SUMMARY

1. Sedimentation and diffusion measurements have been made in the concentration range 0.17–0.8 g./100 ml. on human serum albumins obtained by ethanol fractionation and by electrophoretic separation.

2. The effect of variation in composition of the buffer has been examined with the result that, for a given preparation, sedimentation or diffusion may be affected at finite concentrations, but values extrapolated to zero concentration do not differ significantly.

3. Values of $S_{20,w}^0$ and $D_{20,w}^0$ vary slightly from one preparation to another, indicating differences in average molecular weight. Evidence is presented which suggests that this is due to the separation of groups of molecules of different molecular characteristics rather than to irreversible changes produced by buffer conditions.

4. The faster of the two components which appear on electrophoresis of albumin at pH 4.0 has been isolated. Its sedimentation and diffusion properties were indistinguishable from those of whole albumin.

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The Protective Action of Neutral Solutes against Haemolysis by Freezing and Thawing

By J. E. LOVELOCK

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

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It has been known for many years that sugar solutions have a slight effect in protecting living cells against damage during freezing and thawing (Smith, 1953). Polge, Smith & Parkes (1949) discovered that spermatozoa could be frozen and thawed without loss of motility if glycerol or some similar polyhydric alcohol was included in their suspending medium. Investigation of the protective action of glycerol was facilitated by the observation that red blood cells also were protected by glycerol against the adverse effects of freezing and thawing (Smith, 1950). The nature of the destructive processes which take place when red blood cells are frozen and

thawed, and of the protection given by glycerol was then described (Lovelock, 1953a, b). Briefly, damage on freezing is a consequence of the increase in concentration of electrolytes within and without the cell. In the presence of glycerol the electrolyte concentration at temperatures below the freezing-point is greatly diminished, by an amount sufficient to explain the protection afforded by glycerol.

The effect of glycerol in lowering the concentration of electrolytes in equilibrium with ice at temperatures below 0° is a simple consequence of the colligative properties of solutions. It follows that the protective action of glycerol is unlikely to be

unique, and that other neutral solutes of low molecular weight which are non-toxic and which can permeate the cell should also protect it.

The high efficiency of glycerol in protecting cells against damage attendant on freezing made it of some interest to investigate the protective action of other substances satisfying the requirements given above. This was partly with a view to seeking a still more effective substance, but also to check this prediction arising from the provisional explanation of the action of glycerol.

This paper describes the action of neutral solutes upon the haemolysis which occurs when human red blood cells are frozen to temperatures between 0 and -80° . The compounds tested were mostly those with several hydroxyl groups, including glycols, erythritol and sugars. Methanol, ethanol, acetamide and formamide were also tested.

METHODS

Red blood cells. Human blood to which anticoagulant had been added was stored at 4° until required. The anticoagulant solution was composed of trisodium citrate, 0.073 M; citric acid, 0.035 M; and glucose, 0.01 M. It was added to fresh human blood in the proportion of 15 ml. of anticoagulant to 100 ml. of blood. Blood older than 10 days was not used. The cells were separated by centrifuging and then washed three times in lightly buffered NaCl soln. (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° . The washed cells were always used within 5 hr. of their separation from the plasma.

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° were obtained by means of a thermostatically controlled ethanol bath. This was capable of maintaining the temperature to which it was set to within $\pm 0.1^{\circ}$. Temperatures below -30° were obtained by means of a bath consisting of an open-necked Dewar flask containing 10 l. of ethanol. This flask was adjusted to the required temperature by the addition of solid CO_2 . The heat capacity of the flask was sufficiently great, and its heat losses sufficiently small, for the temperature to remain constant during the 10 min. required for the experiments.

Measurement of haemolysis. In all experiments the damage which occurred to the R.B.C. was assessed by measuring the quantity of haemoglobin released into the suspending solution. After exposure to experimental conditions the R.B.C.'s remaining undamaged were removed by centrifugation. The supernatant solution was diluted with 0.1% Na_2CO_3 , shaken to convert the haemoglobin into oxyhaemoglobin, and the concentration of the latter was measured in terms of the absorption of light at 5200 Å.

Permeability of the cells to the solute. A 0.05 ml. portion of washed R.B.C. was added to 10 ml. of a M soln. of test solute in distilled water, and a second 0.05 ml. portion of cells was added to 10 ml. of a M solute solution in 0.16 M-NaCl. The solutions were placed in a water bath at 37° and observed at 10 min. and 1 hr. The cells were taken as permeable to the

solute if complete haemolysis in the distilled water solutions occurred in 10 min., partially permeable if haemolysis was not complete in 1 hr. and impermeable if no haemolysis occurred in 1 hr. The cells in 0.16 M-NaCl did not haemolyse with any of the solutes tested. They served to indicate that the observed haemolysis in the aqueous solute solution was a consequence of permeation by the solute and not due to some toxic action.

