THE HAEMOLYSIS OF HUMAN RED BLOOD-CELLS BY FREEZING AND THAWING

by

J. E. LOVELOCK

National Institute for Medical Research, Mill Hill, London, N.W.7 (England)

The discovery by SMITH[1] that red blood-cells suspended in a medium containing glycerol can be frozen and thawed without haemolysis revived interest in the nature of the destructive action of these processes. Not only was it of interest to determine the nature of the protective action of glycerol, but the protective action in itself was important experimental evidence relevant to the general problem of the destructive effects of freezing and thawing.

Freezing and thawing provides a convenient laboratory method of haemolysis. In spite of this, the processes which occur when red blood-cells are frozen have only received systematic attention during the last two years. LUYET AND MENZ[2] measured the haemolysis between —3° and —8° C. SMITH, POLGE AND SMILES[3] observed microscopically the damage taking place when red blood-cells are frozen and thawed. No definite suggestions are made in either of these papers concerning the nature of the haemolytic process. In earlier experiments connected with the long-term preservation of red blood-cells, LUYET[4] and STRUMIA[5] attributed the damage which occurs on freezing to the mechanical action of ice crystals formed within or without the cells.

The results reported in this paper suggest that the formation of ice crystals is not in itself damaging to red blood-cells. The physical change which appears to be most important in causing damage on freezing is the concentration of electrolytes within and without the cell, due to the removal of water in the form of ice. This is essentially similar to the process which MORAN[6] suggested was responsible for the damage suffered by muscle on freezing. The effect of salt concentration during freezing is shown to be complex, the damage varying with concentration of salt. In general terms the damage suffered by red blood-cells frozen and thawed to and from a given temperature can be reproduced quantitatively by exposing the cells to a solution of sodium chloride which freezes at the same temperature.

METHODS

Blood

Human blood was collected by venipuncture, using a dry syringe, and transferred immediately to a conical flask containing an anticoagulant solution, the composition of which was sodium citrate 0.073 *M*, citric acid 0.035 *M*, and glucose 0.01 *M*. 7.5 ml of this solution was used for each 50 ml of blood collected. The blood and anticoagulant were mixed by shaking and stored at 4° C; blood more than 5 days old was not used for experimental purposes.

References p. 426.

Red blood-cells

These were separated from the blood by centrifuging, and then washed three times in lightly buffered NaCl solution (NaCl 0.15 *M*, Na_2HPO_4 0.005 *M*, NaH_2PO_4 0.005 *M*). After the final washing the cells were centrifuged, the supernatant solution discarded, and stored at 0° C. The cells were always used within 5 hours of separation from the plasma.

The measurement and maintenance of low temperatures

In all experiments temperatures were measured by means of a calibrated copper constantan thermocouple. The couple was made of thin wires, 40 s.w.g., so that it would occupy only a small volume and would follow rapidly any changes in temperature. The thermocouple potential was measured by means of a potentioneter except where very rapid temperature changes occurred when a short period high resistance galvanometer was used.

Temperatures between 20° C and −30° C were obtained by means of a thermostatically controlled alcohol bath. This was cooled by means of a submerged coil of brass tubing through which alcohol at −78° C was circulated. The circulation of the cooling alcohol was controlled by means of an electrically operated valve which opened when the bath temperature exceeded the level to which the thermostat was set. This apparatus maintained the bath at the temperature for long periods with a deviation not greater than ± 0.1°.

Where rapid freezing was required small volumes of suspension were placed in capillary tubes, diameter 2.0 mm, wall thickness 0.5 mm, and frozen by immersion in a bath of alcohol and solid CO_2 at −78° C. For ultra-rapid freezing, capillary tubes containing a suspension of cells were plunged into a bath of mixed liquid and solid dichlorodifluoromethane at −160° C.

The thawing of suspensions frozen rapidly or ultra-rapidly was carried out by immersing the tube containing the suspension in a bath of water at 42° C. Where rapid thawing was required the tube was crushed while still cold, below −40° C, and the contents shaken vigorously with 0.15 *M* NaCl at 42° C. The time taken by freezing and thawing was measured by observing the fall or rise in temperature of the thin wire thermocouple placed centrally inside the tube.

Photomicrography of red blood-cell ghosts

Photomicrographs of the "ghosts" and debris of red blood-cells after exposure to freezing of concentrated NaCl solutions were taken as follows: One drop of cell suspension was mixed with one drop of a 10% solution of Nigrosine in 0.15 *M* NaCl; approximately 0.01 ml of the mixture was transferred to a quartz coverslip which was placed on a quartz slide, sealed with wax, and photographed with U.V. light at 2750 A.

The 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, and shaken to convert the haemoglobin to oxyhaemoglobin; the concentration of the latter was measured colorimetrically.

