer from stated NaCl concentration to 0.16 M NaCl*		
	1.05	*1.5*	*2.0*
5.2	16	39	—
3.7	15	39	65
2.9	15	38	60
2.2	15	39	62
1.5	12	34	53
0.7	12	34	62
0.0	10	33	51

The permeability of the red blood-cell to glycerol

The human red blood-cell is well known to be permeable to glycerol in dilute solutions. It was of considerable theoretical and practical importance, however, to know whether or not the red cell was permeated by glycerol at the concentrations used to prevent haemolysis on freezing. Also, in order to understand the equilibration process described by SLOVITER[3] it was necessary to know the rate of entry of glycerol into the cell from strong solutions and at various temperatures.

Permeability was measured by observing the time taken for haemolysis to occur when red blood-cells were suspended in 1.5 *M* glycerol in 0.01 *M* phosphate buffer at pH 7.0. It was assumed that lysis occurred when the concentration of glycerol within

References p. 36.

the cell approached that of the medium, *i.e.* when the external osmotic pressure due to the glycerol was too low to prevent plasmoptisis. Measurements were made at various temperatures between $+45°$ and $-10°$ C, and some in the presence of $3 \cdot 10^{-5}$ *M* copper ions. They are shown in Fig. 3. The results indicate that 10 minutes at $+40°$ C is sufficient for the entry of glycerol into the cells, even in the presence of copper. At lower temperatures especially when copper is present longer periods of equilibration may be necessary. At 0° and lower in the presence of copper the cells are from a practical view point impermeable to glycerol.

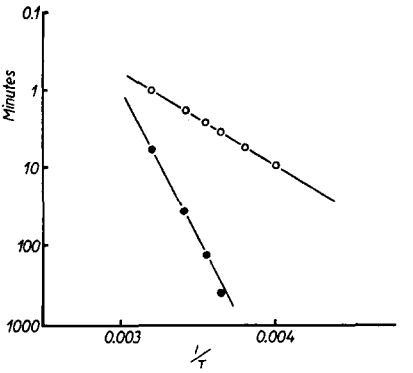

Fig. 3. The permeability of red blood-cells to glycerol at various temperatures, and the effect of copper ions. Ordinate, time taken in 1.5 *M* glycerol for haemolysis, in minutes. Abscissa, reciprocal of the absolute temperature. (o) untreated cells, (●) cells in the presence of $3 \cdot 10^{-5}$ *M* Cu^{++}.

The haemolysis which occurs when red blood-cells impermeable to glycerol are frozen in its presence

The property possessed by copper ions of rendering red blood-cells practically impermeable to glycerol at 0° provides a convenient means of determining the protective action of glycerol within the cell.

Red blood-cells were suspended in glycerol solutions containing $3 \cdot 10^{-5}$ *M* copper ions at 0°. One portion of the suspension was immediately frozen, the other was kept for 20 minutes at $+40°$ C before freezing. With the latter the time at $+40°$ C was sufficient for the entry of the glycerol in spite of the copper. Table II shows the results of freezing cells treated in each of these ways. Cells into which the glycerol had been allowed to penetrate were undamaged by freezing, indicating that treatment with copper ions was not in itself injurious. By comparison, cells which contained no glycerol were severely damaged by freezing even in its presence.

TABLE II

THE HAEMOLYSIS ON FREEZING OF RED BLOOD-CELLS RENDERED IMPERMEABLE TO GLYCEROL BY COPPER

(Cells were suspended in 1.5 and 2.5 *M* glycerol, containing 0.16 *M* NaCl and $3 \cdot 10^{-5}$ *M* Cu^{++}, at 0° C. One portion was frozen immediately and another after 20 minutes at $+40°$ C. Freezing was for 10 minutes at the stated temperatures.)

Glycerol concentration Molar	*Treatment before freezing*	*Per cent. haemolysis at*			
		-15°	*-20°*	*-25°*	*-80°*
2.5	None	11.5	21.5	42	33
2.5	20 mins at 40° C	1.0	1.5	2.0	3.0
1.5	None	18	28	49	37
1.5	20 mins at 40° C	2.0	3.0	7.0	3.5

Haemolysis and time of exposure to low temperatures

The relationship between haemolysis and time of exposure to low temperatures is complex. Very approximately, it was found that damage occurred more slowly at low

References p. 36.

temperatures and high glycerol concentrations. Fig. 4 shows the haemolysis in 1.5 *M* glycerol 0.15 *M* NaCl after various times at temperatures between —15° and —45° C. The temperature at which damage first occurs and the most damaging temperature are seen to be unaffected by the time of exposure.