Permeation of the cells by the solute. Before freezing, the cells were exposed to the test solute for a time at a temperature sufficient to allow permeation of the cell by the solute. With those solutes which readily penetrated the cell membrane, 10 min. at 20° was sufficient. Solutes tested included methanol, ethanol, formamide, acetamide, ethylene and propylene glycols, diethylene and triethylene glycols, glycerol, and monoacetin. With those solutes which penetrated the cell membrane slowly, or not at all, 1 hr. at 37° was allowed for permeation. This was the maximum time and temperature to which the cells could be exposed without appreciable haemolysis. The poorly penetrating solutes included erythritol, xylose, and glucose, and the non-penetrating solutes sucrose, and polyethylene glycol.

Procedure for freezing suspensions of red blood cells. All experiments were made in flat-bottomed tubes (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 suspending medium. After allowing sufficient time for the permeation of the cells by the solute, the suspension was cooled by immersion in a bath set at the required temperature. It was left in the bath for 10 min. after freezing had occurred, either spontaneously or by seeding with an ice crystal. The suspension was thawed by immersion in a water bath at 20° .

RESULTS

Haemolysis of red blood cells on freezing in solutions of NaCl containing various non-ionic solutes

Fig. 1 shows the haemolysis which occurs when suspensions of R.B.C., in 0.16 M-NaCl solution containing various concentrations of non-ionic solutes, are frozen. It illustrates the protective action of those solutes which were most representative of the principal classes of behaviour observed. The class of solute which protects at all temperatures when sufficient is present is represented by glycerol, but includes also ethylene glycol and diethylene glycol. Solutes which protect at temperatures down to -30° , but in which damage takes place at lower temperatures, are represented by methanol and acetamide; similar behaviour is, however, shown by ethanol, formamide, and propylene glycol. Glucose illustrates the behaviour of those substances offering some protection at all temperatures but never complete protection; the behaviour of erythritol and xylose is very similar. Finally, sucrose is chosen to represent the class of solute which affords almost no protection; this also includes polyethylene glycol.

In Table 1 are shown those concentrations of all the solutes tested which are required to prevent

Fig. 1. The haemolysis when R.B.C.'s suspended in 0.16M-NaCl containing various concns. of neutral solute are frozen. The concn. of solute is shown on each curve. In all experiments the cells were frozen for 10 min. at the temperature indicated.

haemolysis at various temperatures. The permeability of the red blood cell to the solute, and the molecular weight of the solute, are also shown.

DISCUSSION

There exists a critical region of temperature in which living cells are rapidly damaged during freezing and thawing. With the human red blood cell this region extends from -3 to -40° , and if more than a few seconds are spent between these temperatures haemolysis takes place. The protective action of the solutes tested is chiefly shown by an ability to reduce this critical region and, if sufficient is used, to

abolish it completely. The results indicate that this action is not a property of glycerol alone. It is shown not only by other polyhydric alcohols, but also by methanol and by acetamide.

The results also show that the prevention of haemolysis in the critical region 0 to -40° is only possible with those solutes which permeate the cells. Glucose and xylose, to which the cell is only partially permeable, offer only partial protection, and sucrose, to which the cell is impermeable, offers little or no protection. Solutes which are toxic, such as methanol and acetamide, are effective in reducing or preventing haemolysis in the region between 0 and -40° , but with these there is a second critical

Table 1. *The protective action of neutral solutes related to their molecular weights and ability to permeate the human red blood cell*

(Measurements were made of the solute concentration needed to prevent haemolysis when 1.0 ml. of a 10% suspension of red blood cells in 0.16M-NaCl was frozen for 10 min. '+' indicates that haemolysis could not be prevented by any concentration of solute. Concn. expressed as g./100 ml. solution.)

Solute	M.W.	Permeability to solute	Concn. to prevent haemolysis at			
			-10°	-20°	-30°	-40°
Methanol	32	Complete	2.8	6.9	10.8	14.7
Formamide	45	Complete	4.8	17.0	+	+
Ethanol	46	Complete	4.2	10.2	+	+
Acetamide	59	Complete	5.0	12.5	20.0	25.0
Ethylene glycol	62	Complete	4.5	11.4	19.0	25.0
Propylene glycol	76	Complete	4.9	14.0	20.0	30.0
Glycerol	92	Complete	5.4	13.0	19.0	24.0
Diethylene glycol	106	Complete	6.2	17.0	25.0	30.0
Erythritol	122	Partial	9.3	24.0	+	+
Monoacetin	134	Complete	8.6	19.0	27.0	35.0
D-Xylose	150	Partial	10.5	+	+	+
Triethylene glycol	150	Complete	8.0	19.0	+	+
Glucose	180	Partial	15.0	+	+	+
Sucrose	342	Impermeable	+	+	+	+
Polyethylene glycol	400	Impermeable	+	+	+	+
(average)						

region with a peak near -55° . The first critical region results from the concentration of electrolytes, and the second presumably from the concentration of solute rising to toxic levels.

