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PERMEABILITY CHARACTERISTICS OF ERYTHROCYTE GHOSTS PREPARED UNDER ISOIONIC CONDITIONS BY A GLYCOL-INDUCED OSMOTIC LYSIS

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Summary

A detailed study has been made of the permeability characteristics of human erythrocyte ghosts prepared under isoionic conditions by a glycol-induced lysis (Billah, M.M., Finean, J.B., Coleman, R. and Michell, R.H. (1976) Biochim. Biophys. Acta 433, 45—54). Impermeability to large molecules such as dextran (average molecular weight 70 000) was restored immediately and spontaneously after each of the 5—7 lyses that were required to remove all of the haemoglobin. Permeabilities to smaller molecules such as $MgATP^{2-}$, [^{3}H]inositol and [^{14}C]choline were initially high but could be greatly reduced by incubation at 37°C for an hour. The extent of such resealing decreased as the number of lyses to which the ghosts had been subjected increased. Both removal of haemoglobin and permeabilities to small molecules were affected significantly by pH, Ca^{2+} concentrations and divalent cation chelators. Maximum resealing was achieved in ghosts prepared in the basic ionic medium (130 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES)) at pH 7.0 (0°C) and with a calcium level around 10^{-5} M. Acidic pH facilitated the removal of haemoglobin whilst the presence of divalent cation chelators slowed down its release. Retention of K^+ by ghosts loaded with K^+ during the first lysis and subsequently incubated at 37°C was substantial but little K^+ could be retained within the haemoglobin-free ghosts. Permeability of the ghosts to K^+ after one lysis was affected by temperature, pH, Ca^{2+} concentrations and by the presence of divalent cation chelators.

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; MES, 2-(*N*-mopholino)ethanesulphonic acid; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid.

Introduction

The resealing (recovery of impermeability to solutes) of erythrocyte ghosts prepared under hypoionic conditions depends on the precise conditions created during haemolysis and subsequent treatments [1—6]. Haemoglobin-free ghosts prepared in cold, low ionic strength buffers lacking divalent cations (Ca^{2+} or Mg^{2+}) are permeable to large molecules and to a variety of smaller molecules [1—12]. However, these ghosts can partially reseal to large molecules on incubation in an isotonic salt solution at temperatures around 30°C [4,5,11]. Such treatment does not reseal the ghosts to small molecules such as sucrose or mannitol [5], but inclusion of a divalent cation (e.g. Ca^{2+}) effect some resealing to mannitol [4] and to $MgATP^{2-}$ [6].

In a previous paper [14], we described a new procedure for preparing human erythrocyte ghosts by glycol lysis under physiological ionic conditions. These will be referred to throughout this paper as isoionic ghosts. These ghosts recovered their impermeability to macromolecules spontaneously and almost completely and to a variety of smaller molecules substantially. These permability characteristics and factors which affect them have now been examined in detail. Solutes selected as permeants encompassed a range of sizes and charges and provided information on both non-mediated and mediated permeation. Some assessments were made at each stage of preparation but particular stress was laid on the characteristics of the final haemoglobin-free preparation and of ghosts after only one lysis.

Materials and Methods

Materials

Radiochemicals were obtained from Radiochemical Centre, Amersham. Other reagents were of analytical grade. Type O Rhesus Positive human blood was obtained from the Midland Blood Transfusion Centre, Birmingham. It was stored at 4°C in the citrate/phosphate/dextrose buffer and used within 3 weeks of donation.

Methods

Preparation of ghosts. The procedure has been described [14]. The basic ionic medium used contained 130 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 10 μM $CaCl_2$ and 10 mM HEPES, pH 7.4, at 0°C. Modifications of this medium were made as required for the purpose of individual experiments.

Permeability assessment. Permeabilities of ghosts obtained by the present procedure were assayed by two methods. The first relied upon the degree of exclusion of a tracer solute and the second upon the rate of efflux of a tracer solute incorporated within the ghosts during lysis. The tracer solutes used for these experiments were [^{3}H]dextran (mol. wt. 73 000), haemoglobin (mol. wt. 68 000), $MgATP^{2-}$ (mol. wt. 600, negatively charged), [^{3}H]ionositol (mol. wt. 180, non-electrolyte), [^{14}C]choline (mol. wt. 88, positively charged), 3-*O*-[^{14}C]methyl-D-glucose (mol. wt. 200, glucose analogue for mediated permeation) and potassium (K^+).

