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MEMBRANE AND INTRACELLULAR MODES OF SUGAR-DEPENDENT INCREMENTS IN RED CELL STABILITY

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SUMMARY

Sugar-dependent increments in red cell stability under osmotic stress can be ascribed to changes either in the membrane or in the intracellular matrix. These two possible modes of action have been tested and characterized.

Rheological investigation of membrane-free haemoglobin solutions has shown that D-glucose, but not D-fructose, promotes the formation of a visco-plastic gel structure. Gel strength is a function of glucose concentration, haemoglobin concentration and temperature. The ability of various sugars to promote gel formation correlates with their solution properties. The existence of gel structure reduces K^+ and haemoglobin leak from red cells whose membranes were partially destroyed by γ-radiation. Reduced osmotic swelling in the presence of glucose is also due to gel formation since the glucose effect is lost in resealed red cell ghosts.

D-Fructose does not protect red cells against radiation damage; its mode of action in increasing red cell stability under osmotic stress is a membrane effect. Cell sizing using the Coulter Counter has shown that fructose, but not glucose, can increase the maximal volume at lysis. At 50 mM, D-fructose expands the red cell ghost volume by 11.2 %; this represents a 7.2 % increase in membrane area. Ghost expansion by fructose is fructose concentration dependent (0–100 mM) and is insensitive to temperature variation (0–37 °C).

INTRODUCTION

Recent proton magnetic resonance studies suggest that D-glucose stabilizes human red cells against osmotic stress by promoting intracellular protein gel formation. When glucose binds to haemoglobin a ternary complex between water, haemoglobin and glucose is formed; this complex is temperature sensitive. This finding correlates with the observation that, on raising temperature above 20 °C, glucose-dependent stabilization of red-cells is lost [1].

When added to gelatin solutions D-glucose promotes gel formation by com-

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plexing with water bound to the protein [2]. Studies on the solution properties of D-glucose suggest that there is a stereospecific interaction between D-glucose and water. This may arise because of the coincidence between the OH spacing of D-glucose and the H-bonding sites of 'bulk' water. It was suggested that D-glucose can shift the conformational equilibrium between the 'dense' and 'bulk' conformations of water [3, 4]. Sugars such as fructose and ribose, which do not have similar OH spacing to D-glucose, do not stabilize bulk water. D-Fructose has no tendency either to promote gelatin gel formation or to form a complex with haemoglobin solutions. It seems likely, therefore, that the gel-promoting effects of glucose are related to the solution properties of this sugar.

Despite the fact that fructose does not promote gel formation, it too can stabilize red cells against osmotic stress. However, this stabilization differs from that seen with glucose, since it is insensitive to changes in temperature in the range 0–37 °C. It was previously suggested [1] that another stabilization mechanism exists; possibly fructose, like certain hydrophobic drugs and also some inorganic ions [5, 6], stabilizes the cells by increasing the cell membrane area and thus increasing the critical haemolytic volume.

These two hypotheses for increased osmotic stability can be experimentally tested. Possible structuring of the red cell intracellular matrix by sugar can be deduced from the rheological behaviour of membrane-free haemoglobin solutions [7] whilst membrane expansion at lysis can be detected by electronic particle sizing using the Coulter Counter. A comparison of whole cell swelling with ghost swelling, using the Coulter Counter, can also demonstrate the importance of the sugar-haemoglobin interaction to red cell stability, since ghosts should not show effects that are ascribed to the presence of intracellular haemoglobin.

An additional means of discriminating between the surface and intracellular modes of sugar-dependent cell stabilization is to determine whether sugars stabilize the cells against radiation damage. γ-radiation is known to alter the permeability properties of human red cell membranes. With low radiation doses intracellular K^+ loss occurs, and with larger doses haemoglobin loss is apparant [8, 9]. Structuring of the intracellular matrix would retard the expulsion of the diffusible intracellular materials, whereas a stabilizing agent acting exclusively at the cell membrane would not prevent loss of the intracellular content following radiation damage.

MATERIALS AND METHODS

All the materials used were of Analar grade. 2,3-Diphosphoglycerate was added as the pentacyclohexylammonium salt (Sigma Chemical Co.). β-Glucose was prepared from α-glucose by the method of Mangam and Acree [10].

Red cell preparation

Human red cells, obtained from outdated blood transfusion stock (by courtesy of Sheffield Regional Hospital Board) were washed by repeated centrifugation and resuspension in 150 mM NaCl with 10 mM Tris · Cl (pH 7.4) until the cells were free of glucose. Cells were loaded with sugar, where appropriate, by incubation for periods sufficient to allow total equilibration [1]. Since the effects of sugars on red cell osmotic fragility are observed in both fresh and cold-stored acid/citrate/dextrose blood

(Simmons, N. L. and Naftalin, R. J., unpublished results) the routine use of citrated blood does not introduce artefacts.

Preparation of membrane-free haemoglobin solutions

Washed glucose-free packed red cells were disintegrated in an M.S.E. ultrasonic disintegrator (maximum power for 30 s). Membrane fragments were removed by either (a) centrifuging the preparation at $100\,000 \times g$ for 120 min at 4 °C or (b) extracting first in 20 % v/v toluene for 14 h at 4 °C and then centrifuging at $100\,000 \times g$ for 60 min at 4 °C. The membrane-free haemoglobin was dialysed overnight at 4 °C against 150 mM NaCl (pH 7.4) to remove organic phosphates [12]. There was no significant difference in behaviour on addition of sugar between haemoglobin solutions prepared by centrifugation alone and those prepared by adding toluene and centrifuging. The concentration of haemoglobin was determined from the $A_{540\,nm}$ of diluted samples using the extinction coefficients as given by Benesch et al. [13].

Membrane preparation

Haemoglobin-free membranes were prepared using the method of Dodge et al. [14]. Washed packed red cells were lysed in 10 times their own volume of ice-cold 5 mM EDTA buffered with Tris · Cl to pH 7.4. The membranes were spun out of solution by centrifuging at $100\,000 \times g$ for 20 min at 4 °C. 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 haemoglobin. Membranes were then freeze-dried and stored at −20 °C.