The damage suffered by red blood cells when they are frozen and thawed occurs almost entirely as a result of exposure to an excessive concentration of electrolyte within and without the cell. In sodium chloride solutions the human red blood cell suffers irreversible damage at salt concentrations exceeding a molar fraction of 0.014 (m.p. -3° approx.). This critical concentration is substantially independent of temperature over the range 40 to -35° and also of glycerol concentration (Lovelock, 1953*a, b*). It follows from the colligative properties of solutions that the mere addition of a neutral solute to a salt solution will lower the concentration of salt in equilibrium with ice at any given temperature below freezing. Thus, it is possible, by adding sufficient neutral solute, to maintain the salt concentration below the critically damaging level at any required temperature. The approximate concentration of solute required for this purpose can be derived as follows:

The depression of the freezing point, ΔT , of an aqueous solution of sodium chloride, containing also a neutral solute, is

$$\Delta T = K(2x + y),$$

when x and y are the concentrations of sodium chloride and of neutral solute respectively. If the concentrations x and y are expressed in molal units, or as mol. fractions, K is a constant and indicates the observed depression of the freezing point of an aqueous solution by unit solute concentration. In practice K varies to some extent with the nature and

concentration of the dissolved substances and to this extent the relationship is approximate.

During freezing, ice separates as a pure substance, and the concentrations of salt and solute therefore increase in the same proportion. If the sodium chloride concentration above which irreversible damage takes place is n times the initial sodium chloride concentration x , the depression of the temperature below 0° at which damage will first occur is

$$\Delta T_D = Kn(2x + y),$$

or, more conveniently, the concentration of solute required to prevent irreversible damage at a temperature given by the depression ΔT_D is to be greater than y , where

$$y = \frac{\Delta T_D - 2Kn x}{Kn}.$$

For a suspension of human red blood cells in 0.16M-NaCl (i.e. $y = 0$), the value of n is 5.0; and if the concentrations are expressed as mol. fractions, x is 0.00286 and K is 105. Substituting these values in the equation above gives

$$y = \frac{\Delta T_D - 3}{525}.$$

The concentration of solute y is here expressed as a mol. fraction.

The line drawn according to this relationship is shown in Figs. 2 and 3 together with the experimentally observed molar fractions of the various solutes found necessary to prevent haemolysis. These figures indicate that with glycerol there is a remarkably close agreement between theory and practice. Solutes which fail to penetrate the cells completely, e.g. glucose and xylose (Fig. 3), and

Fig. 2. The relationship between solute concentration, expressed as a molar fraction, and the temperature to which R.B.C.'s in 0.16M-NaCl can be frozen without haemolysis. The solid line indicates the solute concn. required by theory, and the points the concns. found necessary in practice. The solutes illustrated include only those to which the cell is readily permeable. They are as follows: ○, methanol and ethanol; ●, formamide and acetamide; ●, ethylene and propylene glycols; +, glycerol; ×, di- and tri-ethylene glycols; ●, mono-acetin.

Fig. 3. The relationship between solute concentration, expressed as a molar fraction, and the temperature to which R.B.C.'s in 0.16M-NaCl can be frozen without haemolysis. The straight line indicates the solute concn. required by theory, and the curves the concns. found necessary in practice. The solutes illustrated include only those to which the cell is partially or wholly impermeable; they are: +, erythritol; ×, xylose; ●, glucose; ○, sucrose.

solutes which are toxic at high concentrations, particularly methanol, ethanol, acetamide, and formamide (Fig. 2), show deviations from the theoretical line which increase with increasing concentration of solute. When the molar fraction of solute exceeds 0.04 some deviation occurs with all the solutes tested except glycerol. It is possible that this 'ideal' behaviour of glycerol is a consequence of its unique lack of toxicity. Non-penetrating solutes, e.g. sucrose, show no protection at any concentration.

The rapid decrease in haemolysis at temperatures below -40° has not yet received an explanation in quantitative terms. With glycerol it was shown (Lovelock, 1953b) that a decrease in the rate of haemolysis occurs at temperatures below -35° . At these low temperatures also, the solubility of some of the salts within the cell, e.g. potassium chloride, may be insufficient to cause damage. At sufficiently low temperatures both of these factors must cause a diminution of haemolysis. It remains to determine which, if either of them, is capable of accounting for the observed decrease of haemolysis.