Procedure for exposing red blood-cells to freezing or to salt solutions at low temperatures

All experiments, except those concerned with the rate of cooling, were carried out in flat bottomed test tubes the dimensions of which were—length 10.0 cm, diameter 0.5 cm, wall thickness 0.1 cm. The usual experimental procedure was to place 0.1 ml of packed cells in one of these tubes and immerse it in a bath set to the required temperature. After allowing 2 minutes for the cells and tube to reach the temperature of the bath, 0.9 ml of the suspending solution, also at the bath temperature, was added from a pasteur pipette. The cells were stirred into a uniform suspension by aspirating the mixture in and out of the pipette.

The suspension was frozen when required by touching the surface with a capillary tube containing a very small volume of frozen solution. This caused the temperature of the contents of the tube to rise to the freezing point of the suspending solution. The suspension was therefore left for 7 minutes in the constant temperature bath after freezing occurred to allow a return to the bath temperature and a period of at least 5 minutes at that temperature. Measurements of the rate of cooling of the tubes used when containing 1.0 ml of solution indicated that they reached to within 30% of the bath temperature in 20 seconds and 10% in 60 seconds approximately.

When super-cooling of the suspension was required the same general procedure was followed with the omission of the "seeding" operation. No difficulty was experienced in super-cooling to −10° C and super-cooling to −15° C was frequently obtained for periods as long as 10 minutes. Super-cooling to −15° C or lower is greatly assisted by the careful exclusion of dust from the suspension and apparatus (MASON AND LUDLAM[6]).

After exposure to the experimental conditions the suspension was thawed rapidly, or warmed, by immersing for one minute in a bath of water at 42° C. The haemolysis was measured as described earlier.

References p. 426.

Procedure for exposing red blood-cells to temperature and mechanical shock

Cells were exposed to temperature shock as follows: 0.1 ml of packed cells was suspended in 0.9 ml of salt solution usually 1.1 *M* NaCl. Care was taken to ensure that the temperature of the cells and of the salt were the same before mixing. The mixture was left for 5 minutes and then rapidly added to and mixed with 4.0 or 9.0 ml of a solution of the same salt concentration as the suspension, but at a lower temperature. The suspension was then centrifuged and the quantity of haemoglobin released by the shock measured.

Cells were exposed to mechanical shock in the presence of NaCl solutions as follows: 0.1 ml of packed cells was suspended in 0.9 ml of salt solution, both at the same temperature, and left for 5 minutes. The suspension was then centrifuged and the supernatant solution discarded. The pellet was resuspended in 0.15 *M* NaCl, left for 5 minutes, centrifuged again, and the haemoglobin released by the shock measured. The mechanical shock in these experiments was expressed in terms of the acceleration due to centrifuging, to which the cells were exposed.

Procedure for exposing red blood-cells to strong salt solutions for various times

0.1 ml of packed cells were drawn into a pasteur pipette. 0.3 ml of salt solution was placed at the bottom of a small short test tube. The salt solution was drawn into the pipette as quickly as possible, that is, in less than 0.3 seconds, and the mixture transferred immediately or after a measured interval of time to 5 ml of 0.15 *M* NaCl.

In this experiment and in others where cells and solutions were measured or transferred by means of pasteur pipettes, the glass surfaces were treated to render them non-wettable. This was done by exposing the glass surface to the vapour of dimethyl-dichlorosilane and then washing thoroughly with distilled water. It was possible by using pipettes so treated to transfer completely solutions and suspensions from the pipette.

RESULTS

Observations of the effects occurring during the freezing of 0.9 % NaCl solution

When 0.9 % NaCl solution is frozen in a small vessel such as a test tube or small beaker by immersion in an alcohol bath at —78° C, ice forms as a concentric shell which moves inwards until all the solution has frozen.

The shell formed when salt solutions are frozen is opaque and crystalline in appearance, in contrast with the transparent glass-like shell formed when pure water is frozen. If the freezing process is arrested when only a portion of the solution is frozen and the liquid and solid parts are analysed it is found that their compositions are identical. This is so whether the shell of ice occupies 10 or 90 % of the whole volume. These observations suggest that the inward moving concentric shell, which forms as the solution is frozen, is not ice but a mesh of ice crystals containing strong NaCl solution within its interstices.

This suggestion was confirmed by the following experiment. 10 ml of 0.9 % NaCl solution were frozen in a cylindrical metal vessel 0.5 ins in diameter, with a metal rod supported at its centre. The conductivity was measured during the formation of the shell of ice. It was found that the conductivity between the central rod and the walls of the vessel did not fall to one-half until 90 % of the solution had frozen. This experiment confirms that the shell which advances into the solution during freezing is a mesh of ice crystals with strong NaCl solution held in the interstices and indicates that the liquid phase is continuous throughout the solution until almost all of it is frozen. The lack of "crenation" when red blood-cells are observed during freezing on a microscope slide (SMITH, POLGE AND SMILES[3]) may in part be due to this effect.

Observations of structural changes in the red blood-cell after freezing and thawing and on exposure to strong salt

The appearance of "ghosts" of red blood-cells which have been frozen and thawed in 0.9 % NaCl solution are shown in Figs. 1 and 2. Those from suspensions which have been

References p. 426.

Fig. 1. Ghosts from red blood-cells which have been frozen and thawed to and from −78° C in less than 10 seconds.

