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A SUGAR-DEPENDENT INCREASE IN RED CELL STABILITY

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SUMMARY

The effects of several monosaccharides on the resistance of human erythrocytes to hypotonic stress have been examined. By using probit analysis to determine the 50% response, it is shown that D-glucose reduces cell fragility as a linear function of concentration and that the glucose-dependent increase in cell stability decreases rapidly when the temperature is raised above 20 °C. D-Fructose also reduces cell fragility, but this effect is not sensitive to temperatures in the range 0–37 °C. K^+ loss from cells is insufficient to account for the sugar-dependent cell stabilization. Cell sizing by a multi-channel cell volume analyser shows that the critical haemolytic volume is unaffected by glucose.

By measurement of the relaxation time of cell fragility the bulk visco-elastic moduli can be calculated (see Appendix). It is shown that at 0 °C, glucose increases both the cell elastic and viscous moduli (E_A increased by $1.2 \cdot 10^5$–$3.3 \cdot 10^5$ nM^{-2}, whilst ν_A increased by $1.8 \cdot 10^7$–$9.0 \cdot 10^7$ $nM^{-2} \cdot s^{-1}$).

Proton magnetic resonance studies suggest that glucose, water and intracellular protein form a ternary complex, below 20 °C the ratio of bound water : glucose is 10 : 1. These changes in cell water structure and the cell bulk visco-elastic moduli are consistent with the view that intracellular glucose promotes protein gel formation.

INTRODUCTION

Several sugars and certain other polyhydric reagents protect red cells against hypotonicity, hypertonicity, freeze and shear stress [1–4]. Additionally it has been noted that sugars retard the rate of hypotonic haemolysis [5] and the rate of malonamide induced haemolysis [6]. In this paper the physical basis of sugar-dependent stabilization against hypotonic stress will be examined.

It has been established that in isotonic solutions of low ionic strength K loss from red cells is enhanced [7]. Before any specific stabilizing action can be ascribed to sugars, two possible non-specific mechanisms of cell stabilization must be considered. Firstly, does enhanced K leakage account for the sugar-dependent protection against haemolysis as it does for the stabilizing effect of a slow reduction in external

tonicity [8], and secondly, do the stabilizing sugars increase the area of the cell membrane and thus increase the critical haemolytic volume? [9]. Seeman and Roth [10] have shown these effects with several hydrophobic drugs.

It will be shown that neither of these two mechanisms can account for sugar-dependent protection against hypotonicity and an alternative mechanism derived from experiments which determine the functional dependence of stabilization on sugar structure, concentration and also on temperature and time will be described. Further evidence for this mechanism is obtained from high resolution proton magnetic resonance studies.

METHODS

(A. i) Procedure for determining the effect of sucrose and glucose on the critical haemolytic volume mode

Human erythrocytes (Red Cross blood stored for 1 week in acid–citrate–dextrose medium) were washed twice in 154 mM NaCl containing 0.1 M NH_4Cl and 5 mM sodium phosphate buffer (pH 7.3). All subsequent solutions contained 0.1 M NH_4Cl–phosphate medium. The NH_4Cl rapidly and completely equilibrates across the erythrocyte membrane and thus serves to maintain electrical conductivity for cell-size monitoring without shrinking the cells [3, 11]. A second batch of erythrocytes was washed twice in 300 mM sucrose in NH_4Cl–phosphate, and a third batch in 154 mM NaCl with 20 mM glucose in NH_4Cl–phosphate. The final concentration of of erythrocytes in each suspension was approx. 20 000 cells per ml. All solutions and procedures were carried out a room temperature (21–22 °C). The solutions had all been previously filtered through a millipore filter (0.45 μm diameter pores) in order to remove dust.

The erythrocytes were then sized by a specially constructed electronic cell detector with a 100-channel pulse-height analyzer [12, 13]. After recording the volume distribution of the cells suspended in their starting isotonic media, each cell suspension was then diluted with NH_4Cl–phosphate in progressive amounts. The cell volume distribution was monitored after each dilution. Although the final osmolarities of NaCl and of sucrose progressively dropped with each dilution, the concentration of glucose remained fixed since a diluting solution of 20 mM D-glucose in NH_4Cl–phosphate was added to the cells suspended in D-glucose. Because of the drop in total ionic strength, the electrical conductivity of the suspending media fell by about 1%; the presence of 0.1 M NH_4Cl throughout prevented greater reductions in conductivity. Each sampling after each dilution consisted of 10 ml removed from a total volume ranging from 200 ml (before dilutions) to 500 ml (after the dilutions). Each multi-channel analysis was done at least in duplicate, and both sets of records are given in Results. In this method, the time interval between each progressive dilution step was about 4 min.

(ii) Procedure for determining the effect of glucose on final ghost volume

It is known that approx. 1–2 h after osmotic haemolysis, the erythrocyte ghosts become sealed and spherical [14, 15]. The effect of D-glucose on the sealed ghost volume was studied, therefore, two hours after the hypotonic lysis of erythrocytes in the presence of varying concentrations of D-glucose.

Human erythrocytes (Red Cross acid-citrate–dextrose blood) were washed twice in 154 mM NaCl in NH_4Cl–phosphate and resuspended to make a final stock suspension of about 4 000 000 erythrocytes per ml. Aliquots of 0.1 ml suspension were pipetted into 20 ml of NH_4Cl–phosphate, containing 0, 5, 10, 20, 40 and 80 mM D-glucose, while mixing the cells vigorously on a vortex mixer. The cells thus underwent hypotonic haemolysis in the presence of low concentrations of D-glucose. 1.5–2 h later the cells were sized in the electronic cell detector with pulse–height analyser.

The distribution of the cell volumes were fitted by computer to a Gaussian by a least squares iterative method. The computer analysis thus provided the modal channel of the fitted Gaussian, as well as the standard deviation of the fitted Gaussian. The fitted Gaussian ignores the dust particles and debris which have very low "volume".

(B) Procedure for haemolysis experiments

(i) Cell preparation. 50-ml aliquots of cold stored human red cells (by courtesy of the Sheffield Regional Hospital Board) were washed free of acid–citrate–dextrose by a repeated cycle (4 times) of centrifugation and resuspension in isotonic saline (150 mM NaCl–10 mM Tris–Cl, pH 7.4). The cells were equilibrated with test sugar by incubation for an appropriate time in saline to which crystalline sugar had been added to bring the overall concentration to the required level. For glucose alone, equilibration was at room temperature for 2 h; for experiments involving relatively impermeant sugars, e.g. fructose, equilibration was carried out overnight (15 h) at 4 °C. Equilibration under the conditions of temperature and time used for preloading was tested by measuring the distribution ratios of ^{14}C-labelled sugars to ^{3}HHO between cells and supernatant at various sugar concentrations. No significant differences were obtained for the ratio between cells and supernatant at increasing concentrations of sugar, both in the case of fructose and glucose, showing that equilibration was complete.

(ii) Drastic (fast haemolysis). For "drastic haemolysis", aliquots of packed cell suspension were rapidly pipetted by automatic syringe (Repette Marlow Ltd) into a series of tubes containing 10 ml of saline of increasing hypotonicity (with the test-sugar at the same concentration as in the pre-incubation suspension). The tubes were immediately shaken to disperse the cells, left for a standard time then centrifuged ($1500 \times g$) for 5 min. Samples of cell-free supernatant were then removed for determination of Hb and K^+.

(iii) Slow haemolysis. The method used was essentially that given by Seeman et al. [8]. An aliquot of the packed cell suspension was pipetted into 5 ml of isotonic saline contained in a 50 ml beaker in which dispersion was effected by a magnetic stirring bar. A given volume of distilled water (buffered with 10 mM Tris–Cl, pH 7.4) was then delivered via a flexible siliconplastic tube at a constant rate by an "H.R. Flow inducer" (Watson-Marlow Ltd, Marlow, Bucks.). The cells were stirred throughout the addition period, and the time of stirring was constant. After cessation of stirring, the solution was left for a standard time and then centrifuged ($1500 \times g$) for 5 min. Samples of cell-free supernatant were then removed for Hb and K^+ determination.