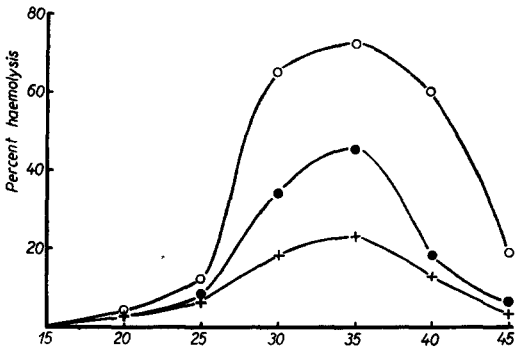

Fig. 4. The haemolysis of cells in 0.16 *M* NaCl and 1.5 *M* glycerol after freezing for 5 minutes (×), 10 minutes (●), and 80 minutes (o).

The composition of the liquid phase when solutions containing glycerol and 0.16 M NaCl are frozen

Fig. 5 shows the increase in NaCl concentration as freezing progresses in solutions containing initially 0.16 *M* NaCl. In the figure the concentrations of NaCl are expressed as mole fractions. The concentrations of glycerol initially present were chosen to be the same as those used to suspend red blood-cells in the experiment illustrated in Fig. 1. For each initial glycerol concentration shown in Fig. 5, from 0 to 3.0 *M*, the temperature at which haemolysis occurs (shown in Fig. 1) is marked by an "x". It will be noted that haemolysis first occurs at a critical NaCl concentration (mole fraction 0.014). This critical concentration is apparently unaffected by either temperature or glycerol concentration.

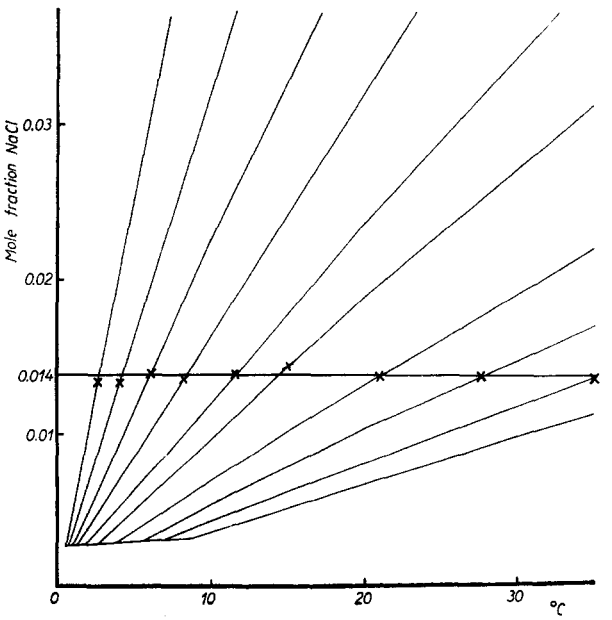

Fig. 5. The NaCl concentration of the liquid phase when 0.16 *M* NaCl, with various initial concentrations of glycerol also present, is frozen. The lines shown are from left to right those for solutions containing initially 0.0, 0.15, 0.3, 0.5, 0.75, 1.0, 1.5, 2.0, 2.5, and 3.0 *M* glycerol. The point marked "X" on each line is the observed temperature at which haemolysis first occurs with cells suspended in a medium whose initial glycerol concentration is the same as that of the line. The intersecting horizontal line at NaCl concentration Mole fraction 0.014, *i.e.* 0.8 *M* in water.

References p. 36.

DISCUSSION

There exists a critical region of temperature during freezing and thawing in which living tissue is rapidly damaged. With the red blood-cell this region extends from —3° to —40° C and if more than a few seconds are spent between these temperatures during freezing and thawing haemolysis occurs. The experimental results indicate that the addition of glycerol to suspensions of red blood-cells before freezing causes a reduction in the extent of this critical region. The range of temperatures in which haemolysis occurs decreases with increasing concentration of glycerol up to 2.5 *M*; at this concentration the critical region vanishes and red blood-cells may then be frozen to any temperature without appreciable haemolysis.