Fig. 2. Ghosts from red blood-cells which have been frozen and thawed to and from −78° C in approximately 60 seconds.

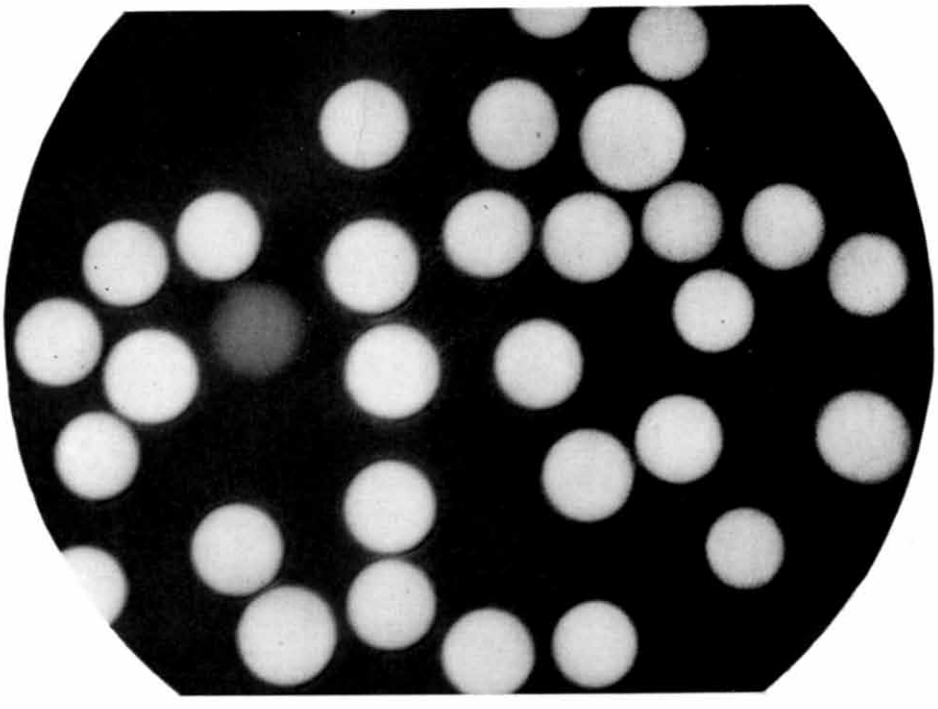

Fig. 4. Untreated human red blood-cells.

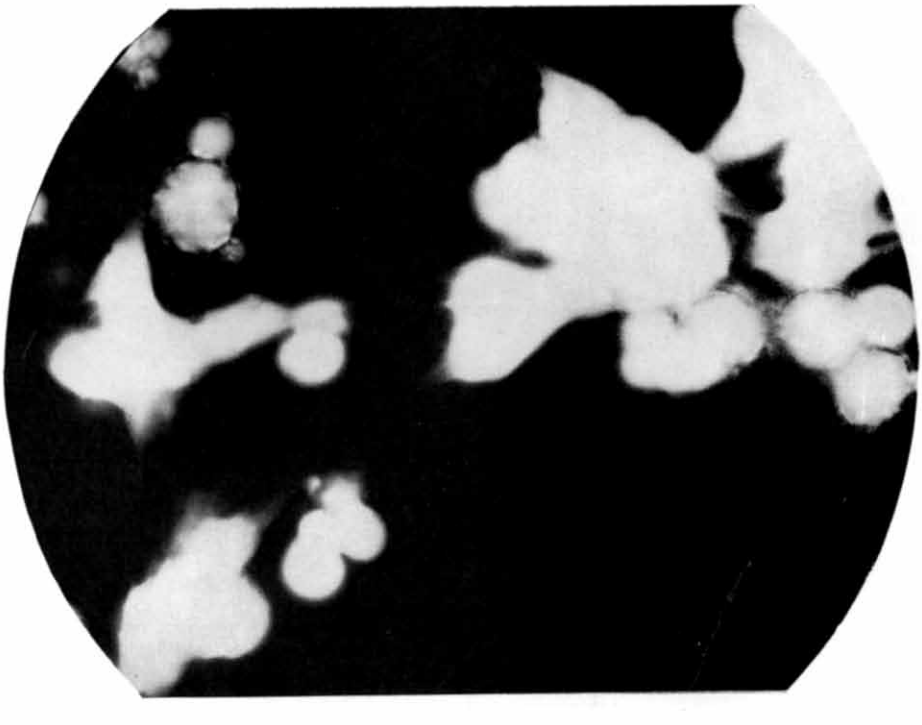

Fig. 3. Ghosts from red blood-cells which have been exposed to 4 *M* NaCl at 0° C.

rapidly frozen and thawed, that is to say have spent less than 10 seconds in the region —4° to —40°C, appear similar in size and shape to the unhaemolysed cells. In contrast, cells which have been slowly frozen and thawed and have spent more than 30 seconds in the region —4° to —40°C are greatly damaged. The "ghosts" are distorted in size and shape, agglutinated, and each clump of agglutinated "ghosts" is connected to other clumps by a fine network of threads. A very similar picture of damage occurs when cells are exposed to strong solutions of lyotropic salts. In Fig. 3 are shown "ghosts" distorted and agglutinated by exposure to 4.0 *M* NaCl. In Fig. 4 are shown normal red blood-cells for comparison.