Preparation of resealed red cell ghosts

Once washed membranes prepared as described above were resealed in isotonic saline (150 mM NaCl, 5 mM ATP Tris-buffered, pH 7.4) for 2 h at 37 °C. Where appropriate, glucose of the required concentration was included in the resealing medium.

Determination of the shear behaviour of haemoglobin solutions with and without sugar

The cone-plate viscometer (Brookfield Laboratories) allows the application of a range of shear rates to a solution contained between the plate and cone of the instrument [15]. The shear stress developed is constant throughout the sample, and is directly related to the torque on the cone (which is measured). Since the cone constants are known the absolute values for the shear stress can be calculated. The η (viscosity) of the solution is then, by definition, equal to shear stress/shear rate.

For a simple Newtonian fluid, η is independent of shear rate. The development of structure within a fluid, synonomous with gel formation, will be characterized by visco-plastic (visco-elastic) behaviour. Three criteria for visco-plasticity are (1) shear dependence of η (shear thinning), (2) non-zero intercept of the stress/shear rate relationship (i.e. a finite yield stress), and (3) dependence of shear stress on shear history (hysteresis in the shear stress/shear rate relationship.)

2 cm^3 of haemoglobin solution were placed in the cup (plate) and subjected to a range of shear values, 3 torque readings being recorded for each shear rate.

The temperature of the plate/cone was maintained to ± 0.1 °C by forced circulation of thermostated water through conduits in the cup structure.

Procedure for determining red cell and ghost volumes during osmotically induced swelling

Red cell volumes were measured with a Coulter Counter (Model Fn) fitted with a 100 μm orifice tube. Electronic cell sizing was performed by a 100 channel pulse-height analyser (Channelyser Coulter Electronics Ltd.). Absolute calibration was achieved using latex beads of known diameter and hardened red cells (4C cells Coulter Diagnostics Inc.). The output of the pulse-height analyser was monitored by an X-Y recorder.

In order to maintain the conductivity of solutions at low tonicities, all solutions contained 0.1 M NH_4Cl [1]. Prior to use all solutions were filtered through a 0.45 μm diameter millipore filter to remove dust.

Cells were pipetted into solutions of varying tonicity to give a final cell concentration of approx. 20 000 cells/cm^3. Since NH_4Cl freely penetrates the red cell membrane, and as equilibrated sugars do not contribute to the osmotic pressure gradient, the effective tonicities of the solutions are determined only by their [NaCl]. After mixing, cell sizing was immediately initiated. Sizing stopped when the modal channel accumulated a pre-set number of cells ($<$ 1 min after mixing).

Mean cell volumes ($\pm$ S.D.) were calculated from the size histograms taking each interval as a grouped measure of X. In order to conform to the observed fructose-dependent cell stabilization, membrane expansion, if observed, has to be temperature insensitive [1]. It was therefore necessary to determine the effect of temperature variation (0–37 °C) on the accuracy of the volume measured. To this end, the aperture resistance in 0.1 M NH_4Cl was determined as a function of the temperature. The variation in aperture resistence affects aperture current by $< 1\%$. Additionally, latex beads of known diameter showed $< 1\%$ variation in measured 'volume' in 0.1 M NH_4Cl over the temperature range of interest.

Procedure for radiation damage experiments

Following equilibration of sugars, red cell suspensions were adjusted to 10% haemotocrit and exposed for varying periods to a ^{60}Co radiation source giving 90 krad $\cdot$ min^{-1}. Following irradiation the cells were held for a standard 20 min period at room temperature before centrifugation and removal of the supernatant (preincubation) fluid. The fragility of the cells was determined using the Probit analysis method as previously described at 22 °C [1]. When the cell fragility was measured in media containing different sugar concentrations from those in the preincubation conditions, the cells were incubated for up to 1 h in an intermediate isotonic saline solution containing the required [sugar] before transfer to the hypotonic sugar-saline solutions for fragility determination.

Phase transition temperature of membrane lipids

The method described by Zimmer and Schirmer [18] was used. 50 mg of freeze-dried membranes were dissolved in 1 cm^3 150 mM NaCl buffer by homogenization followed by sonication. Viscosity measurements at constant shear were made using the Brookfield Viscometer. Membrane solutions were Newtonian. Viscosity measurements were made at 0.5 °C intervals over the required temperature range.

RESULTS

(1) Evidence for intracellular haemoglobin gel formation

Rheological properties of membrane-free haemoglobin solutions. When haemoglobin is added to saline, the shear stress/shear strain relationship becomes curved towards the stress axis at the lowest values of shear rate (Fig. 1A). At higher values of shear rate pronounced shear-thinning of the solution occurs (Figs. 1A and 1B) and at shear rates above 15 s^{-1} the haemoglobin solution behaves as a simple Newtonian fluid.

Addition of 100 mM D-glucose to the haemoglobin solutions results in an exaggeration of the observed non-Newtonian behaviour at low shear rates. The curvilinearity of the stress/shear rate relationship is such that when extrapolated to zero shear there is a significant positive intercept on the stress axis, indicating that before the solution is set in motion by the viscometer a yield stress must be overcome. Additionally, when glucose is present, hysteresis is consistently noted on returning to