It has been shown (LOVELOCK[7]) that the greater part of the damage suffered by red blood-cells on freezing and thawing can be attributed to the destructive action of strong salt solutions. In particular, the exposure of the human red blood-cell to a solution of NaCl stronger than 0.8 *M* is damaging. The concentration of salt which occurs when a solution of NaCl is frozen is greatly reduced by the addition of glycerol. It follows that this salt buffering capacity of glycerol offers a possible explanation of its protective action during freezing.

In Fig. 5 are shown the NaCl concentrations in equilibrium with ice at various temperatures and in the presence of different concentrations of glycerol. It is of particular interest to note that the temperatures at which the mole fraction of NaCl is 0.014 (0.8 *M* in water) are always the same as the temperatures where damage first occurs. Also, when the initial concentration of glycerol exceeds 25% by weight, this critical NaCl concentration is not reached in the temperature range 0 to —40° C.

In the presence of glycerol the concentration of NaCl continues to increase below —35° C. The rapid decrease in the destructive effect of freezing at temperatures below —35° C indicates that the lower limits of the critical range of temperatures is determined by some other factor than the external NaCl concentration.

Two items of experimental evidence offer a possible explanation of this discrepancy. Firstly, it has been shown that if glycerol is prevented from entering the cells by treatment with copper ions before freezing, they are no longer protected against damage. Secondly, it has been shown that damage occurs at a steadily decreasing rate as the temperature falls and becomes very slow at temperatures below —35° C. The fact that glycerol must be present within the cell before it can exert its protective action suggests that the internal KCl concentration is at least as important as the external NaCl concentration in causing damage. Unlike NaCl, KCl becomes less soluble as the temperature falls and at temperatures below —35° C is insufficiently soluble to maintain the critically damaging concentration, mole fraction 0.014. While it is not possible to offer a precise quantitative explanation of the lower limits of the critical region in terms of salt concentration, the two factors outlined explain reasonably closely the observed decrease in damage when cells are frozen to temperatures below —35° C.

It has been suggested (LUYET[10]; STRUMIA[11]) that mechanical injury resulting from the crystallisation of ice within and without the cell is the principal cause of damage on freezing. Glycerol undoubtedly modifies the shape of ice crystals formed in its presence, and its addition to a solution considerably decreases the volume of ice formed on freezing to any given temperature. It is almost certainly true that glycerol greatly reduces the mechanical stress of freezing. The experimental evidence, however, does not

References p. 36.

offer support to the suggestion that glycerol acts principally by protecting the red blood-cells from mechanical injury on freezing. Thus damage occurs not at low temperatures below —40° C where the cells are crushed by ice, but at relatively higher temperatures where there is still sufficient unfrozen fluid to maintain them freely in suspension. Furthermore, when glycerol is prevented from entering the cells, by treatment with copper ions, they are destroyed on freezing, notwithstanding the increase in lebensraum due to the glycerol.

It has been suggested that ice formation is only damaging when it occurs within the cell, and that glycerol protects by dehydrating the cell and thereby preventing ice formation within its bounds. This suggestion also is incompatible with the experimental evidence. Red blood-cells are only transiently dehydrated by suspension in glycerol, and are rapidly restored to their original degree of hydration as the glycerol permeates the cell.The cells will only remain dehydrated in the presence of glycerol if it is prevented from permeating them, as by treatment with copper. Cells so dehydrated do not survive freezing.

The hypothesis that glycerol protects red blood-cells during freezing by acting as a "salt buffer" has certain practical consequences. In particular, it suggests that a lowering of the salt concentration of the suspending medium before freezing should result in an improved recovery of red blood-cells. It also suggests that where slow freezing is unavoidable, such as in the bulk preservation of red blood-cells, a glycerol concentration of at least 2.5 *M* is necessary in a medium containing 0.16 *M* NaCl if haemolysis is to be avoided. A combination of these two suggestions provides the basis for a practical medium in which large volumes of red blood-cells may be frozen as slowly as desired without damage. This is 2.0 *M* glycerol in 0.12 *M* NaCl. In this medium the critical NaCl concentration, mole fraction 0.014, is not reached at temperatures above —35° C where rapid damage can occur. It is gratifying to note that according to CHAPLIN AND MOLLISON[8] red blood-cells in bulk have been frozen and thawed in this suggested medium without recourse to special vessels or techniques of freezing.