Haemolysis and the time taken during freezing and thawing

It has long been recognised that the time taken to pass through a critical region of temperature is an important variable in determining the degree of damage sustained by living organisms during freezing and thawing. Experiments described later in this paper indicate this critical region to be bounded by the temperatures —4° and —40°C. In Table I is shown the extent of haemolysis occurring when human red blood-cells are taken at varying rates through this region. The variation of temperature with time, during the freezing or thawing of a suspension of red blood-cells in 0.9 % NaCl, is far from linear. It was necessary, therefore, to express the rate of freezing and thawing in terms of the time taken to cross the critical temperature region. The results shown in the table confirms that the time spent between —4° and —40°C has a profound influence on the damage sustained by the red blood-cells. When it is less than 5 seconds damage is slight, and if it is more than 30 seconds almost all the cells are haemolysed.

TABLE I

THE HAEMOLYSIS WHICH OCCURS WHEN RED BLOOD-CELLS ARE FROZEN AND THAWED AT DIFFERENT RATES

Volume frozen (ml)	*Time spent between −3° and −40°C (seconds)*		*Per cent. haemolysis*
	Freezing	*Thawing*	
0.1	0.3	1.5	7
0.1	0.3	50	90
0.5	3.0	2	10
0.5	2.8	24	52
1.0	9.0	2	35
1.0	9.0	43	93

The critical region of temperature during freezing and thawing

The boundaries of the critical region of temperature in which alone damage takes place rapidly during freezing and thawing have been stated to be —4° and —40°C. The lower boundary was determined as follows. A 10 % suspension of red blood cells in 0.9 % NaCl was sealed in a number of glass capillary tubes, and rapidly frozen to —160°C by immersion in a bath of dichlorodifluoromethane. The tubes were then transferred to an alcohol bath at —60°C which was heated at a rate of 1° per minute; tubes were removed and thawed in water at 42°C at regular intervals until the alcohol temperature was −35°C. The results of this experiment indicated that the lower boundary of the critical region of temperature was between —41° and —39° C. Tubes taken out when the bath temperature was —41° and —39° were respectively less than 20% and more than 80% haemolysed. Further experiments showed that frozen red blood-cells in saline could be

References p. 426.

kept at —45° C for 30 minutes without more damage than others kept at —180° C for the same time and that at —39° C haemolysis was complete within one minute.

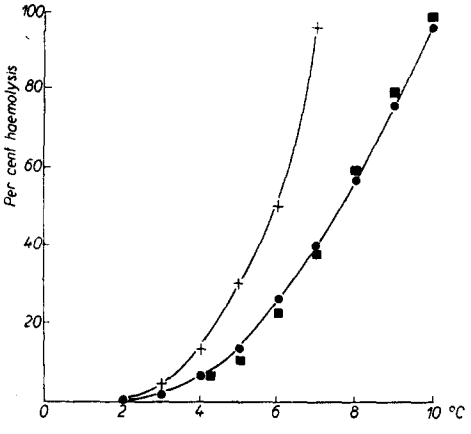

Fig. 5. The haemolysis which occurs when red blood-cells are frozen for 5 minutes at various sub-zero temperatures in solutions of 0.15 *M* LiI, 0.15 *M* NaCl, and 0.08 *M* Na_2SO_4, and then rapidly thawed
+——+ LiI ●——● NaCl
■——■ Na_2SO_4

The upper boundary of the critical region was established by freezing a 10 % suspension of red blood-cells in saline at a series of temperatures between —2° and —10° C and maintaining them for 5 minutes at the chosen temperature. The suspensions were then thawed at 42° C, centrifuged, and the haemolysis measured. The results of these measurements are shown in Fig. 5, together with results of similar experiments in which 10 % suspensions of red blood-cells in isotonic solutions of LiI and Na_2SO_4 were used instead of 0.9 % NaCl. Suspensions of red blood-cells in 0.15 *M* NaCl stronger and weaker than 10 % were also frozen, and the haemolysis measured. It was found that from tightly packed cells to a 5 % suspension the haemolysis which occurs on freezing was unaffected by the strength of the suspension.

The action of strong NaCl solution at low temperatures

The temperature range over which damage to the red cells occurs, namely —3° to —40° C, is consistent with the hypothesis that much of the injury results from the exposure of the cell to concentrated salt solution. To test this hypothesis, 10 % suspensions of red blood-cells in solutions of NaCl of various concentrations were made and maintained at low temperatures for a period of 5 minutes. In order to avoid temperature shock the cells and solution were separately cooled to the required temperature and mixed at that temperature. The results of this experiment are shown in Fig. 6, where it may be seen that the mere exposure to concentrated NaCl solution below 3.0 *M* is not alone sufficient to explain the damage occurring during freezing.