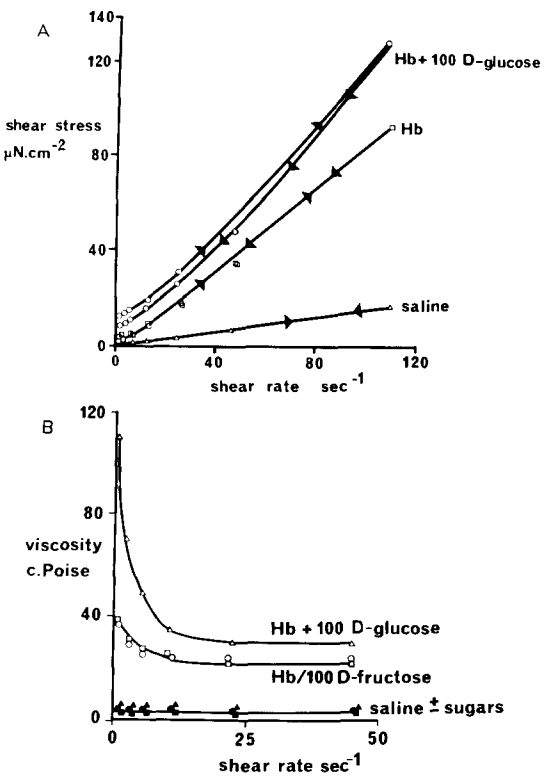

Fig. 1. (A) Shear stress/shear rate diagram. Points represent single determinations of torque after 15 s of shear. Arrows indicate the direction of variation of shear rate, (Hb) = 22 %, w/v. Temperature = 4 °C. (B) Viscosity (η) is plotted against shear rate. Three determinations per point are made (15 s of shear per point). The values are the means for each determination. The determinations are made from low to high shear rates [Hb] = 23.4 %, w/v. Temperature = 4 °C. (□) Haemoglobin solution; (○) haemoglobin+100 mM fructose; (△) haemoglobin+100 mM glucose; (■) saline; (●) 100 mM fructose in saline; (▲) 100 mM glucose in saline.

low shear rates after subjection of the solution to high shear rates (Fig. 1A).

Confirmation that the haemoglobin solutions with glucose have a finite yield stress comes from observing that following shear the indicator of the torque meter does not return to zero (Stiction). The magnitude of the stress measured in these conditions, approximating to zero shear rates, is virtually identical to the yield stress obtained from the extrapolated stress/shear rate diagram (i.e. 7.5 $\mu N \cdot cm^{-2}$).

In contrast to the effects of D-glucose, addition of D-fructose at 100 mM has no effect upon the rheological behaviour of haemoglobin solutions (Fig. 1B).

The data shown in Figs 1A and 1B indicate that D-glucose induces the formation of a gel when added to haemoglobin solutions, since three criteria for viscoplasticity (finite yield stress, shear thinning and hysteresis) are satisfied. It may be deduced that gel formation by glucose must involve the reversible interaction between haemoglobin molecules. As previously suggested [1] this may be mediated through a ternary glucose-water-haemoglobin complex.

Characterization of the haemoglobin-sugar interaction using the Brookfield viscometer. (i) D-Glucose concentration dependence. Glucose-dependent reduction in red cell osmotic fragility is a linear function of glucose concentration in the concentration range 0–150 mM [1]. Additionally, glucose stabilizes gelatin gels as a linear function of glucose concentration [2]. Fig. 2 shows that the viscosity changes due to the addition of sugar to haemoglobin are a linear function of glucose concentration in the range 0–150 mM at both high (114.5 s^{-1}) and low (2.29 s^{-1}) values of shear rate.

(ii) Haemoglobin concentration dependence. Since the proposed mechanism for gel formation [1, 2] invokes a ternary protein-glucose-water complex, the formation of the gel will be critically related to the protein concentration. In the erythrocyte, the haemoglobin concentration is very high (34 % w/v) [19]. At this concentration the centre to centre distance for haemoglobin molecules is not much greater than their sphere of rotation [20].

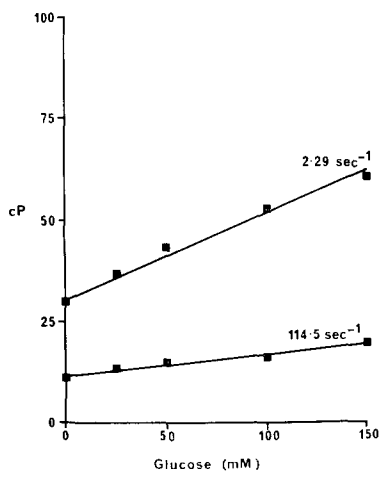

Fig. 2. The effects of variation of glucose concentration on the viscosity of haemoglobin solutions 21 % w/v measured at 4 °C. The viscosity was measured at high (114.5 s^{-1}) and low (2.29 s^{-1}) shear rates.

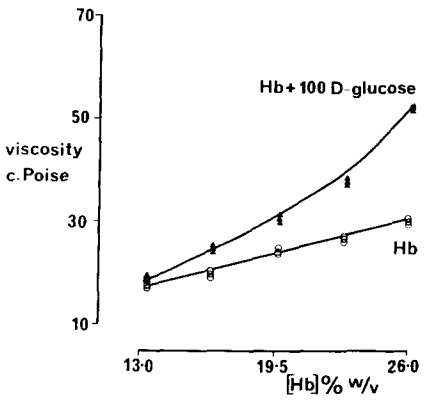

Fig. 3. Haemoglobin concentration dependence for haemoglobin alone (○) and haemoglobin+100 mM D-glucose (▲) shear rate 5.73 s^{-1}. Temperature = 4 °C.

Fig. 3 shows the dependence of viscosity on [Hb] for haemoglobin alone and haemoglobin+100 mM D-glucose, 4 °C. The most concentrated solution of haemoglobin used was 26 % w/v haemoglobin.

Haemoglobin viscosity is not directly related to [Hb] since the reduced viscosity (viscosity/concentration) is slightly greater at 26 % w/v haemoglobin than at 13 % w/v. It is probable that haemoglobin viscosity has a second order relationship with [Hb]. The increment in reduced viscosity on raising [Hb] is very much greater in the presence of glucose, indicating enhanced protein-protein interaction.

(iii) Temperature sensitivity. The glucose-haemoglobin interaction is temperature sensitive [1] Fig. 4a shows the temperature dependence of viscosity for haemoglobin and haemoglobin+100 mM glucose. The glucose-dependent increment in viscosity decreases exponentially as the temperature is raised (4 to 37 °C). These data are

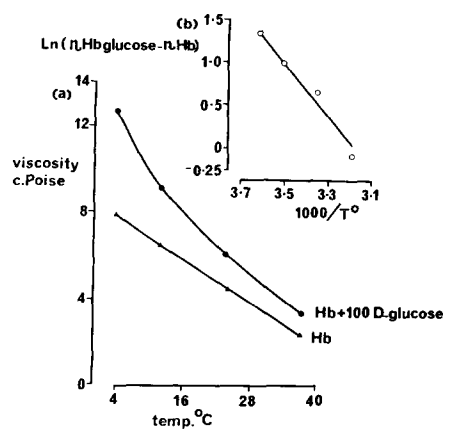

Fig. 4. (a) Temperature sensitivity of viscosity at a shear rate of 22.93 s^{-1} for haemoglobin (▲) and haemoglobin+100 mM D-glucose (●). [Hb] = 21.5 % w/v. Each point represents the mean of three determinations. Standard deviations lie within the points. (b) (insert) 'Arrhenius' plot for the data of (a). Ln (ηHb 100 mM glucose − ηHb) is plotted against $1000/T^{0}$.