Note: All haemolysis experiments were at room temperature (20 °C) unless otherwise stated.

(*iv*) *Haemoglobin determinations.* Percentage haemoglobin release was determined by measuring the optical density of the supernatant solutions at 540 nm in a Unicam SP 600 spectrophotometer.

(*v*) *K^+ determinations.* K^+ was determined by using an E.E.L. 227 integrating flame photometer, employing Li^+ as an internal standard. All supernatant solutions were diluted to the range 0 to 1.0 mM K^+ and adjusted to 15 mM Li^+.

(*C*) *Preparation of cells, haemoglobin and haemoglobin-free membranes for proton magnetic resonance spectroscopy*

(*i*) *Preparation of cells.* The cells were washed as in Section B(i), and equilibrated with glucose for at least 2 h at 20 °C before NMR spectroscopy.

(*ii*) *Preparation of membranes.* Haemoglobin-free membranes were prepared using the method of Dodge et al. [16] Washed packed cells were lysed in 10 times their volume of ice cold 5 mM sodium EDTA buffered with Tris–HCl to pH 7.4. The membranes were spun out of solution by centrifuging at 18 000 $\times g$ for 20 min at 4 °C, using an M.S.E. 18 centrifuge. The supernatant solution was removed and the precipitated membranes were washed by resuspension and recentrifugation a further four times in Tris buffered EDTA which removed the bulk of the haemoglobin. The membranes were then stored at −20 °C in 5 mM EDTA until required. 40 h before required the membranes were thawed for PMR and dialysed against 200 mM KCl, with D-glucose added to give the appropriate equilibrium concentration at 4 °C. The concentration of membranes was determined by measuring the dry weight of a known volume of membrane suspension.

(*iii*) *Preparation of membrane-free haemoglobin.* Glucose-free packed cells were disintegrated in an M.S.E. ultrasonic disintegrator. The membrane fragments were spun out of suspension by centrifuging the preparation at 18 000 $\times g$ for 120 min at 4 °C, and the supernatant solution was pressure dialysed against 200 mM KCl, or 200 mM KCl supplemented with sufficient D-glucose to bring the equilibrium concentration to 200 mM. The concentration of the haemoglobin solution remaining was estimated from the $A_{540\,nm}$ of diluted samples.

(*D*) *Nuclear magnetic resonance studies*

NMR spectra were run at 56 MHz on a Varian D.A. 60 spectrometer, fitted with a constant temperature device, and calibrated by the side-band technique. When required, chloroform was used as an external reference. For wide-line measurements at high gain, in order to avoid spinning sidebands the tubes were not spun.

In order to obtain an approximate estimate of the positions of the broad-band increments, curves were decomposed as shown in Figs 16 and 17 using a Du Pont Curve Resolver.

(*E*) *Statistical methods*

(*i*) *Probit analysis.* The essence of this method is the transformation of the symmetrical sigmoid response curve, by means of the probit, to a rectilinear form upon which regression analysis can be performed (Fig. 1). The probit Y for any proportional response, p, for normal dosage tolerance, μ, is given by:

$$p = \frac{1}{\sqrt{2\pi}} \int_{\infty}^{y-5} e^{-\frac{1}{2}\mu^2} d\mu$$

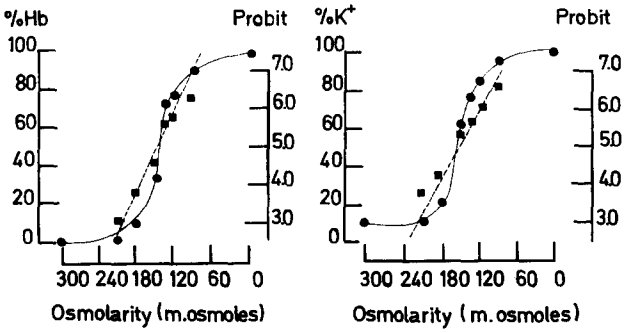


Fig. 1. The effect of the probit transformation on characteristic fragility curves for haemoglobin and K^+. ●, percentage release of haemoglobin/K^+; ■, the percentage points transformed to probits. - - -, maximum likelihood least-squares line for probit versus osmolarity.

The analysis gives a maximum likelihood solution by successive regression after assignation of weights and working probits to the initial probit regression line. The dosage required to give any particular value of probit, and hence value of proportionate response, can be easily determined from this final linear relationship between probit and dosage.

The characteristic sigmoid fragility curves (Fig. 1) obtained by plotting either percent release of haemoglobin or K^+ against external NaCl concentration result from the quantal response of the cells, to stress i.e. the all or none nature of lysis [17]. It could be argued that K^+ release is not truly quantal because of prelytic K^+ release, but it is treated as such for the purpose of this analysis since a good fit to the probit transformation is actually obtained for the K^+ data. The fragility curves were symmetrical about the mid point indicating a normally distributed population of red cell tolerance to hypotonicity obviating the necessity of applying a normalizing transform to the hypotonicity scale.

A program for probit analysis [18] written in Fortran IV was run on the University of Leicester ICL 4130 computer. The output gave the NaCl concentration for 50% response (LD_{50}) together with an estimate of its variance, the slope and intercept of the probit regression line, and a χ^2 statistic which allowed testing for heterogeneity of response.

From paired analyses of haemoglobin and K^+ leakage, the percentage K leakage at the hypotonicity required to give 50% haemoglobin release may be calculated, and this quantitates the pre-haemolytic K loss.

(*ii*) *Bartlett's analysis.* The van Stevenick plot [20] of Fig. 6 illustrates the prelytic loss of K^+ in drastic haemolysis with or without glucose. Clearly, if there is a difference in the amount of prelytic K^+ loss with glucose, this should be most evident in the lower half of the curve. By making a linear approximation for this part of the curve (0 to 40% lysis), it is possible to quantify any differences that exist with or without glucose from differences in the slopes and intercepts. Both variables are subject to experimental error so that neither the regression line of X on Y nor Y on X will give a sufficiently good estimate of the best fit line. The best fit line is therefore calculated using the method suggested by Bartlett (see ref. 19). The data is divided into 3 ranked groups 1, 2, 3, the groups being of equal size, then:

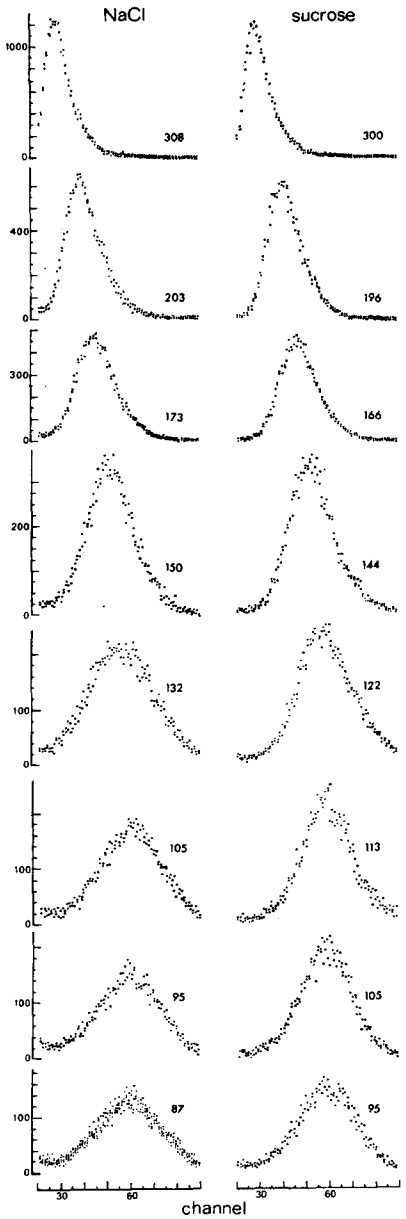


Fig. 2. Volume distribution of red cells as monitored by the pulse height analyser (see Method Ai) figures beside each plot are the external osmolarities.

$$\text{slope} = \frac{\overline{Y}_3 - \overline{Y}_1}{\overline{X}_3 - \overline{X}_1} = \beta$$

and intercept $= \overline{Y} - \beta\overline{X}$.