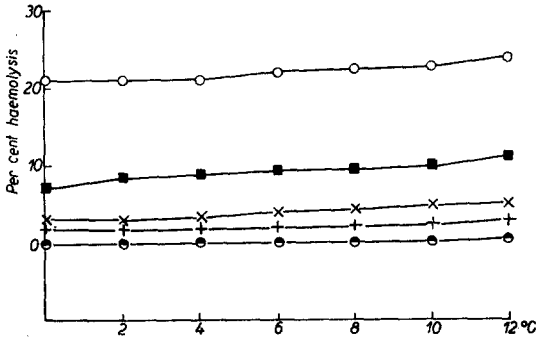

Fig. 6. The haemolysis which occurs when red blood-cells are suspended for 5 minutes in NaCl solutions of various strengths at different sub-zero temperatures. NaCl concentrations:
○——○ 4 *M*; ■——■ 3 *M*; ×——× 2 *M*;
+——+ 1 *M*; ◒——◒ 0.15 *M*.

The effect of exposure to strong salt solution followed by resuspension in 0.15 M NaCl

It has been shown that although some damage results from the exposure of red blood-cells to concentrated NaCl solutions it is not sufficient to explain the haemolysis which occurs when cells are frozen to —10° C or less. During freezing and thawing, however, the cells are exposed successively to strong NaCl and then to dilute NaCl. The possibility that cells exposed to

References p. 426.

strong NaCl would haemolyse on re-suspending in 0.15 *M* NaCl was therefore examined.

Cells were suspended in NaCl solutions at various temperatures for 5 minutes, and then diluted by mixing with distilled water so as to give a final concentration of 0.15 *M*. The diluted suspension was centrifuged and the haemolysis resulting from this operation measured. The results of this experiment are shown in Table II.

TABLE II

THE HAEMOLYSIS WHICH OCCURS WHEN RED BLOOD-CELLS ARE RESUSPENDED IN 0.15 *M* NaCl AFTER EXPOSURE FOR 5 MINUTES TO STRONG NaCl SOLUTIONS

Temperature (°C)	*Per cent haemolysis after exposure to NaCl solution* — *Molar concentration*					
	1.0	*1.5*	*2.0*	*2.5*	*3.0*	*4.0*
20	2.7	11.3	38	74	79	81
0	3.7	13.3	41	81	80	81
−5	3.5	12.8	41	80	80	81

The effect of temperature and mechanical shock on red blood-cells exposed to strong salt solutions

Red blood-cells are not normally affected by sudden chilling or by centrifuging. In the presence of NaCl solutions stronger than 0.8 *M*, however, they appear to become temporarily unstable and haemolyse if suddenly cooled, or if subjected whilst in suspension to an acceleration greater than 20 g, and then resuspended in 0.15 *M* saline. Fig. 3 shows the haemolysis which occurs when red blood-cells suspended in NaCl solutions of increasing concentration are cooled suddenly from 30° to 5°C. Fig. 8 shows the haemolysis which occurs when the cells are cooled at various rates over various temperature intervals when suspended in 1.0 *M* NaCl. Fig. 9 shows the haemolysis occurring when cells suspended in NaCl solutions of increasing strength are centrifuged at various rates and the pellet resuspended in 0.15 *M* NaCl.

These results are sufficient to indicate the considerable complexity of the phenomena which occur when red blood-cells are subjected simultaneously to strong NaCl solutions and to some form of shock. They cannot, however, conveniently be investigated in the temperature range relevant to the effects of freezing, that is below 0°C, and the detailed observation of these effects forms the subject of a separate investigation. In general terms, however, the sensitivity of the red blood-cells to shock when suspended in strong NaCl solutions appears to increase with decreasing temperature. Fig. 7 shows that the chilling of cells in 1.0 *M* NaCl at 0°C causes more haemolysis over a given temperature interval than the chilling of cells initially at a higher temperature.

Haemolysis and time of exposure to salt solutions

The fact that red blood-cells are destroyed within a few seconds on freezing requires that the destructive action of salt solutions must be equally rapid if it is to explain the damage which occurs. The haemolysis which occurred when red blood-cells were exposed to strong salt solutions for various times at 0°C are shown in Table III. The rate of destruction by strong salt solution is seen to be similar to that which occurs on freezing and thawing (Table I).

References p. 426.

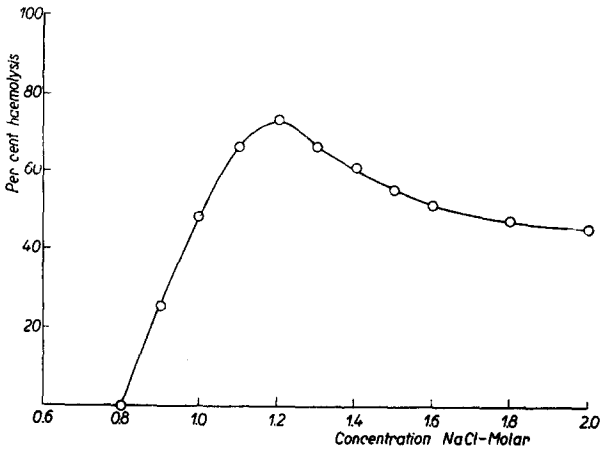

Fig. 7. The haemolysis which occurs when red blood-cells suspended in NaCl solutions of various strength are suddenly cooled from 30° to 5° C.