On the basis of the results it is possible to confirm the desiderata for an ideal agent for protection against damage during freezing and thawing. They are as follows: (1) high solubility in aqueous salt solutions at temperatures from $+40$ to -40° ; (2) ability to permeate the cell rapidly and completely; (3) low molecular weight; and (4) absence of toxicity, even when in great excess.

Of all the substances tested, glycerol alone is capable of fulfilling all these requirements. Indeed, it is unlikely that any substance exists which is as effective as glycerol in protecting the human red blood cell against the adverse effects of freezing and thawing. In the practical preservation of human red blood cells other considerations than protection during freezing and thawing are important, e.g. the ease with which the protective agent can be removed from the cells, and the prolonged survival at low temperatures. In choosing a substance for the practical preservation of red blood cells these factors also must be taken into account. Even so, it seems unlikely that a substance better than glycerol will be found.

SUMMARY

1. The protective action of fifteen neutral solutes against the haemolysis of human red blood cells by freezing and thawing has been observed. The solutes tested included mono-, di- and poly-hydric alcohols, amides, and sugars.

2. The results of these observations indicate that protection only occurs when the solute is able to permeate the cell and that the weight concentration of solute required for protection is proportional to its molecular weight.

3. The mechanism of the protective action is explained in terms of the colligative properties of solutions, and desiderata for an ideal protective agent are listed. For human red blood cells the substance most closely fulfilling these criteria is glycerol.

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Biochemical Studies of Toxic Agents

6. THE CONVERSION OF NAPHTHALENE INTO 1:2-DIHYDRO-2-HYDROXY-1-NAPHTHYL GLUCOSIDURONIC ACID IN THE RABBIT

By E. D. S. CORNER, F. S. BILLET AND L. YOUNG

Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1

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Rats and rabbits dosed with naphthalene excrete 1:2-dihydronaphthalene-1:2-diol (Young, 1947; Booth & Boyland, 1949), and by analogy with anthracene it might be expected that excretion of a glucuronide of this diol also takes place, for Boyland & Levi (1935, 1936) have shown that in rats and rabbits anthracene is converted into 1:2-dihydroanthracene-1:2-diol, some of which is excreted as a glucuronide (see also Boyland & Wiltshire, 1953). This possibility has been studied in the present work and the results obtained provide good evidence that in the rabbit naphthalene is converted into a glucuronide of 1:2-dihydronaphthalene-1:2-diol, namely 1:2-dihydro-2-hydroxy-1-naphthyl glucosiduronic acid.

EXPERIMENTAL

The animals used were male rabbits weighing from 2.5 to 3.0 kg. They were housed in metabolism cages designed to permit the collection of urine separate from the faeces. The animals were fed on a diet consisting of cabbage together with a mixture of bran and chaff and they had access to water at all times.

Quantitative experiments

A quantitative study was made of the excretion of glucuronic acid and ethereal sulphate by rabbits before and after they had been dosed with naphthalene. Two experiments were carried out. In the first the urine of four rabbits was analysed for glucuronic acid and ethereal sulphate on the three consecutive days immediately before the administration of a single dose of naphthalene to each rabbit. Naphthalene (1 g./kg. body wt.) was administered by stomach tube as a 15% (w/v) soln. in liquid paraffin. Daily analysis of the urine was then continued until the glucuronic acid and

ethereal sulphate excretion had returned to the pre-dosing level. The second experiment was carried out on another four rabbits in the same way as the first except that 24 hr. after the first dose of naphthalene had been given, each rabbit received a second dose of the same size as the first.

Table 1. *Excretion of inorganic, ethereal, and total sulphate S (IS, ES and TS) and of glucuronic acid in the urine of four rabbits before and after the administration by stomach tube of naphthalene*

(Each dose 1 g./kg. body wt. as a 15% (w/v) soln. in liquid paraffin.)				
	Sulphate S (mg./kg. body wt.)			
Day	IS	ES	TS	Glucuronic acid (mg./kg. body wt.)
Expt. 1. Single dose of naphthalene				
1	24.6	1.7	26.3	13.4
2	34.1	5.2	39.3	22.1
3	22.2	1.7	23.9	31.2
(Naphthalene administered)				
4	12.1	7.9	20.0	473.0
5	6.0	5.4	11.4	297.0
6	26.2	0.2	26.4	52.6
7	25.3	2.1	27.4	29.0
Expt. 2. Two doses of naphthalene				
1	30.4	3.0	33.4	26.9
2	24.7	4.2	28.9	20.0
3	21.8	0.8	22.6	29.2
(Naphthalene administered)				
4	23.3	10.6	33.9	588.0
(Naphthalene administered)				
5	4.9	6.2	11.1	825.0
6	7.6	4.7	12.3	448.0
7	27.1	3.6	30.7	112.0
8	25.8	2.8	28.6	29.8