TABLE I

The viscosity increments in haemoglobin solutions, 23 % w/v, are normalized around the increment observed with 100 mM α-D-glucose measured at 4 °C at a shear rate of 2.29 s^{-1}. All measurements were taken 3 min after addition of the sugar to the haemoglobin solutions.

Sugar	Normalized viscosity increment (%)
100 mM α-D-glucose	100
100 mM methyl β-D-glucose	71
100 mM β-D-glucose	134
100 mM 2 deoxy-D-glucose	101
100 mM D-galactose	64
100 mM L-sorbose	86
100 mM D-fructose	2.4
100 mM D-ribose	30
100 mM D-xylose	47.5
100 mM sucrose	27.8

plotted according to an empirical 'Arrhenius' equation in Fig. 4b. The activation energy is 16.61 kcal/mol.

(iv) Specificity. Various sugars protect against osmotic lysis [1]. Those with high temperature coefficients for protection can be thought to stabilize exclusively by promoting gel formation, i.e. D-glucose and L-sorbose. Table I shows the relative viscosity increments observed with various sugars at 100 mM at 4 °C and a shear rate of 2.29 s^{-1}. L-Sorbose and D-glucose have similar affinities for haemoglobin. It is of particular interest that D-fructose and D-ribose should have a low affinity for gel formation, since neither interact with (bulk) water [3, 4]. D-Xylose has an intermediate position. These results correlate with the observed temperature sensitivity for protection of these sugars. Sucrose has a low affinity for haemoglobin gel formation, which differs from its effect in stabilizing gelatin gels [2]. In contrast to the observed effects on fragility, galactose has a relatively high affinity for haemoglobin gel formation. This anomolous finding is the subject of further investigation.

Haemoglobin binds β-D-glucose preferentially to the α-isomer; mutarotation subsequently abolishes the increment compared to α-glucose. The affinity of 2-deoxyglucose is no different from that of D-glucose alone. β-methylglucose has a reduced affinity compared to glucose.

Effect of modifiers of haemoglobin structure on the glucose-haemoglobin interaction. (i) Oxygenation/deoxygenation. Haemoglobin exists in 2 structural tertiary forms, namely the oxy and deoxy forms [21]. These two forms are in thermodynamic equilibrium [22]. Deoxygenation results in an open structure stabilized by salt linkages. Recent dilatometric measurements indicate a positive volume change on deoxygenation [23]. Oxygenation results in the breakage of the salt linkages, thus destabilizing the deoxy structure in favour of the oxy form. Human oxyhaemoglobin is more soluble than deoxyhameoglobin, indicating an enhanced haemoglobin-water interaction.

Table II shows that either gassing with N_2 or addition of sodium dithionite virtually abolish the glucose-haemoglobin interaction. Deoxyhaemoglobin solution is more viscous than oxyhaemoglobin, in agreement with the dilatometric measurements. Conversely, gassing with 100 % O_2 significantly increases the glucose-depen-

TABLE II

EFFECT OF VARIOUS MODIFIERS OF HAEMOGLOBIN TERTIARY STRUCTURE ON THE GLUCOSE DEPENDENT INCREMENT IN VISCOSITY OF HAEMOGLOBIN SOLUTIONS

	Viscosity (cP) at 2.29 s^{-1} ±S.D.	Ratio (Hb+glucose)/Hb
Hb	26.03±0.30	2.66
Hb+100 mM glucose	70.43±0.60	
Hb+dithionite (0.2 mM)	53.60±1.01	1.18
Hb+dithonite (0.2 mM)+100 mM glucose	63.40±1.20	
Hb+N_2	97.00±1.06	1.15
Hb+N_2+100 mM glucose	112.00±2.00	
Hb+O_2	27.04±0.58	3.55
Hb+O_2+100 mM glucose	96.70±0.30	
Hb+2.5 mM 2,3-diphosphoglycerate	32.06±0.40	1.42
Hv+2.5 mM 2,3-diphosphoglycerate+ 100 mM glucose	45.60±0.32	

dent increment in viscosity. 2,3-diphosphoglycerate, a substance known to decrease the oxygen saturation of haemoglobin at a given partial pressure of O_2 [24] significantly reduces the glucose effect.

(ii) Effect of urea denaturation. Urea at 5–6 M concentration will disrupt tertiary structure and unfold protein chains [25]. It was of interest, therefore, to observe what effects, if any, there were on the glucose-haemoglobin interaction. Fig. 5 shows that urea has a two-phase effect on haemoglobin solutions. Large increments in viscosity at 5–6 M urea probably indicate protein disaggregation and unfolding. In the presence of 100 mM glucose there is significant protection of the native haemoglobin against denaturation by urea. At 2 M urea the glucose-dependent increment in viscosity is unaffected.

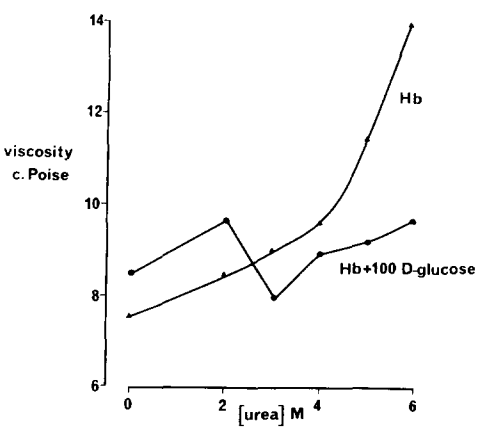

Fig. 5. The effect of urea denaturation on haemoglobin (▲) and haemoglobin+100 mM D-glucose (●). All measurements were made 20 min after addition of glucose/urea. Temperature = 4 °C. Shear rate = 114 s^{-1}. [Hb] = 19.0 % w/v.