The significance of the differences between the slopes with or without D-glucose is given by Student '*t*'-tests.

RESULTS

(*Ai*) *The effect of variation of external tonicity on cell volume*

The data shown in Fig. 2 are the cell volume distribution as monitored by the cell detector and pulse-height analyzer, using Method (Ai). The volume distributions on the left are those for the NaCl series, and those on the right are for cells in sucrose. The final osmolarity of NaCl or sucrose is noted beside each spectrum of cell volumes. It can be seen that as the osmolarity of NaCl or sucrose decreased the cell volumes shifted up-channel and remained up-channel until the most dilute solutions. This indicates that the cells did not collapse or shrink when they became ghosts. The ordinates in Fig. 2 indicate that the cell concentrations fell with progressive dilution.

A plot of the modes of each volume distribution versus the osmolarity is presented in Fig. 3. The modal erythrocyte volume under isotonic conditions was 90 μm^3. The modal critical haemolytic volume was about 188 μm^3 for cells suspended in NaCl or in sucrose.

(*ii*) *Effect of* D-*glucose on critical haemolytic volume.* Using Method (Aii) the effects of different concentrations of D-glucose were tested on the sizes of spherical ghosts formed during hypotonic haemolysis. These results are shown in Fig. 4 where the cell volume distributions were monitored starting from the top. At 0 mM D-glucose

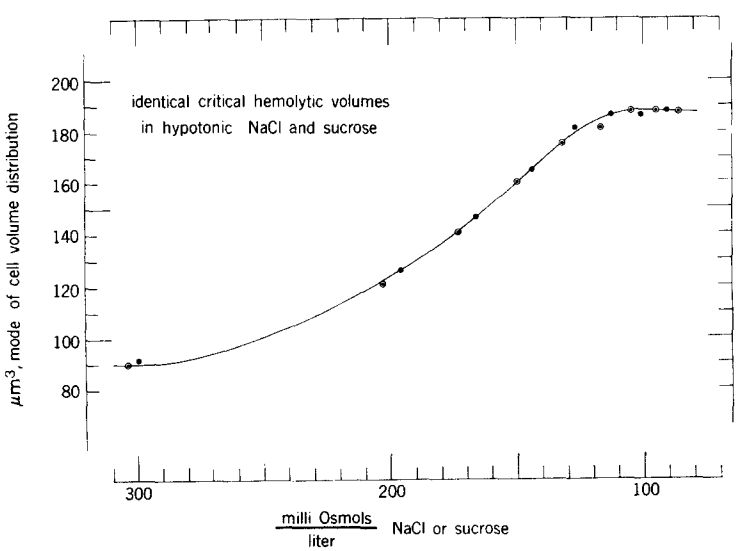


Fig. 3. Plot of model volumes of red cell suspensions at differing external tonicities of NaCl and sucrose.

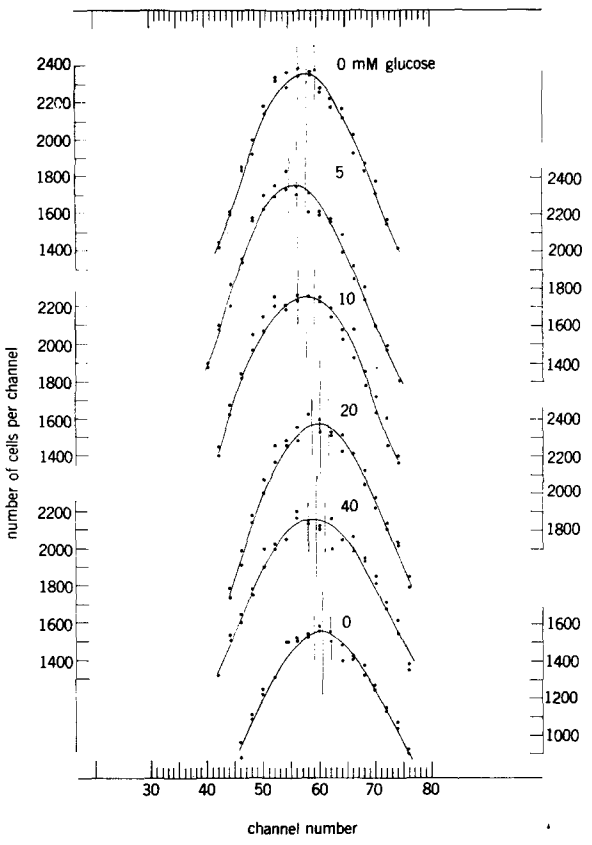


Fig. 4. Plot of volume distribution of resealed ghosts following hypotonic haemolysis (see Method Aii) figures beside each plot are the equilibrium concentrations of D-glucose in the suspensions.

(i.e. just NH_4Cl–phosphate medium) the modal volume was 57.5 ± 1.5 (standard deviation of the fitted Gaussian to the raw data) channels, where each channel represents 3 μm^3. The modal volumes for 5, 10, 20 and 40 mM D-glucose were 56.0, 57.5, 60.0 and 59.1, respectively, all having the same standard deviation of 1.5 channels. The bottom spectrum in Fig. 4 was a re-run of the cells in 0 mM D-glucose, but where the cells were diluted with NH_4Cl–phosphate, the modal volume was $60.3 \pm$ 1.5 channels. D-Glucose did not significantly affect the spherical ghost volumes.

The results shown in Figs 2–4 indicate that sucrose or D-glucose have no effect on the critical haemolytic volume, V_c, or the spherical volume of erythrocyte ghosts. Meryman [3, 11] on the other hand, using a microhaematocrit method found that a wide variety of impermeant solutes substituted for extracellular saline increased V_c. He observed no change in V_c when D-glucose was substituted for saline. Confirmatory experiments (Simmons, N. L. and Naftalin, R. J., unpublished results) using the haematocrit method also showed no change in V_c in cells equilibrated with 50 mM D-glucose even though marked protection was observed in these suspensions. With sucrose+0.1 M NH_4Cl replacement of saline, some increase was observed in the packed volume of sphered cells. A possible explanation of the difference in results obtained by

the haematocrit and channel analyser methods with sucrose replacing saline as the suspending medium, may be that the packing properties of sphered cells in high concentrations of sucrose differ from those in saline. A factor of some importance may be the decrease in buoyant density of cells suspended in sucrose+0.1 M NH_4Cl compared with cells suspended in saline solutions of equivalent tonicity.

(Bi) Effect of D-*glucose at 25 mM on drastic haemolysis and prelytic* K^+ *loss*

Figs 5a and 5b show lysis as monitored by haemoglobin and K^+ release. The effect of glucose on the prelytic loss of K^+ is shown in the van Stevenick plots. Fig. 6b shows the lower part of Fig. 6a on an extended scale; it being clear that differences, if any, will be most evident in this region. No difference in the prelytic losses with or without D-glucose at 25 mM is evident. This finding is confirmed firstly, by the probit analysis of the data, and secondly, by the Bartlett's analysis of the data between 0% and 40% of the van Stevenick plots.

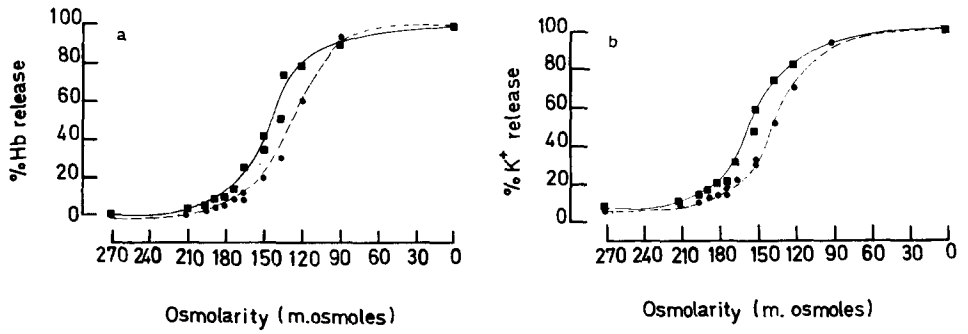


Fig. 5. The effect of 25 mM glucose at 20 °C after 5 min test incubation on fragility as monitored by (a) haemoglobin release, (b) K^+ release. ■ (—), zero-glucose; ● (- - -), +25 mM glucose.