For the exclusion experiments the ghost suspensions were adjusted to a stan-

dard concentration of membrane phospholipid of 2.0 μmol/ml. To 0.2 ml of this ghost suspension, 0.2 ml of the ionic medium containing the tracer solute with or without Triton X-100 was added. The suspension was mixed well. After a specified period of time either at 0°C or at 20°C, the suspension was centrifuged at 20 000 × *g* for 15 min in the cold. In the experiments using haemoglobin as the tracer solute, 0.10-ml aliquots of the supernatant were sampled and their haemoglobin contents were determined according to Dodge et al. [15]. For radioactive tracer experiments 0.10-ml aliquots of the clear supernatant were transferred into scintillation vials and mixed with 10 ml of 1 : 1 (v/v) Triton X-100/toluene-based scintillator. Radioactivity was measured in a Philips liquid scintillation counter.

The percentage of the total volume of the ghost suspension that was inaccessible to the tracer solute was then computed from the following relationship:

$$\% V_i = \frac{X - Y}{X} \times 100$$

where $\% V_i$ = percent volume of the ghost suspension inaccessible to the tracer, X = amount of the tracer per unit volume of the supernatant, Y = amount of the tracer in the supernatant in the presence of 0.05% Triton X-100 in the suspension (i.e. no barrier to diffusion exists).

The exclusion of $MgATP^{2-}$ was assessed by measuring the extent of latency of Mg^{2+}-ATPase. ATPase activity occurs only at the cytoplasmic face of the membrane and the observed enzyme activity is, therefore, a measure of the degree of impermeability to the externally supplied substrate [1,16]. Latency was measured in the present experiments by comparing the measured Mg^{2+}-ATPase activity of a ghost suspension with that obtained after freezing and thawing. The Mg^{2+}-ATPase activity was determined according to Dunham and Glynn [17] and the freezing and thawing was performed by the method of Garrahan and Glynn [18].

For efflux experiments the ghosts were loaded with a particular tracer solute (for further details, see Results) during lysis and the external tracer was then washed away. The pellet was suspended in 20 volumes of the tracer-free medium and 2-ml aliquots in duplicate were removed at intervals and the ghosts sedimented at 20 000 × *g* for 15 min in the cold. The supernatant was discarded and the pellet treated with 0.1 ml cold 10% trichloroacetic acid and centrifuged. The clear supernatant was sampled for the measurement of radioactivity in a Philips liquid scintillation counter. For some experiments, ghosts loaded with tracer were not washed with tracer-free medium but were directly diluted 70—100-fold in the tracer-free medium. The leakage of the tracer solute was then followed in the same way. In the case of K^+ efflux experiments, the ghost suspension (2.0 μmol lipid phosphorus/ml) initially containing 130 mM K^+/l was suspended in 70 volumes of 140 mM NaCl, 10 mM HEPES, pH 7.4 (or 7.0) at 20°C. 7-ml aliquots in duplicate removed at intervals, were centrifuged at 20 000 × *g* for 15 min in the cold. Retention of K^+ in the pellet was then measured by flame photometry. In some experiments, ghosts were washed twice in 20 volumes of ice-cold 140 mM NaCl, 10 mM HEPES, pH 7.4 (or 7.0) before measuring K^+ efflux. Efflux of 3-*O*-[^{14}C]methyl-D-glucose was followed using the procedure described by Jung et al. [19]. Ghosts were loaded with F

mM D-glucose in the presence of tracer amount of 3-*O*-[^{14}C]methyl-D-glucose and transferred to 60 volumes of a tracer-free medium containing D-glucose at the same level but without the radioactive tracer. 6-ml aliquots in duplicate were removed at intervals into centrifuge tubes containing 2 ml of 5% $HgCl_2$ in the ionic medium. This treatment stopped the mediated permeation of sugars. The suspension was centrifuged at 20 000 × *g* for 15 min in the cold. The radioactivity was then measured in the pellet. Efflux of [^{3}H]inositol was measured under identical conditions.