(2) *Evidence for a fructose-membrane interaction resulting in an increased haemolytic volume*

Effects of D-*glucose and* D-*fructose on red cell swelling and ghost volumes in hypotonic media.* Decreasing the tonicity of the external solution causes the red cell volume distributions to shift progressively to larger volumes. At the lowest tonicities the cells lyse and become ghosts. As in previous studies [1] no collapse of volume was observed with controls or cells incubated plus sugar as they become ghosts. Ghost volume was also invariant up to 45 min after lysis at 0, 20 and 37 °C. Fig. 6B (insert) shows the volume distribution of cells in isotonic NaCl and in zero NaCl. Isotonic cell volumes of equilibrated cells are identical irrespective of sugar type and concentration in the suspending medium. This confirms that the cells are fully equilibrated with sugar [1]. The ghost volume distribution with 50 mM D-fructose is symmetrically larger than that of control cells. No difference is observed with 50 mM D-glucose compared to control.

Fig 6A shows the mean cell volumes plotted as a function of the external tonicity for control cells and those with 100 mM D-fructose. The mean volume of control cells in isotonic saline is 86.79 $\mu m^3 \pm 14.38$(S.D.). The maximal volume to which the cell population swells is 140 $\mu m^3 \pm 23.6$(S.D.). With 100 mM D-fructose the maximal volume attained is 170.07 $\mu m^3 \pm 30.6$(S.D.). No difference in volume is seen between ghosts in 100 mM D-glucose and control values.

Temperature sensitivity of ghost expansion by D-*fructose.* Fig. 7 shows the effect of variation of the ambient temperature on the maximal volume attained by cell ghosts (zero NaCl). D-Fructose increases ghost volumes at all temperatures in the range 0–37 °C. This increased volume is fructose concentration dependent. The ghost volume of controls increases as the temperature is raised. This result agrees with that of Hoffman et al. [26] who showed that the ratio of cell haemolytic volume to that of

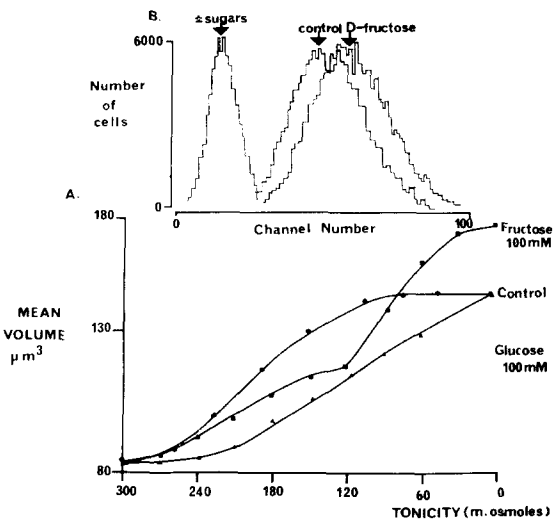

Fig. 6. (A) The isotonic and ghost volume distributions for control cells and cells with 100 mM D-fructose. Volume distributions for cells + 100 mM D-glucose are coincident with control values and are omitted for clarity. (B) The effect of tonicity on mean cell volume for control cells (●); + 100 mM D-fructose (■) and + 100 mM D-glucose (▲).

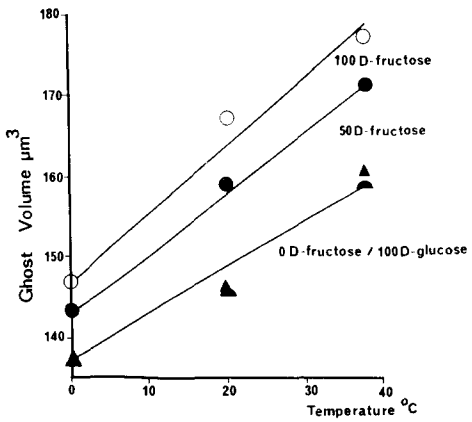

Fig. 7. The effect of temperature on the ghost volume for control cells (▲); cells + 100 mM D-glucose (◓); +50 mM D-fructose (●) and +100 mM D-fructose (○).

cell isotonic volume increased with temperature. It also supports the view that a membrane effect (eg. an increased membrane fluidity)[18] is associated with the reduction in fragility at elevated temperature (vide infra).

Magnitude of the ghost expansion by D-*fructose.* Table III shows that the fructose-dependent increase in ghost volume is linearly related to fructose concentration. Some saturation is evident at 100 mM (see also Fig. 7). The magnitude of the membrane expansion resulting from D-fructose can be compared to that of a neutral anaesthetic, octanol, which is anti-haemolytic and also expands the cell membrane at lysis [5].

Effects of D-*fructose on membrane phase transition as indicated by viscometry.* As reported by Zimmer and Schirmer [18] a kink in the viscosity temperature coefficient is seen at approx. 18 °C with a sonicated erythrocyte membrane suspension (Fig. 8). D-Fructose at a concentration of 50 mM shifts this turning point to lower temperatures. Glucose at an equivalent concentration has no effect. This observation

TABLE III

Comparative effects of D-fructose and octanol on membrane expansion. Figures in parentheses represent data from published work [5].