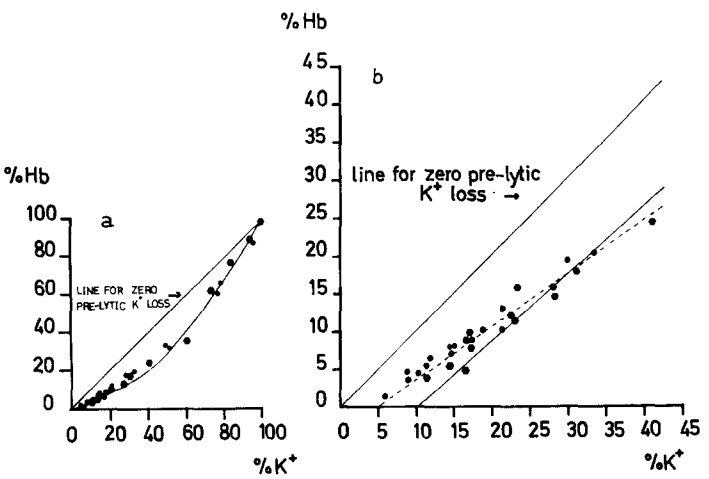


Fig. 6. Van Stevenick plot for data of Fig. 2. —, theoretical line for zero prelytic loss of K^+, (b) is the lower half of (a) drawn on an extended scale. Hexagons, zero glucose; ●, +25 mM glucose. In (b) — and - - -, Bartlett best-fit lines for zero glucose and +25 mM glucose, respectively.

TABLE I

Summary of probit analysis for paired haemoglobin and K^+ data from 6 experiments at 20 °C following 5 min incubation ±25 mM glucose. Errors shown are the standard errors (S.E.) of the means of the six experiments. Within each experiment there were at least 36 degrees of freedom.

	LD_{50} haemoglobin (mosmoles)	LD_{50} K^+ (mosmoles)	Protection (LD_{50} haemoglobin + sugar − LD_{50} haemoglobin zero sugar) (mosmoles)	% K^+ leakage at hypotonicity giving 50 % haemoglobin loss
Zero glucose	149.09±4.59 (S.E.)	142.65±5.32 (S.E.)	12.714±2.57 (S.E.)	62.75±4.33 (S.E.)
25 mM glucose	161.77±3.16 (S.E.)	156.27±4.75 (S.E.)		57.92±2.78 (S.E.)

TABLE II

Summary of Bartlett's analysis for best-fit line for data of van Stevenick plots for 4 experiments at 20 °C following 5 min test incubation ±25 mM glucose.

Experiment (±25 mM glucose)	Slope of Bartlett's line	t	Degrees of freedom	P	Intercept	t	P
−	0.305				12.704		
1		−0.1590	42	>0.8		1.572	<0.10
+	1.186				8.576		
−	1.286				5.518		
2		0.1170	47	>0.9		−3.182	<0.01
+	0.944				9.805		
−	1.105				9.790		
3		0.1270	42	>0.9		2.116	<0.05
+	1.332				3.700		
−	1.055				10.170		
4		0.003	48	>0.9		1.148	<0.3
+	1.027				8.39		

Probit analysis (Table I) confirms the protection afforded by D-glucose against haemoglobin and K^+ loss. A t-test on the pairs of calculated K percentage losses at 50% haemolysis with or without 25 mM D-glucose shows no significant difference ($P<0.5$).

The Bartlett's analysis (Table II) shows no significant difference, $P>0.8$, between slopes with or without 25 mM D-glucose (4 experiments). A significant difference ($P<0.05$) does exist between intercepts in 2 experiments. This difference is due to a protection against basal lysis in isotonic solution; the conclusion that 25 mM D-glucose does not affect the prelytic loss of potassium due to hypotonic stress is unaffected.

(*ii*) *Effect of membrane strain rate on glucose-dependent protection and prelytic K^+ loss.* Table III shows that glucose-dependent protection is independent of membrane strain rate. Protection is observed when lysis is induced either by drastic or slow reduction in external tonicity. Although an increased rate of prelytic K^+ loss is observed during slow haemolysis, confirming previous observations [8, 21], the presence of glucose makes no substantial difference to this leakage rate. Thus, although the increased prelytic K^+ leakage is sufficient to account for the decrease in apparent fragility of cells with slow changes in external tonicity, increased leakage does not account for the increased protection observed in the presence of glucose during both slow and drastic haemolysis.

(*iii*) *Effect of D-glucose concentration on drastic haemolysis and prelytic K^+ loss.* Fig. 7 shows the glucose concentration dependence of protection against haemolysis following one hour incubation in hypotonic solution at 0, 20 and 32 °C. Prior to the experiment the cells had been equilibrated with glucose at the appropriate concentration for several hours at room temperature. At 0 and 20 °C protection increased as a linear function of glucose concentration, whereas at 32 °C glucose-dependent stabilization is not observed until the concentration is raised to 100 mM. In four out of six

TABLE III

Probit analysis of data showing the effect of reduction in membrane strain-rate on glucose-dependent protection against haemoglobin and K^+ loss.

Treatment		LD_{50} haemoglobin (mosmoles)	Standard error	LD_{50} K^+ (mosmoles)	Standard error	% K^+ release at the hypotonicity required to give 50 % haemoglobin release
'Drastic'	Normal	87.66	2.70	69.40	4.94	64.01
	+20 mM glucose	103.64	1.86	90.38	4.44	60.01
	+60 mM glucose	154.74	2.24	128.50	5.18	66.98
'Slow' change in membrane strain rate	Normal	141.80	4.90	26.22	7.44	91.76
	+20 mM glucose	211.42	5.10	76.68	0.96	87.62
	+60 mM glucose	294.56	6.38	105.48	2.54	92.69

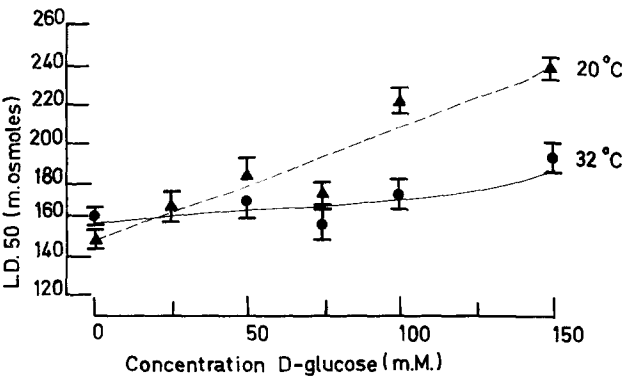


Fig. 7. The effect of temperature and glucose concentration on fragility monitored by the hypotonicity required to give decrease from 300 mosmoles 50 % Hb release (i.e. LD_{50} Hb) for each treatment. I bars indicate ±standard error for each point. Data for 0 °C are omitted for clarity since these are not significantly different from those at 20 °C.

experiments at 0 °C above 50 mM there was a departure from linearity in the relationship between concentration and protection (Table IV). Protection in this case is best fitted to an exponential of glucose concentration. It is clear that protection is not a saturable function of glucose concentration. The glucose-dependent protection against prelytic K^+ leakage has a similar form of concentration dependence. However, at concentrations above 75 mM an increased prelytic K^+ loss is observed. This K^+ loss is calculated to be insufficient to account for the observed stabilization, but may partially account for the non-linear increase in protection at high concentration.

(*iv*) *Effect of other sugars on haemolysis and prelytic K^+ loss.* Good [6] has shown that sugar stereoisomers have widely different activities as inhibitors of haemolysis in malonamide. In the experiments described in this paper, haemolysis is recorded after incubation for 1 h when lysis is virtually complete, hence the observed changes in cell fragility are due to an increase in the stability of the cell structure and cannot be ascribed to an alteration in membrane permeability to solutes and water. Fig. 8 shows the effect of varying concentrations of D-fructose, L-sorbose, D-xylose, D-ribose and D-galactose on cell fragility.