Results

Permeabilities to haemoglobin and to dextran

Substantial exclusion of macromolecules from isoionic ghost preparations was indicated by measurements of volumes inaccessible to [^{3}H]dextran and to haemoglobin in suspensions of ghosts adjusted to a constant phospholipid concentration (Table I). The extent of exclusion from freshly isolated haemoglobin-free ghosts suspensions was unchanged by a subsequent 1 h incubation at 37°C and was not significantly different from that of ghosts after one lysis which had been resealed to small molecules by incubation (see later). These observations indicate that recovery of impermeability to macromolecules by the ghosts is spontaneous.

This indication was further substantiated by efflux experiments. As can be seen from Table II, (for experimental details see the legend), the efflux rates of both haemoglobin and [^{3}H]dextran incorporated into the haemoglobin-free ghosts during an additional lysis were negligible.

Permeability to $MgATP^{2-}$

Fig. 1 shows the latency of Mg^{2+}-ATPase activity, a measure of impermeability to $MgATP^{2-}$, as a function of cycles of loading and lysis. The total Mg^{2+}-ATPase activity of the ghosts as observed after freezing and thawing remained unchanged throughout the preparative procedure, but the latency of the enzyme appeared to decrease with increasing number of cycles of load-

TABLE I

EXCLUSION OF MACROMOLECULES BY ISOIONIC GHOSTS

Each ghost sample was adjusted to a phospholipid concentration of 2 μmol/ml. The ghost suspension was mixed with an equal volume (0.2 ml) of ice-cold, isoionic medium containing either haemoglobin (0.5%) or [^{3}H]dextran (10^4 cpm/ml) and centrifuged immediately at 20 000 × *g* for 15 min at 0—4°C. For further details, see Materials and Methods.

Ghost samples	Percent volume of ghost suspension inaccessible to	
	Haemoglobin	[^{3}H]dextran
Ghosts after one lysis incubated (37°C, 1 h)	Not determined	33
Haemoglobin-free ghosts		
freshly isolated (at 0°C)	31	30
incubated (37°C, 1 h)	34	33
Triton X-100 treated	0	0

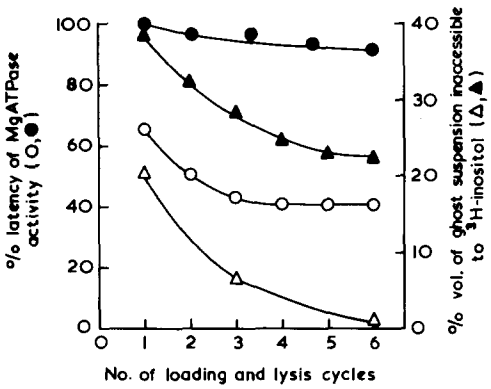
TABLE II

TIME-COURSE OF EFFLUX OF MACROMOLECULES INCORPORATED INTO HAEMOGLOBIN-FREE ISOIONIC GHOSTS

Haemoglobin-free, isoionic ghosts were loaded at 20°C with 1.5 M glycol for 10 min, chilled and then diluted with 19 volumes of ice-cold, isoionic medium containing 0.5% haemoglobin. The suspension was centrifuged at 20 000 × *g* for 15 min at 0°C and the pellet was washed once in the same medium to remove glycol. Ghosts were either kept at 0°C (freshly isolated) or incubated at 37°C for 1 h and then washed twice in 20 volumes haemoblobin-free, isoionic medium. [^{3}H]Dextran was incorporated under the same conditions. Efflux of the tracer solutes was followed by resuspending the ghosts in 20 volumes of tracer-free medium at 0°C.

Time of efflux (min)	Retention of incorporated			
	Haemoglobin (mg/ml)		[^{3}H]Dextran (10^{-3} × cpm/ml)	
	Freshly isolated	Incubated	Freshly isolated	Incubated
0	1.2	1.2	22	23
15	1.0	1.2	20	23
60	1.0	1.2	20	22

ing and lysis. The loss of latency was most rapid during initial cycles. Freshly isolated, haemoglobin-free ghosts retained their impermeability to $MgATP^{2-}$ to the extent of 30–50%. However, these ghosts recovered their impermeability to $MgATP^{2-}$ almost completely when they were incubated at 37°C for an hour (Fig. 1).