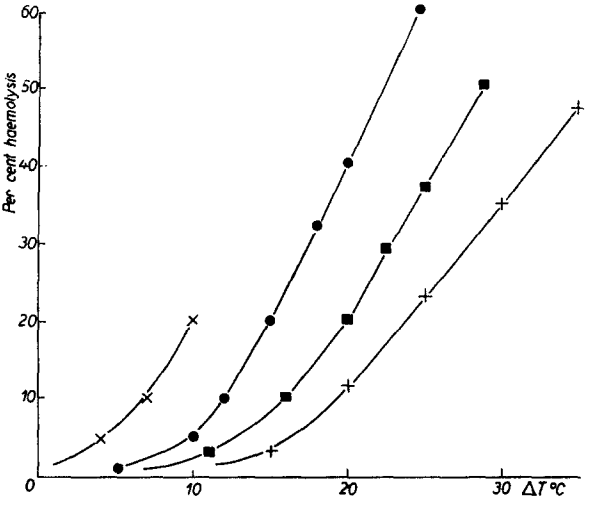

Fig. 8. The haemolysis which occurs when red blood-cells suspended in 1.0 *M* NaCl are suddenly cooled through various temperature intervals (Δ T), and from different initial temperatures.
Initial temperature:
0° ×——×;
+20° ●——●;
+30° ■——■;
+45° +——+.

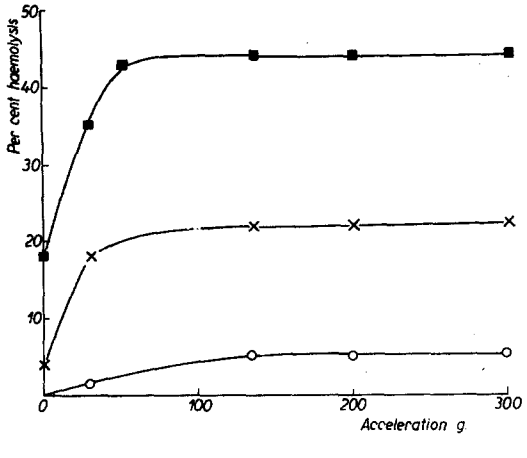

Fig. 9. The haemolysis which occurs when red blood-cells suspended in NaCl solutions are subjected to an acceleration by centrifuging and then resuspended in 0.15 *M* NaCl.
Cells in 0.8 *M* NaCl ○——○;
1.0 *M* NaCl ×——×;
1.5 *M* NaCl ■——■.

TABLE III

THE HAEMOLYSIS WHICH OCCURS WHEN RED BLOOD-CELLS ARE EXPOSED FOR VARYING TIMES TO STRONG NaCl SOLUTIONS AT 0° C AND THEN TRANSFERRED TO 0.15 *M* NaCl

NaCl concentration (Molar)	*Per cent haemolysis after exposure for*					
	1	*2*	*5*	*10*	*30*	*60*
			(seconds)			
1.5	2	2	4	8	11	11
3.0	8	15	53	60	64	64
4.0	10	22	60	80	80	80

TABLE IV

A COMPARISON OF THE EFFECTS OF FREEZING AND THAWING WITH THOSE OF TRANSFER FROM ISOTONIC NaCl TO STRONG NaCl AND BACK AGAIN. COLUMN 1 GIVES THE HAEMOLYSIS WHICH OCCURS WHEN RED BLOOD-CELLS IN 0.15 *M* NaCl ARE FROZEN AT THE STATED TEMPERATURES FOR 5 MINUTES – COLUMN 2 THAT WHICH OCCURS WHEN THE CELLS ARE EXPOSED TO THE STATED NaCl CONCENTRATION – COLUMN 3 THAT WHICH OCCURS WHEN THE CELLS ARE TRANSFERRED TO 0.15 *M* NaCl AFTER EXPOSURE TO THE STRONG NaCl – COLUMN 4 THE ADDITION OF COLUMNS 2 AND 3

Temperature (°C)	*NaCl solution with this freezing point M*	*Haemolysis per cent*			
		1	*2*	*3*	*4*
2	0.6	0	0	0	0
3	0.86	1.4	0	0	0
4	1.15	6.0	0	3.3	3.3
5	1.43	13	0	11.3	11.3
6	1.7	26	0	25	25
7	2.0	40	0	38	38
8	2.2	57	0	56	56
9	2.4	76	2	74	76
10	2.6	96	11	78	89
12	3.0	100	13	80	93
15	3.6	100	23	80	100

DISCUSSION

It has long been recognised that exposure to low temperatures alone is not rapidly lethal to living organisms. The experiments just described indicate that the red blood-cell is not exceptional in this respect. Thus, red blood-cells have been super-cooled to —15° C whilst suspended in 0.15 *M* NaCl solution for 10 minutes without the occurrence of haemolysis. Further, suspensions of red blood-cells have been rapidly frozen to temperatures below —40° C and kept without suffering damage for several hours.