All but the last mentioned protect against haemolysis as a linear function of

TABLE IV

Probit analysis for a single experiment at 0 °C following 1 h test incubation and 2 h pre-equilibration at room temperature showing the concentration-dependence of protection.

Concentration of D-glucose (mM)	Protection (LD_{50} haemoglobin + sugar − LD_{50} zero sugar) (mosmoles)	% K^+ release at the hypotonicity required to give 50 % haemolysis
Zero	–	66.03
30	11.92± 2.57 (S.E.)	65.85
75	26.40± 2.51 (S.E.)	78.59
100	81.86±12.20 (S.E.)	81.72

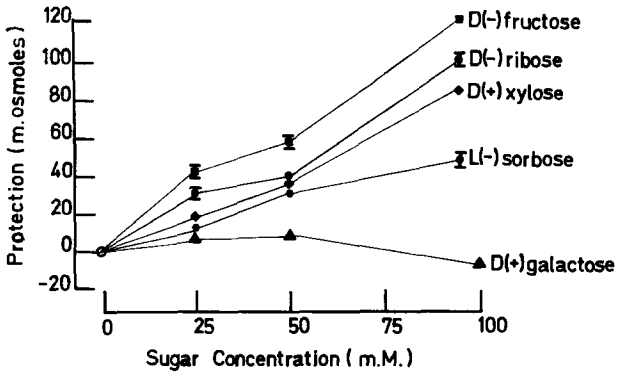


Fig. 8. The effect of various sugars at 0 °C after hour test incubation, and 15 hour pre-equilibration at 4 °C. Protection (LD_{50} of test sugar–LD_{50} Hb normal (mosmoles)) is plotted against sugar concentration. I bars indicate ±standard error of protection.

concentration. The extent of protection is also dependent on the sugar structure.

The calculated percentage of K^+ release at the external tonicity giving 50% of the total haemoglobin release, i.e. LD_{50} haemoglobin, is insufficient to account for the protection against haemoglobin release observed with fructose, ribose, xylose and sorbose, as already shown with glucose, whilst galactose does not protect cells against haemoglobin loss (as shown above) or K^+ loss in the concentration ranges tested (unpublished data).

At low concentrations, D-fructose is a more effective stabilizer against hypotonic haemolysis than is D-glucose (see Table V). The relative stabilizing activities as indicated in Table V are similar to the relative activity of the sugars in inhibiting the rate of malonamide induced haemolysis as previously reported by Good [6].

(*v*) *Effect of temperature variation on glucose-dependent protection against hypotonic haemolysis.* In the absence of glucose, cooling cell suspensions from 37 to 0 °C increases cell fragility [1, 6], with 50 mM glucose present, lowering the temperature slightly increases the cell stability (Fig. 9). On cooling, protection, as measured

TABLE V

The relative stabilizing activities of test-sugars at 25 mM expressed as the fractional protection observed relative to that for glucose (= 1.0). All data from experiments at 0 °C following 1 h test incubation and pre-equilibration for 15 h at 4 °C. Errors shown are the standard error of the fraction in each case.

Sugar	Fractional protection (relative to glucose = 1.0)		
	Expt 1	Expt 2	Expt 3
D-Glucose	1.0	1.0	1.0
D-Fructose	2.46±0.09	1.60±0.06	1.230±0.18
D-Xylose	1.60±0.08	0.99±0.075	
D-Ribose	1.29±0.09	0.88±0.06	
L-Sorbose			0.908±0.20
D-Galactose			0.540±0.23
D-Mannose			0.340±0.24

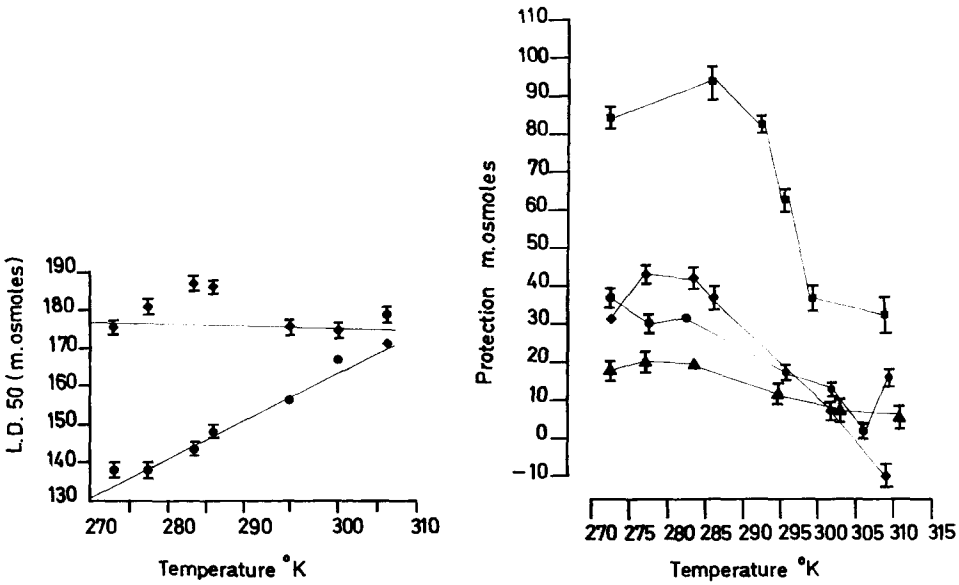


Fig. 9. Data from a single experiment following 1 h test incubation and 2 h pre-equilibration at room temperature, showing the effect of temperature (°K) on LD_{50} (the hypotonicity from 300 mosmoles) to give 50% lysis. ●, zero glucose; ◆, +50 mM glucose. I bars indicate ±standard error of the determination of LD_{50} (—, least square linear regression line).

Fig. 10. Protection at 50 mM glucose (LD_{50}+glucose−LD_{50} zero glucose (mosmoles) is plotted against temperature (°K) for 4 experiments. I bars indicate ±standard error of protection. I bars lie within the points when not shown.

by the difference in tonicity required to cause 50% lysis, increases from zero at 37 °C to a maximum at 0 °C. Mean protection at 0 °C is approx. 50±10 mosmoles with 50 mM glucose (Fig. 10). In order to reduce the large amount of variation between experiments the data is normalized by expressing protection at any temperature in the range 0–37 °C as a fraction of the protection obtained at 0 °C. By graphing the $\log_e$ of fractional protection against the reciprocal of temperature (°K) for four sets of results, a functional relationship between temperature and protection is obtained (Fig. 11). It is seen that the temperature coefficient of fractional protection changes sharply at 25 °C. This transition may possibly be due to a phase change occurring within the cells.

(*vi*) *The contrasting effects of temperature variation on protection from hypotonic haemolysis by* D-*glucose and* D-*fructose.* Fig. 12 shows the contrasting effects of temperature variation on glucose and fructose-dependent stabilization. The cells were fully equilibrated with 50 mM glucose or fructose before exposure to hypotonicity. It is observed that the fructose-dependent stabilization in the temperature range 0–37 °C is independent of temperature, whereas the glucose-dependent stabilization increases on cooling from zero at 37 °C as previously indicated. A qualitative difference can also be seen between the absolute stability of the cells in the presence and absence of glucose. Whereas cooling decreases cell stability in the presence or absence of fructose, cooling increases cell stability when glucose is present.