The red blood-cell is exceptional in its simplicity of structure and permeability to water. It is probable that these properties are connected with the ease with which, in the presence of glycerol, it may be frozen and thawed. In spite of the unusual structure of the red blood-cell certain general conclusions applicable to other cells are suggested by the experimental evidence just reported. Firstly, it appears that the protective action of glycerol is only shown when it has permeated the cell before freezing. It follows that cells which are impermeable to glycerol are not likely to survive freezing even in its presence. Secondly, glycerol does not appear to protect against osmotic or thermal shock, and where these are likely to occur it is necessary to establish controlled rates of freezing for the successful revival of the cells. Finally, the concentration of glycerol needed for the successful freezing and thawing of a given cell will depend upon the salt content of the suspending medium and the rate of freezing and thawing found necessary or practicable. In general terms, the faster the rate of freezing and thawing and the lower the concentration of salt the less glycerol will be needed.

ACKNOWLEDGEMENTS

I am indebted to Dr A. S. PARKES, F.R.S., for his interest and advice, and I should like to acknowledge the helpful suggestions of Dr H. A. SLOVITER. I also wish to thank Dr T. D. JAMES, Director of the North London Blood Supply Depot, for his interest and assistance.

References p. 36.

SUMMARY

The effects of including glycerol in suspensions of human red blood-cells during freezing and thawing have been observed. The results of these observations confirm that damage on freezing results largely from the concentration of the electrolytes within the cell. In the presence of glycerol this concentration of electrolytes by freezing is greatly reduced, and is sufficient to explain its protective action. From this interpretation of the action of glycerol improvements are suggested in the technique of preserving red blood-cells at low temperatures.

RÉSUMÉ

Etude des effets de la glycérine sur le comportement des globules rouges humains pendant la congélation et le dégel de suspensions de ceux-ci. Les résultats des observations confirment que les dommages causés par la congélation résultent dans une large mesure de la concentration des électrolytes à l'intérieur des cellules. En présence de glycérol, la concentration des électrolytes causée par la congélation est réduite considérablement, et ceci suffit à expliquer l'action protectrice du glycérol. Cette interprétation suggère des améliorations à la technique de conservation des globules rouges aux basses températures.

ZUSAMMENFASSUNG

Die Wirkung von zugefügtem Glyzerin in Suspensionen menschlicher roter Blutkörperchen beim Frieren und Auftauen wurde beobachtet. Die Ergebnisse dieser Beobachtungen bestätigen, dass die Zerstörungen beim Einfrieren weitgehend von der Konzentration der Elektrolyte innerhalb der Zelle herrühren. Die Konzentration der Elektrolyte beim Einfrieren wird durch die Gegenwart von Glyzerin stark herabgesetzt und dies ist ausreichend, um seine Schutzwirkung zu erklären. Ausgehend von dieser Darlegung der Wirkungsweise des Glyzerins werden Verbesserungen der Technik der Konservierung roter Blutkörperchen bei niedrigen Temperaturen vorgeschlagen.

REFERENCES

1 C. POLGE, A. U. SMITH AND A. S. PARKES, *Nature*, 166 (1949) 666.
2 A. U. SMITH, *Lancet*, II (1950) 910.
3 H. A. SLOVITER, *Lancet*, I (1951) 823.
4 P. L. MOLLISON AND H. A. SLOVITER, *Lancet*, II (1951) 862.
5 A. U. SMITH, C. POLGE AND J. SMILES, *J. Roy. Microscop. Soc.*, 71 (1951) 186.
6 A. S. PARKES, *Proc. Soc. Study of Fertility*, No. 2 (1951) 12.
7 J. E. LOVELOCK, *Biochim. Biophys. Acta*, 10 (1953) 414.
8 H. CHAPLIN, JR. AND P. L. MOLLISON, *Lancet*, 264 (1953) 215.
9 M. H. JACOBS, *Ann. N.Y. Acad. Sci.*, 50 (1950) 824.
10 B. J. LUYET, *Preservation of the formed elements and of the proteins of the blood*, American National Red Cross, 1949.
11 M. STRUMIA, *Preservation of the formed elements and of the proteins of the blood*, American National Red Cross, 1949.

Received October 25th, 1952