The fact that the cells can survive freezing and thawing if these processes are sufficiently rapid also suggests that the formation of ice is not in itself damaging. This last hypothesis is also supported by the observation that very little damage occurs if suspensions of red blood-cells are frozen to a temperature not lower than —4° C where nearly 90 % of the water of the suspension is frozen.

The experimental evidence demonstrates that there is a critical region of temperature in which the cells are irreversibly damaged if they remain longer than a few seconds. The boundaries of this region are —3° and —40° C for red blood-cells suspended in 0.15 *M* NaCl. The upper limit of the critical region is the freezing point of 0.8 *M* NaCl solution, the lower limit is that of the eutectic temperature for the mixture of salts and other

References p. 426.

substances present within the red blood-cell. The critical region of temperature therefore coincides with that region in which the cell is exposed both on the outside and on the inside of its membrane to solutions of NaCl at concentrations in excess of 0.8 *M*.

The results confirm that the exposure of red blood-cells to salt solutions stronger than 0.8 *M* is damaging. The destructive action of salt solutions is complex, and manifests itself in several ways which appear to be qualitatively different.

Firstly, in NaCl solutions stronger than 0.8 *M* the cells appear to become permeable to sodium ions, and they are not always shrunken as in mildly hypertonic solutions. The increased permeability does not immediately cause haemolysis although at concentrations as high as 2.5 *M* it occurs within a few hours at 37°C; presumably a new osmotic equilibrium is reached with a relatively high internal concentration of sodium ions. The cells in this condition tend to haemolyse if resuspended in "physiological" saline, possibly as a result of excessive internal osmotic pressure due to their burden of sodium ions. Freezing and thawing carries the cells through such a cycle of exposure to strong and then weak NaCl solution.

A second consequence of the exposure of cells to salt concentrations stronger than 0.8 *M* is the development of sensitivity to thermal and mechanical shock. Normally, the red cell may be cooled over wide ranges of temperature and subjected to considerable mechanical stress without haemolysis occurring. In moderately strong salt solutions, however, sudden cooling through 10° to 50°C, according to the initial salt concentration and temperature, causes haemolysis. Similarly, cells in moderately strong salt solutions are damaged by centrifuging and haemolyse when resuspended in 0.15 *M* NaCl. These effects are very complex and form the subject of a separate investigation. Their importance as sources of damage during freezing and thawing cannot be assessed in quantitative terms. Thermal shock is not likely, however, to be an important cause of damage at temperatures above —10°C during freezing. Mechanical shock is likely to play some small part in causing damage during the early stages of freezing when cells will be subjected to distortion and movement during the growth of ice crystals.

The third way in which salt solutions are damaging appears when concentrations are greater than 3.0 *M*. Exposure of the cells to these very strong solutions causes their complete destruction. The process appears to be connected with the lyotropic properties of NaCl solutions and is shown at lower concentrations by more lyotropic salts, *e.g.* LiI and KSCN. The appearance of the "structures" remaining after the "ghosts" of red blood-cells have been destroyed by this process are highly characteristic and have been described fully by FURCHGOTT[7]. Thread and bead-like forms predominate. These can be seen in Fig. 2 and 3, which are photographs of frozen and of salt-treated red blood-cells respectively. This effect is not important as a source of damage to frozen cells at temperatures above —10°C. The salt concentration is insufficient until the temperature falls below —10°C.

The extent to which red blood-cells are damaged by exposure to NaCl solution under conditions similar to those occurring during freezing are shown in Table IV. Also shown for comparison is the haemolysis occurring when the cells are frozen at temperatures between —2° and —15°C, and the NaCl concentrations corresponding to these temperatures of freezing. The table illustrates the relative importance of the two principal processes of damage at the various temperatures, and shows that the aggregate damage due to these effects is sufficient to explain quantitatively that which occurs on freezing and thawing.

References p. 426.

The "survival" of red blood-cells when rapidly frozen and thawed appears to be due to the relative slowness with which the destructive action of NaCl occurs. The results show that, if freezing and thawing take place within 5 seconds or less, damage is slight. Exposure to strong NaCl solutions for similar times is also harmless. LUYET[4] has suggested that red blood-cells may survive rapid cooling to —180°C if the suspending medium vitrifies instead of freezes. This may be so with the rapid cooling rates and relatively viscous media used by LUYET. It must be emphasised, however, that with the suspending medium used in the experiments described in this paper, 0.15 *M* NaCl, vitrification would not occur. The lowest temperature of super-cooling with a dilute solution of this nature is —40°C (MASON AND LUDLAM[6]).