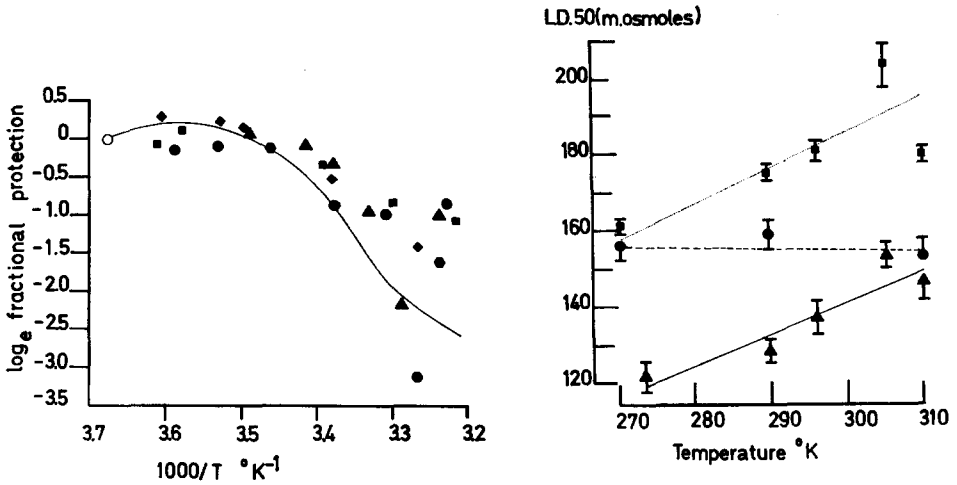


Fig. 11. The functional relationship between temperature and protection (expressed as $\log_e$ fractional protection versus $100/T$ K^{-1}). Data is from 4 separate experiments, ●, ▲, ■, ◆, shown in Fig. 10 and is fitted to the least square 4th order polynomial.

$y = 6487 - 63.85x + 199.72x^2 - 18.6x^3 - 0.419x^4$

Fig. 12. Data from a single experiment (1 h test incubation, 15 h pre-equilibration at 4 °C), showing the contrasting effect of temperature on LD_{50} of zero sugar (▲), 50 mM glucose (●) and 50 mM fructose (■). I bars indicate standard error of LD_{50} (10 degrees of freedom). Lines are best fit linear regression lines.

The temperature coefficients of a variety of other sugars have been determined (Table VI).

Galactose is no more effective at stabilizing cells at 0 °C than at 37 °C.

Three types of sugar–cell interaction emerge from these studies. Firstly, sugars such as galactose and mannose which do not affect cell stability; secondly, with fructose the temperature coefficient of protection is low; and thirdly, glucose, sorbose and xylose stabilize cells better at 0 °C than at higher temperatures.

(vii) Time dependence of fragility ±glucose and characterization of viscoelastic

TABLE VI

The temperature coefficient of protection (i.e. the protection observed at 37 °C to protection observed at 0 °C), for various sugars. Galactose does not show significant protection either at 37 °C or at 0 °C. All data are for experiments at 0 °C following 1 h incubation and 15 h pre-equilibration at 4 °C. Errors shown are the standard error for each estimate of the temperature coefficient.

Sugar	Temperature coefficient of protection (Protection at 37 °C/protection at 0 °C)
D-Glucose	12.00±1.24 (S.E.)
D-Fructose	1.17±0.06 (S.E.)
D-Ribose	1.79±0.19 (S.E.)
D-Xylose	4.98±0.47 (S.E.)
D-Sorbose	9.45±2.72 (S.E.)
D-Galactose	—

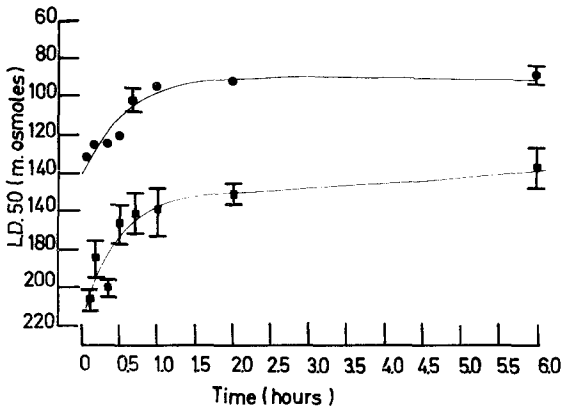


Fig. 13. The time dependance of fragility, as given by the LD_{50}, at 0 °C, following 2 h pre-equilibration at room temperature with ●, zero glucose; ■, +50 mM glucose. I bars are ±standard error of LD_{50} (10 degrees of freedom).

behaviour of red cells. Fig. 13 shows the time dependent increase in fragility ±50 mM glucose. The difference in LD_{50} values in the presence and absence of glucose represents the glucose-dependent increase in cell stability. Minus glucose it is assumed that the mechanical strength of the cell is solely dependent on membrane properties. The elastic modulus E_B of the membrane is calculated by applying Eqn 3 (see Appendix) to the observed changes in cellular volume shown in Fig. 2. P_{os}, the osmotic pressure difference, is calculated from the difference in external and internal tonicities; the ratio of cell radius:membrane thickness (r:d) is taken tc be 100.

On cooling cells in the absence of glucose an increased fragility is observed indicating an apparent decrease in E_B as calculated from Eqn 3, assuming that the relationship between volume and external tonicity is independent of temperature [8].

The NMR spectra show (vide infra) that glucose interacts mainly with the intracellular protein, from which it may be deduced that glucose-dependent decreases in osmotic fragility are due to increases in E_A, the elastic modulus of the intracellular material. No significant interaction of glucose with membrane associated water is observed, so E_B, the elastic modulus of the membrane, remains unchanged. From Eqns 6, 7 and 3 (see Appendix) it can be shown that;

$$E_A = \left(\frac{P_{os(glucose)} - 1}{P_{os}}\right) \cdot \frac{4d}{3r} \cdot E_B$$

where $P_{os(glucose)}/P_{os}$ is the ratio of osmotic pressure differences existing across the cell membrane immediately prior to lysis in the presence or absence of glucose.

The time-dependence of fragility (Fig. 13) is interpreted as a visco-elastic relaxation where:

$$-\frac{t}{\tau} = \ln\left(\frac{P_{(t)} - P_{(\infty)}}{P_{(0)} - P_{(\infty)}}\right)$$

By plotting ln $((P_{(t)} - P_{(\infty)})/(P_{(0)} - P_{(\infty)}))$ against time, the slope of the line obtained is equal to $-1/\tau$, where τ is the characteristic time for relaxation (Fig. 14). $P_{(t)}$ and

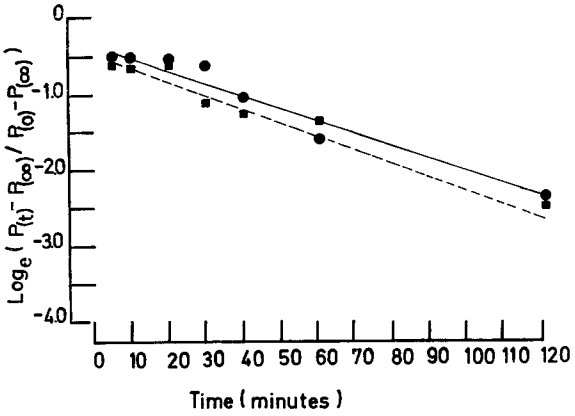


Fig. 14. Data of Fig. 13 plotted as $\log_e[P_{(t)}-P_{(\infty)})/P_{(0)}-P_{(\infty)})]$ against time. ●, zero glucose; ■, +50 mM glucose. —, least square line for zero glucose points, - - -, 50 mM glucose.

$P_{(\infty)}$ are defined as the osmotic pressure differences across the cell membrane at time t and infinity respectively. The intracellular tonicity is calculated assuming that the critical haemolytic volume is constant for all conditions ($V_c = 1.8 \times$ isotonic volume) (see Fig. 2). $P_{(0)}$, the pressure difference at time zero (i.e. immediately following the step change in tonicity), is equal to (internal tonicity/1.5 − external tonicity). 1.5 is the relative cell volume at which sphering occurs. It is assumed that there is negligible mechanical resistance to volume change between biconcave disc and sphering.

ν_B, the viscous modulus of the membrane alone, may now be calculated from Eqn 7;

$$\bar{\nu} = \nu_A + \frac{3r}{4d}\nu_B$$

where ν_A is the viscous modulus of the intracellular material plus glucose, and $\bar{\nu}$ is the bulk viscous modulus.

Table VII summarises values obtained for elastic and viscous moduli at varying temperatures ± glucose. It was not possible to observe relaxation in cell fragility at elevated temperatures. Further work using an alternative method of measuring fragility is in progress.

Values for membrane visco-elasticity given here agree well with previously published data [22, 23]. Values for the intracellular viscoelastic moduli E_A and ν_A are approximately two orders of magnitude greater than those reported for sea urchin eggs [24].