	Isotonic volume μm^3 $\pm$S.D. μm^3	Ghost volume μm^3 $\pm$S.D. μm^3	% increase in ghost volume	% increase in membrane area
Control	86.59 $\pm$ 14.38	142.04 $\pm$ 27.24 (145)	–	–
Fructose 50 mM	84.74 $\pm$ 13.85	158.92 $\pm$ 34.60	11.21	7.19
Fructose 100 mM	85.17 $\pm$ 13.83	174.27 $\pm$ 35.01	21.90	14.35
Octanol 1 mM (0.74 mM)	84.97 $\pm$ 16.69	177.60 $\pm$ 37.40 (165)	24.71 (13.80)	15.96 (9.37)

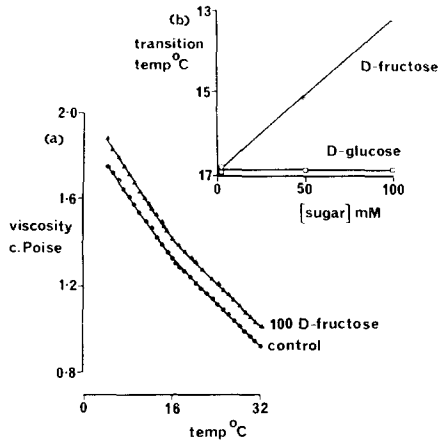

Fig. 8. (a) Viscosity of a solution of sonicated erythrocyte membranes as a function of temperature for control (●) and plus 50 mM D-fructose (▲). (b) Membrane phase transition temperature is plotted against sugar concentration for glucose (□) and fructose (○).

is consistent with the localization of the fructose effect to the membrane and indicates that its mode of action may be associated with increased membrane fluidity. D-Fructose decreases the membrane phase transition as a linear function of concentration (Fig. 8b). D-Glucose has no comparable effect within the equivalent concentration range.

(3) *Additional evidence discriminating between the membrane and intracellular modes of sugar-dependent stabilization*

Red cell swelling in the presence of D-*glucose correlated with intracellular gel formation.* There are clear differences in red cell swelling in control media, media with 100 mM fructose and media with 100 mM glucose. (Fig. 6B). In the presence of D-fructose, swelling occurs in two phases. Although the maximal volume to which cells can swell is dependent on D-fructose concentration, no dependence of swelling at intermediate volumes on fructose concentration is observed. In the presence of glucose, cells do not swell as much as controls until the lowest tonicities. This reduction in swelling is D-glucose concentration dependent. This is shown in Fig. 9A which shows cell swelling at 0, 50 and 100 mM glucose plotted according to the Boyle van't Hoff law (i.e. $\pi(V-b) =$ constant) [27]. Since glucose is fully equilibrated and there is no excess K^+ loss from cells incubated in the presence of glucose [1], this result shows that a stabilizing force acting within the cells prevents the cells from swelling to nullify the osmotic pressure gradient across the membrane, i.e. this is evidence for structure formation within the cells. Similar findings have been reported for frog oocytes [34, 35].

Removal of haemoglobin from red cells should abolish the glucose-dependent decrease in swelling. Resealed red cell ghosts containing less than 5 % of their original haemoglobin were swollen in the presence of glucose. The resulting size distribution were monitored with the Coulter Counter. Fig. 9B shows the ghost swelling data plotted according to the Boyle van't Hoff relationship. No glucose-dependent decrease in ghost swelling is observed.

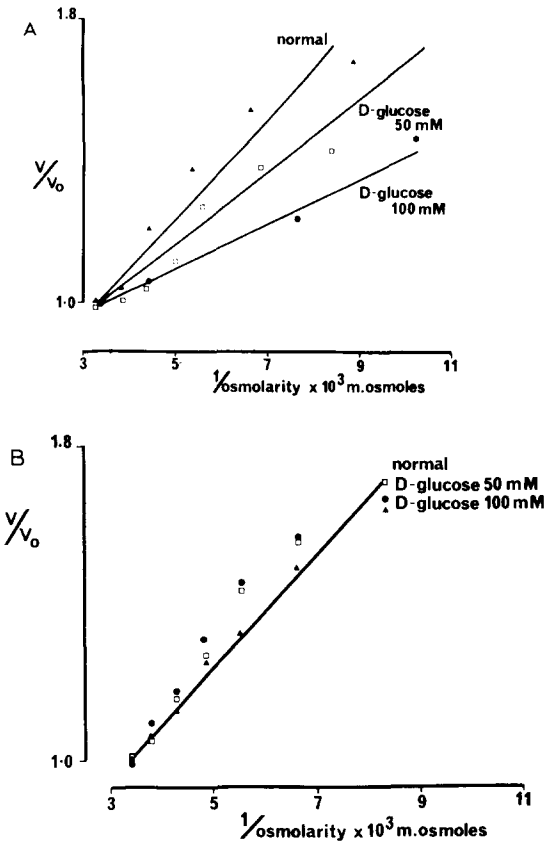

Fig. 9. (A) The effect of tonicity on mean cell volume plotted according to the Boyle van 't Hoff relationship. Volume is expressed relative to isotonic. Control cells (▲); +50 mM D-glucose (□); +100 mM D-glucose (●). (B) The effect of tonicity on mean volume in resealed ghosts. Volume is expressed relative to isotonic. (□) control; (●) +50 mM D-glucose; (▲)+100 mM glucose.

Effects of sugars on radiation-induced release of haemoglobin and K^+. The effects of radiation on the release of intracellular K^+ and haemoglobin from human red cells suspended in isotonic saline at 22 °C are shown in Fig. 10. Following radiation doses in the range 0.2–0.5 Mrad most of the intracellular K^+ is released from control suspensions. K^+ loss during the first 20 min period following irradiation is retarded if 100 mM D-glucose is present in the suspension medium ($P < 0.001$). 100 mM D-fructose or sucrose added to the suspension does not retard K^+ loss ($P > 0.8$).

The radiation dose required to release 50 % of the cell haemoglobin within the 20 min post-irradiation period is approximately 5 times greater than that required to release 50 % of the cell K^+.

With 100 mM glucose present in the cell, haemoglobin loss is significantly retarded following a radiation dose in the range 1–1.5 Mrad ($P < 0.001$). Neither fructose nor sucrose retarded haemoglobin loss ($P > 0.8$).