There is a considerable amount of indirect evidence which suggests that the destructive effect of strong salt solutions is upon the cell as a whole and not merely upon the surface structures directly in contact with the suspending medium. Cells suspended in isotonic solutions of lyotropic salts such as LiI or KSCN are more readily damaged on freezing than cells in NaCl. Cells suspended in salts less lyotropic than NaCl such as Na_2SO_4, or which do not concentrate on freezing such as sodium oxalate, are, however, not less damaged than those suspended in NaCl. This evidence is consistent with the hypothesis that the internal medium of the cell, isotonic KCL, concentrates and damages the cell during freezing. Further, and more direct evidence in support of this hypothesis arises from the nature of the protective action of glycerol in preventing damage on freezing and thawing. It is shown (LOVELOCK[8]) that one effect of adding glycerol to the suspending medium is to lessen the rise in NaCl concentration during freezing. It is further shown that, if glycerol is prevented from permeating the red blood-cells before freezing, they are destroyed although the NaCl concentration of the external suspending medium does not rise unduly during freezing.

The red blood-cell is unusual among cells in its simplicity of structure and high permeability to water. It is probable that the picture of destruction by freezing shown to occur with the red blood-cells is connected with these properties. More complex and less permeable cells are likely to succumb to other destructive processes before the NaCl concentration had risen to as high as 0.8 *M*. The amoeba, for example, has been shown by CHAMBERS AND HALE[9] to be destroyed by the disruptive effect of ice formation within the cell when cooled to —0.6°C. Other cells, rabbit spermatozoa for example, are sensitive to osmotic shock (EMMENS[10]). They would undoubtedly perish when the concentration of the molecules in the medium increased suddenly as on freezing.

ACKNOWLEDGEMENT

I am indebted to Dr. A. S. PARKES, F.R.S., for his interest and advice. I wish also to express my thanks to Mr. J. HARMER, of the National Institute for Medical Research, who took the photomicrographs.

SUMMARY

The destructive processes which occur when red blood-cells are frozen and thawed have been observed. The results of these observations suggest that the principal damaging effect associated with freezing is the concentration of the electrolytes present within and without the cell. The destructive action of exposure to concentrated salt solutions is not instantaneous and the survival of rapidly frozen and thawed cells is attributed to this factor. The nature of the damage caused by

References p. 426.

exposure to strong salt solutions is complex. At moderate concentrations the red blood-cell becomes sensitive to thermal and mechanical shock; at high concentrations the cell structure is dispersed by an apparently lyotropic effect.

RÉSUMÉ

L'auteur a observé les processus destructifs qui ont lieu lorsque les globules rouges sont congelés et dégelés. Il résulte de ces observations que l'effet destructif principal qui accompagne la congélation semble être la concentration des électrolytes présents à l'intérieur et à l'extérieur de la cellule. L'action destructive que les solutions salines concentrées exercent sur les cellules n'est pas instannée, ce qui permet d'expliquer le fait que des cellules ayant été congelées et dégelées rapidement peuvent survivre à ce traitement. Les dommages causés aux cellules par les solutions salines fortes sont de nature complexe. Sous l'action de solutions de concentration modérée, les globules rouges deviennent sensibles aux chocs thermiques et mécaniques; dans les solutions très concentrées, la structure cellulaire est détruite par un effet apparemment lyotrope.

ZUSAMMENFASSUNG

Es wurde der zerstörende Prozess, der beim Einfrieren und Auftauen roter Blutkörperchen stattfindet, beobachtet. Die Ergebnisse dieser Beobachtungen lassen vermuten, dass der hauptsächlich schädigende Effekt, der das Enfrieren begleitet, die Konzentration der innerhalb und ausserhalb der Zelle anwesenden Elektrolyte ist. Der Zerstörungsprozess beim Einbringen in konzentrierte Salzlösungen verläuft nicht momentan und das Überleben schnell eingefrorener und wieder aufgetauter Zellen ist diesem Faktor zu zuschreiben. Die Natur der durch Einbringen in konzentrierte Salzlösungen verursachten Schädigung ist verwickelt. Bei mässigen Konzentrationen werden die roten Blutkörperchen für thermische und mechanische Stösse empfindlich; bei hohen Konzentrationen wird die Zellstruktur durch einen offenbar lyotropen Effekt zerstört.

REFERENCES

[1] A. U. SMITH, *Lancet*, 11 (1950) 910.
[2] B. J. LUYET AND L. J. MENZ, *Biodynamica*, 7 (1951) 25.
[3] A. U. SMITH, C. POLGE AND J. SMILES, *J. Roy. Micr. Soc.*, 71 (1951) 186.
[4] B. J. LUYET, *Preservation of the formed elements and of the proteins of the blood*, American National Red Cross, 1949.
[5] M. STRUMIA, *Preservation of the formed elements and of the proteins of the blood*, American National Red Cross, 1949.
[6] T. MORAN, *Proc. Roy. Soc., B*, 105 (1929) 177.
[7] R. F. FURCHGOTT, *Cold Spring Harbor Symposia Quant. Biol.*, 8 (1940) 224.
[8] J. E. LOVELOCK, (1953) in press.
[9] R. CHAMBERS AND H. P. HALE, *Proc. Roy. Soc., B*, 110 (1932) 336.
[10] C. W. EMMENS, *J. Physiol.*, 107 (1948) 129.

Received July 23rd, 1952