(C) Proton magnetic resonance studies

In the absence of blood cells the water spectrum of an isotonic solution remained essentially unchanged in the presence of glucose in the concentration range of interest. However, at low temperatures in the presence of blood cells there was a detectable fall in the intensity of the water band when glucose was added (Fig. 15). At 20 °C this was accompanied by the appearance of a broad band shifted slightly to lowfield (Fig. 16), but at 0 °C no such band could be detected. At elevated temperatures (35 °C)

TABLE VII

Summary of calculated values for the viscoelastic moduli E_B, ν_B, E_A and ν_A at 0, 20 and 35 °C, 50 mM glucose. The range of values obtained is due to the non-linearity of cellular volume changes with external tonicity following sphering (Fig. 2).

Temperature (° C)	Zero glucose (E_A, ν_A negligible)			+50 mM glucose (E_B, ν_B unchanged)		
	E_B (nm^{-2})	$1/T$ (min^{-1})	ν_B ($nm^{-2} \cdot s^{-1}$)	E_A (nm^{-2})	$1/T$ (min^{-1})	ν_A ($nm^{-2} \cdot s^{-1}$)
0	$2.60 \cdot 10^5$–$1.01 \cdot 10^7$	0.01707	$9.0 \cdot 10^7$–$3.54 \cdot 10^9$	$1.2 \cdot 10^5$–$3.3 \cdot 10^5$	0.01528	$4.8 \cdot 10^7$–$1.32 \cdot 10^8$
20	$2.89 \cdot 10^6$–$1.72 \cdot 10^7$	0.03550	$5.4 \cdot 10^8$–$3.08 \cdot 10^9$	$1.1 \cdot 10^4$–$3.3 \cdot 10^4$	0.01054	$1.8 \cdot 10^7$–$9.00 \cdot 10^7$
35	$4.35 \cdot 10^6$–$2.12 \cdot 10^7$	no estimate	–	$8.8 \cdot 10^3$–$2.5 \cdot 10^4$	no estimate	–

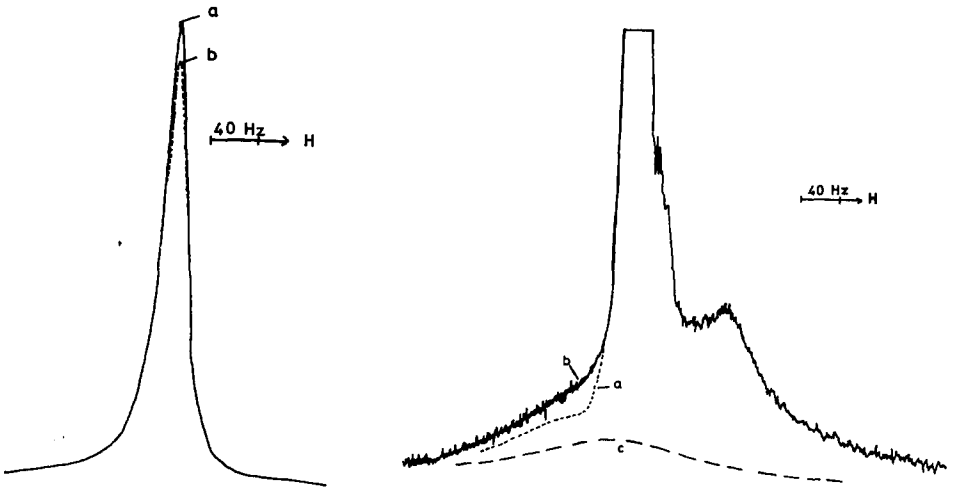


Fig. 15. Proton magnetic resonance spectrum for blood cell suspension at 20 °C at low instrument gain, (a) in the absence of glucose, (b) after the addition of glucose (200 mM). Haematocrit 90 %.

Fig. 16. As in Fig. 15, but at high gain, (a) no glucose, (b) +glucose, (c) difference curve.

this fall was greatly reduced, and no extra broad band could be detected.

The fall in the main water band corresponds, approximately, to a "loss" of ten water molecules per glucose molecule. We postulate that these water molecules are retained for relatively long times in an environment in which the water molecules are strongly hydrogen-bonded (causing the down-field shift) and relatively slowly rotating, thus causing a width enhancement. At 0 °C this band is so broad that it becomes lost in noise under the broad lines caused by organic protons. However, on warming this band narrows, becoming detectable in the room-temperature region. On further warming, the rate of interchange between bulk and bound water increases to the point

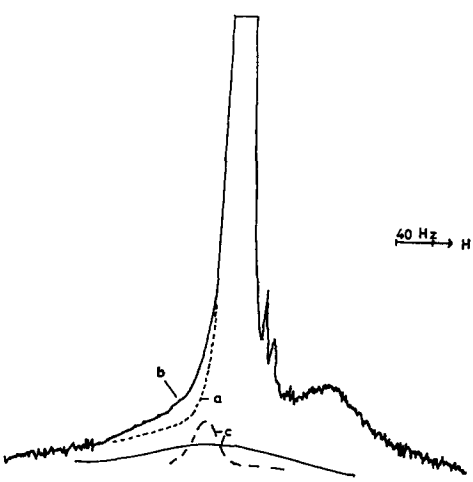


Fig. 17. As in Fig. 16, but with the cells replaced by membrane free haemoglobin, concentration 35 mg · ml^{-1}.

at which an average band is detected. When cell and membrane-free haemoglobin solutions were used, the same type of result was obtained, except that it seemed to be necessary to invoke the presence of two additional bands to obtain a good fit for the different spectra (Fig. 17). No changes were apparent on addition of glucose to haemoglobin-free membranes.

These results must mean that the glucose facilitates binding between intracellular organic material and water, forming a long-lived, slowly tumbling complex involving all three components. It is hoped that relaxation studies will help to ellucidate these effects further.

Other experiments have shown that the fall in height and increase in width of the main water band also takes place when D-glucose is added to solutions containing bovine serum albumin, dextran and particularly gelatin. These effects are dependent on the protein and sugar concentrations and also critically dependent on temperature (in preparation).

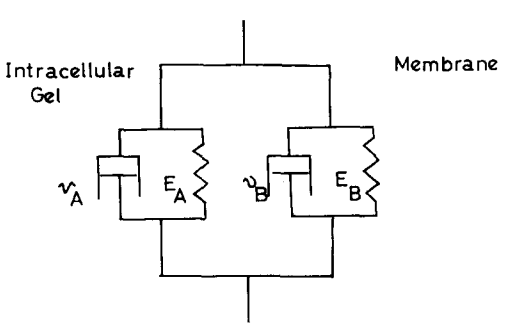
DISCUSSION

The results detailed in the previous section show that the increase in red cell stability arising from interaction with D-glucose is not due either to loss of internal K^+ or to a change in cell membrane area.

Glucose-dependent stabilization is an approximately linear function of concentration and increases on cooling from 37 °C. In addition to increasing the equilibrium stability glucose also increases cell bulk viscosity.

From the proton magnetic resonance studies of red cell suspensions it may be deduced that at 25 °C and below there is an increased amount of water immobilised within the cell by the cell protein. The extent of this increase is directly related to glucose concentration. At 37 °C no difference is observed between the PMR spectrum of cell suspensions containing glucose in the concentration range 0–200 mM. Similar findings to these have been made recently with PMR studies of water binding to starch [25]. In the temperature range -40 to $+20$ °C, water is immobilized due to multilayer adsorption to the glucose-OH groups of the starch.

PMR studies of scl-gel transformation in agar solutions show that the abrupt change in the stability of the solution at the critical temperature is accompanied by a small increase in the fraction of immobilized water surrounding the agar fibres. The change in gel stability is a consequence of the immobilization of the fibre network which encloses large volumes of freely exchangeable and mobile water [26].



A similar mechanism may give rise to glucose-dependent stabilization when glucose is adsorbed to the intracellular protein matrix and water is immobilised by multilayer adsorption to the glucose–protein complex. This hypothesis accounts for the critical sensitivity of the stabilization to temperature.