The large difference between the radiation doses required to release 50 % of

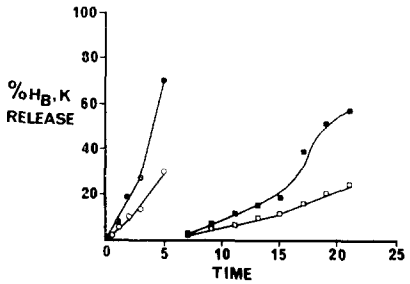

Fig. 10. Effect of increasing doses of radiation 90 krad · min^{-1} on percentage of total release of cell K^+ and haemoglobin. NaCl (pH 7.4) at 22 °C. The cells have preincubated with 100 mM glucose and 100 mM fructose for 4 h to allow equilibration of sugars. Release of K^+ and haemoglobin from cells is measured 20 min after irradiation. Suspensions containing 100 mM sucrose (not shown) have identical responses to controls or suspensions containing 100 mM fructose. The figure shows results from one of 4 similar experiments. Percentage K^+ release from cells incubated in the presence (○) or absence (●) of 100 mM glucose; and percentage of total haemoglobin loss from cells incubated in the presence (□) and absence (■) of 100 mM glucose.

the cell K^+ and 50 % of the cell haemoglobin illustrates that the colloid osmotic pressure difference across the cell membrane cannot be the sole factor determining haemolysis in isotonic media.

As neither sucrose nor fructose has any retarding influence on K^+ or haemoglobin loss from irradiated cells, this indicates unequivocally that the mechanism of cell stabilization with these sugars differs from that with glucose.

Effects of radiation pretreatment on osmotic fragility with and without glucose. Lower doses of radiation increase red cell osmotic fragility. Fig. 11 shows the effects of increasing levels of irradiation on the fragility of irradiated cells as measured by the hypotonicity required to give 50 % haemolysis. With glucose present the cell fragility is decreased by an invariant amount over a wide dose of radiation.

If the site of action for the glucose-dependent stabilization were the cell membrane, then its progressive destruction by radiation would also reduce the glucose-dependent stabilization.

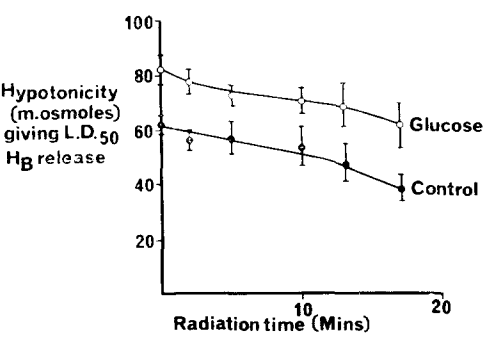

Fig. 11. Effects of increasing radiation doses at 90 krad · min^{-1} on the fragility of cells incubated ±100 mM D-glucose at 22 °C. (●) Control; (○) +100 mM glucose. Bars are S.E.M. of LD_{50} (at least 5 degrees of freedom for each point). The figure shows the results from one of 4 similar experiments. The hypotonicity referred to in the ordinate scale = (300− tonicity of saline giving 50 % cell lysis).

Reversibility of the glucose-dependent stabilization of human red cells. Since the stabilizing effects of sugars have been ascribed to physical interactions with the intracellular matrix [1] it follows that the stabilizing effect will be entirely lost on removal of the sugar. Hence the stabilizing effect of D-glucose should be fully reversible following the removal of the sugar.

Table IV shows that the effects of glucose on red cell stability are indeed entirely reversible since (a) no significant difference in fragility exists between cells which have been preincubated in glucose/saline and subjected to hypotonic stress in the absence of glucose and cells which have not been incubated with glucose (b) there is no significant difference in cell fragility between cells irradiated in the presence of glucose then subjected to hypotonic stress in the absence of glucose and cells which have been both irradiated and then subjected to hypotonic stress in the absence of glucose and (c) there is no difference between the fragility of cells irradiated in the presence of glucose and subjected to hypotonic stress in the presence of the same glucose concentration and cells irradiated without glucose present, but added to the suspension following irradiation prior to measurement of fragility. This same experiment shows that radiation increases the fragility of cells both with and without glucose present and that glucose reduces the fragility of both control and irradiated cells by a similar amount.

TABLE IV

EFFECT OF γ RADIATION ON RED CELL FRAGILITY

Probabilities calculated from Student's *t*-test paired means solution. N.S., not significant.

	Preincubation solution	Intermediate solution	*n*	LD_{50} (mosmol)	% control
A	NaCl	NaCl	5	63.69±0.74	100
B	100 mM glucose+NaCl	NaCl	4	61.31±3.40	96.3±5%
C	100 mM glucose+NaCl	100 mM glucose+NaCl	5	96.40±4.99	151.00±8%
Irradiated cells (0.9 Mrad)					
D	NaCl	NaCl	5	46.72±6.24	73.0±10%
E	100 mM glucose+NaCl	NaCl	4	50.65±1.76	80.0±3%
F	100 mM glucose+NaCl	100 mM glucose+NaCl	5	73.04±1.84	115.0±3%
G	NaCl	100 mM glucose+NaCl	4	76.53±3.02	120.0±5%

System		*n*	*p*	
Radiation effect	(A−D)+(B−E)+(C−F)	28	> 0.001	
Glucose effect	(A−B)+(D−F)+(E−G)	26	> 0.001	
Glucose effect on irradiated cells	(D−F)+(E−G)	18	> 0.001	
Preincubation effect of glucose on irradiated cells	(D−E)	8	> 0.2	N.S.
Preincubation effect of glucose on control cells	(A−B)	8	> 0.5	N.S.
Preincubation effect	(D−E)+(A−B)	16	> 0.5	N.S.
Preincubation effect (addition of glucose)	(F−G)	8	< 0.2	N.S.
Preincubation on glucose effect	(A−C)−(D−F)	8	< 0.2	N.S.

DISCUSSION

The mechanical stability of the red cell is usually discussed in terms of its membrane properties; the red cell interior is assumed to have no stabilizing function, being represented as a homogeneous fluid [28]. Indeed there is good evidence to suggest that membrane stiffness as measured by the cell elastimeter [7] is related to red cell osmotic fragility, filterability and post-transfusion in the micro-circulation.