An approximate analogue of the mechanical behaviour of the cell is shown in the above diagram. The cell membrane may be viewed as a viscoelastic body. The elastic modulus of the membrane is calculated from the relationship between cell volume and external tonicity. The membrane viscosity is calculated from the relaxation of fragility. It is assumed that in the absence of D-glucose the intracellular components make a negligible contribution to the bulk stability of the cell. It is noted that the membrane strength decreases on cooling from 37 °C whereas membrane viscosity rises. A possible explanation of the loss of membrane strength due to cooling is that following phase changes in the membrane lipid the membrane fluidity decreases and it becomes brittle. An identical Voigt body analogue has been used previously to describe the mechanical behaviour of red cells to hypotonic stress [22]. The calculated value of the membrane elastic modulus and viscosity in this paper are similar to those found by Kalchalsky et al. [22]. The more complex model proposed by Rand [23] in which an additional spring and dashpot are added in series to the parallel spring and dashpot array is inappropriate as this model predicts that the equilibrium pressure across the membrane should always fall to zero. The findings reported here exclude this. In contrast to the effect of cooling on membrane stability in glucose-free solutions, addition of glucose results in an increase in cell stability on cooling. This, taken together with the direct evidence from PMR of glucose-haemoglobin interaction suggests that the site of glucose-dependent stabilization is other than the membrane, hence the second Voigt body in parallel to the membrane array. Hiramoto [24] has measured directly the viscoelasticity of sea urchin egg cytoplasm and found that it increases considerably during spindle formation following fertilization. The elastic modulus of glucose stabilized red cell cytoplasm is calculated to be approximately two orders of magnitude greater than that of sea urchin eggs.

It is clear that stabilization by promotion of intracellular gel formation can only occur with sugars which actually penetrate the cell membranes. Sucrose, and other higher molecular weight cell stabilizers [22], do not easily penetrate cells so it is likely that another mechanism by which sugars may stabilize the cell membrane exists and it is possible that D-fructose stabilizes by this alternative mechanism, since the temperature and concentration dependence of cell stabilization by fructose differs from that of D-glucose.

The stereo-specificity of sugar-dependent stabilization is different from the specificity of sugars for their membrane transport sites [27]. Fructose, sorbose, xylose and ribose are all good stabilizers, yet have lower operational affinities for the transport binding sites than galactose and mannose, which do not stabilize the cells. Additionally there is no evidence of any kind for saturation of the stabilization process which would be expected if the sugars were bound exclusively to the transport site. Nevertheless, the formation of a stable intracellular gel may be of some importance in the interpretation of the asymmetric operational sugar flux parameters, particularly kinetic analysis using the integrated rate equation for efflux from cells loaded with high concentrations of D-glucose, since the real cell volume changes due to osmotic pressure variation at 20 °C will fall short of the calculated changes assuming ideal solution

properties for the sugar present in the intracellular water.

The increase in cell stability following gelation of the intracellular proteins may also account for the increased cell resistance to hypertonic [2] and shear [4] stress in the presence of glucose.

A possible mechanism whereby sugars and other polyhydric solutes at high concentration protect cells from freeze damage [3] is by formation of a stable gel (glass) within the cells, thus preventing cell dehydration [28], which causes membrane damage due to accumulation of higher intracellular ionic concentrations. The results described in the paper on the effects of cooling cell suspension at temperatures above freezing lend support to this view.

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APPENDIX (Received November 28th, 1973)

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The analysis is concerned with the deformation of the cell from the time when it first achieves a spherical shape to final rupture of the cell wall. The cell is modelled as a spherical core of radius r and composed of visco-elastic material A, enclosed by the membrane of thickness d and composed of visco-elastic material B.

The spherical symmetry of the model implies that the analysis may be conducted in terms of the proportional increase in volume of the core $\delta v/v$ and the pressure p which the core exerts upon the bounding skin.

At time $t > 0$ the radial pressure p may be idealized as arising from two parts:

$$p(t) = -p_1(t) + p_{os}(t) \tag{1}$$

where p_{os} denotes the osmotic pressure within the core and p_1 the restraining pressure of the core material.

Both the materials A and B are modelled as linear visco-elastic materials of the Voigt type [1]. Thus within the core the relationship between the volume increases and the restraining pressure p_1 is given by:

$$p_1 = E_A \frac{\delta v}{v} + v_A \frac{\delta \dot{v}}{v} \qquad \delta \dot{v} = \frac{\mathrm{d}}{\mathrm{dt}} \delta v \tag{2}$$

where E_A and v_A are elastic and viscous moduli respectively of the bulk behaviour of the material. The shear behaviour of the core material A does not enter into the discussion.

The appropriate relationship for the membrane material may be best derived by first considering purely elastic behaviour. The membrane is subject to a uniform biaxial state of stress with components σ giving rise to strains ε in the surface of the membrane.

Consideration of the equilibrium of one half of the spherical membrane (Fig. A1) gives rise to the relationship:

$$p\pi r^2 = 2\pi r d\sigma$$

i.e.

$$\sigma = \frac{pr}{2d}$$

and hence the strains are given by:

$$\varepsilon = \frac{(1-\rho)}{E_B}\sigma$$

in all directions, where E_B denotes Young's Modulus and ρ Poisson's ratio.

The relationship between the increase in volume and p may now be obtained

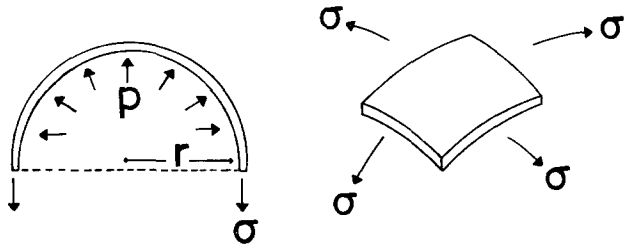

Fig. A1

by equating the work done by the pressure p to the increase in elastic strain energy stored within the membrane:

$$\tfrac{1}{2}p\frac{\delta v}{v} = (2 \cdot \tfrac{1}{2}\sigma\varepsilon \cdot 4\pi r^2 d)/v$$

Hence the following relationship is obtained:

$$\frac{\delta v}{v} = \frac{3}{4}\frac{r}{d}\frac{p}{E_B} \tag{3}$$

where elastic incompressibility has been assumed ($\rho = 1/2$).

Eqn 3 may now be generalized to a Voigt material, yielding:

$$p = \frac{4}{3}\frac{d}{r}\left(E_B\frac{\delta v}{v} + \nu_B\frac{\delta \dot{v}}{v}\right) \tag{4}$$

where ν_B denotes a viscosity coefficient.

The relationship between the osmotic pressure p_{os} and the increase in volume may now be obtained from Eqns 1, 2 and 4:

$$p_{os}(t) = \left(E_A + \frac{4}{3}\frac{d}{r}E_B\right)\frac{\delta v}{v} + \left(\nu_A + \frac{4}{3}\frac{d}{r}\nu_B\right)\frac{\delta \dot{v}}{v} \tag{5}$$

Eqn 5 may be solved for:

$$p_{os}(t) = p_{os}H(t)$$

where $H(t)$ denotes the Heaviside step function;

$$H(t) = 0 \qquad t < 0$$
$$= 1 \qquad t \geqslant 0$$

to yield the solution;

$$\frac{\delta v_{(t)}}{v} = \frac{\delta v_{(\infty)}}{v} \cdot (1 - e^{-t/\tau}) \tag{6}$$

where;

$$\frac{\delta v_{(\infty)}}{v} = p_{os} \Big/ \left(E_A + E_B\frac{4d}{3r}\right)$$

and the characteristic time τ is given by;

$$\tau = \frac{\nu_A + \frac{4}{3}\frac{d}{r}\nu_B}{E_A + \frac{4}{3}\frac{d}{r}E_B} \tag{7}$$

The pressure on the membrane $p(t)$ may now be obtained from Eqn 4;

$$p(t) = p(\infty) + ((p(0) - p(\infty))e^{-t/\tau})$$

where

$$p(\infty) = \frac{\delta v_{(\infty)}}{v}\frac{4}{3}\frac{d}{r}E_B$$

and

$$p(0) = \frac{\delta v_{(\infty)}}{v}\frac{\nu_B}{\tau}\frac{4}{3}\frac{d}{r}$$

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