However, it is evident that the state and organisation of haemoglobin within the cell can have important consequences in determining red cell structure and resistance to stress. Studies on the attachment of haemoglobin to red cell membranes show that a substantial fraction of haemoglobin is loosely bound and that a portion of this is more strongly bound [29]. In sickle cell anaemia deoxygenation results in gelation of the intracellular matrix [30] even when haemoglobin S forms only a small proportion of the total [38]; increased amounts of haemoglobin are then associated with the membrane [31]. The gelation as a result of deoxygenation of sickle cells results in tactoid formation [32] and an increased cell resistance to shear-induced rupture. Ponder [33] has remarked that the extremely high concentration of haemoglobin within normal erythrocytes brings the contents to the 'verge of gelation' and has suggested that the anomalous swelling observed in rat red cells at low temperatures results from gelation of the haemoglobin.

The effects of various monosaccharides on red cell osmotic fragility have been explained within a framework of two separate loci of action, namely the membrane and the intracellular matrix [1].

The evidence presented in this paper differentiates between these two loci, demonstrates their relative affinities for sugars and extends the observations of sugar-dependent increased stability to include the effects of radiation damage.

It is apparent from the swelling behaviour of cells in hypotonic solutions (Figs. 6B, 9A) that addition of glucose causes gelation of haemoglobin. Cells plus 100 mM glucose do not swell until there is a 60 mosmol pressure difference, implying the existence of a finite yield stress. The cells thus behave as visco-plastic (visco-elastic) bodies since at low values of stress, below the yield value, a regime of high elasticity exists. On exceeding the yield stress swelling occurs, accompanied by breakdown and flow of the intracellular matrix. Furthermore, no yield stress is observed on swelling resealed ghosts in the presence of glucose, since no divergence in the Boyle van't Hoff plot is observed compared to controls (Fig. 9B). An additional characteristic of visco-plastic bodies observed in whole red cells plus glucose is the exponential decrease in the elastic modulus [1] as the temperature is raised.

The behaviour of membrane-free haemoglobin parallels that of whole cells, in that at low temperatures concentrated solutions have characteristics which typify visco-plastic dispersions [16], namely a finite yield stress, shear thinning, hysteresis of the shear stress/shear rate diagram and an exponential decrease of elastic and viscous moduli with increasing temperature.

Since at limitingly high values of shear rate a glucose-dependent increase in viscosity is observed, it may be deduced that the volume occupied by the haemoglobin molecule together with its surrounding layer of immobilized water is increased by glucose. This is entirely consistent with the formation of a ternary glucose-water-haemoglobin complex as was previously suggested [1] on the basis of PMR data.

Urea at 4–6 M denatures haemoglobin by disrupting secondary bonds responsible for the maintenance of secondary and tertiary structure. Since glucose reduces the urea effect it follows that the strength of secondary bonding must be increased.

The glucose-dependent increase in secondary bonding also leads to increased intermolecular haemoglobin interaction, since viscosity in the presence of glucose at constant shear rate is a second-order function of haemoglobin concentration (Fig. 3). The time required for the formation of a significant proportion of such bonds is > 0.1 s since (a) marked shear thinning occurs below shear rates of approx. $10\ s^{-1}$ (Fig. 1B) and (b) hysteresis is observed on returning to low shear values from high (Fig. 1A). It is characteristic of gel formation that gel strength develops over a period of hours [16, 37].

Comparison of the yield-stress observed in whole cells (Fig. 6B and ref. 1) to that observed in haemoglobin solutions indicates a large discrepancy (at least 5 orders of magnitude). Since 5% gelatin gels have elastic moduli of $10^4 - 10^5\ Nm^{-2}$ [16] similar to that required for the haemoglobin gel in situ, this discrepancy may be partially due to the experimental conditions in which the yield stress for haemoglobin is determined. For instance, the concentration of haemoglobin solutions tested is less than that in situ (approx. 20%, compared to 34%). Both haemoglobin viscosity and the increment due to glucose are power functions of haemoglobin concentration, hence the glucose-dependent effect will be greatly enhanced at the protein concentration of the red cell. Also the plate-cone viscometer may underestimate the 'true' yield stress since determinations of yield values are subject to slippage [16]. However, it is possible that the reticular network within the cells could reinforce the strength of the intracellular matrix in much the same way as addition of interacting fillers to several types of visco-plastic materials can dramatically increase their yield stress. This increased strength is caused by the dispersion of localized stresses which would otherwise weaken the overall structure. An alternative explanation for the apparent increase in the red cell elastic modulus may be that sugars reduce the osmotic pressure across the red cell membrane by reducing the activity of the intracellular constituents, however the bulk of the results presented argue against this explanation. Thus, despite the large quantitive discrepancy between the yield stress of haemoglobin compared to whole cells, there are sufficient parallels to suggest that within the compacted intracellular environment a significant increase in cell stability can result from glucose-dependent increases in the strength and number of intermolecular bonds.

This particular point can be emphasised by reference to the radiation damage experiments where the membrane is progressively destroyed by γ-radiation. Glucose, but not fructose, protects against K^+ and haemoglobin leakage. Also varying degrees of radiation pretreatment do not affect the glucose-dependent increment in stability under osmotic stress. These effects are completely attributable to an intracellular mode of action for glucose.

Since D-fructose does not interact with haemoglobin (Fig. 1B and Table I) and does not protect against radiation damage, the observed protection against osmotic lysis [1] must be associated with a membrane interaction. The results presented in section 2 show that fructose protects red cells against hypotonic lysis by increasing the membrane area. This effect is directly dependent on fructose concentration in the range tested and is insensitive to changes in temperature. The mode of action for

protection by D-fructose may be similar to hydrophobic drugs such as chlorpromazine and octanol [5], since these agents are anti-haemolytic and expand the red cell membrane at lysis. The evidence presented in Fig. 8 shows that the fructose effect may be associated with an increased membrane fluidity.

The biological importance of sugar-macromolecular interactions is not confined to stabilization of red cells: Sigler [36] has suggested that the uptake of sugars into agar gels may be correlated with transport phenomena observed in yeast cells. Furthermore the current kinetic analyses of sugar transport across red cell membrane assume that the red cell interior may be treated as a single compartment. The evidence presented in section I of the Results make this assumption untenable: some of the consequences of the interaction between glucose and haemoglobin to the interpretation of the kinetics of red cell glucose transport are being investigated.

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