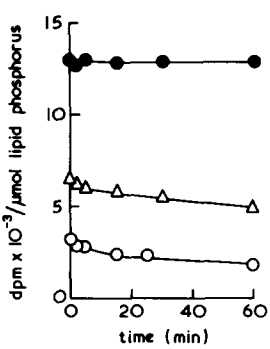


Fig. 1. Resealing of isoionic ghosts (pH 7.4) as a function of number of loading and lysis cycles. The ghosts were prepared by the standard procedure. Resealing to $MgATP^{2-}$ (mol. wt. 605) was expressed as percent latency of Mg^{2+}-ATPase activity, which determines the degree of exclusion of externally supplied $MgATP^{2-}$: ○, freshly isolated ghosts; ●, ghosts incubated at 37°C for 1 h. Resealing to inositol (mol. wt. 180) was assessed from the extent of exclusion of [^{3}H]inositol by the ghost suspension. The ghost suspension was mixed with an equal volume (0.2 ml) of ionic medium containing [^{3}H]inositol and centrifuged immediately for 15 min at 0°C. △, freshly isolated ghosts; ▲, ghosts incubated at 37°C for 1 h. Note the effect of temperature on resealing.

Fig. 2. Effect of number of loading and lysis cycles on the efflux of [^{3}H]inositol incorporated into the isoionic ghosts. Ghosts at the appropriate stage were lysed in the isoionic medium (pH 7.4) containing [^{3}H]inositol. The packed ghosts were again suspended in the same medium, incubated at 37°C for 1 h and then washed twice in 20 times their volume of [^{3}H]inositol-free, ice-cold isoionic medium. Efflux was then measured by suspending in 20 volumes of the isoionic medium at 20°C. Lysis stage: first (●); third (△), and sixth (○).

Permeability to [^{3}H]inositol

Fig. 1 shows the resealing characteristics of the ghosts to [^{3}H]inositol as a function of number of cycles of loading and lysis. Immediately after the first lysis, the ghosts showed substantial permeability to [^{3}H]inositol, and they became completely permeable at the final stage when the ghosts were free of haemoglobin. However, on incubation at 37°C for 1 h, haemoglobin-free, isoionic ghosts substantially recovered their impermeability to [^{3}H]inositol. Since the ghosts were completely impermeable to dextran, the volume inaccessible to dextran in the ghost suspension could be taken as the volume occupied by the ghosts. The proportion of this volume which excluded [^{3}H]inositol was taken as a measure of impermeability of the ghosts to [^{3}H]inositol. On this basis, the final haemoglobin-free ghost preparation was calculated to be 60 ± 10% impermeable to inositol.

For efflux experiments, ghosts after first, third and sixth lyses were loaded with [^{3}H]inositol, as described for macromolecules above (Table II), and incubated at 37°C for 1 h. The extracellular solute was removed by washing the ghosts twice in 20 volumes of tracer-free, ice-cold, isoionic medium. The efflux was then followed at 20°C and the results are presented in Fig. 2. The initial concentration of [^{3}H]inositol entrapped within the ghosts was much lower at the later stages of lysis than it was after the first lysis but the leakage rate was much higher.

Effects of pH on permeabilities

In order to study the effects of pH on the characteristics of haemoglobin-free, isoionic ghost preparations, the pH of the ionic medium was varied within the pH range 6—8 and a specified pH was maintained throughout the preparative procedure. A 10 mM MES (2-(*N*-morpholino)ethanesulphonic acid, pK_a at 20°C = 6.15) buffer was used to achieve acid pH values. Compared to neutral pH (pH 7.0), acidic pH appeared to expedite the removal of haemoglobin whilst alkaline pH seemed to slow down the release of haemoglobin (Table III). Freshly isolated ghost preparations obtained at various pH, were examined by phase contrast microscopy (see ref. 14) for values impermeability to dextran and were all found to exclude dextran completely. However, resealing to small mol-

TABLE III

EFFECTS OF pH ON HAEMOGLOBIN RELEASE FROM GHOSTS

A specified pH was maintained throughout the preparative procedure. Basic ionic medium contained 130 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 10 μM $CaCl_2$ and 10 mM HEPES or MES as appropriate (see the text). Glycol-induced lyses were affected under standard conditions described [14]. Lipid phosphorus was determined by King's method [13] and haemoglobin by the method of Dodge et al. [15]. Numbers in parentheses indicates number of loading and lyses cycles required to reach the final stage of preparation.

pH maintained during preparation	Haemoglobin content (mg/μmol lipid phosphorus)	
	After fourth loading and lysis	At final stage
6.0	0.025	0.025(4)
7.0	0.050	0.020(5)
8.0	0.100	0.040(6)

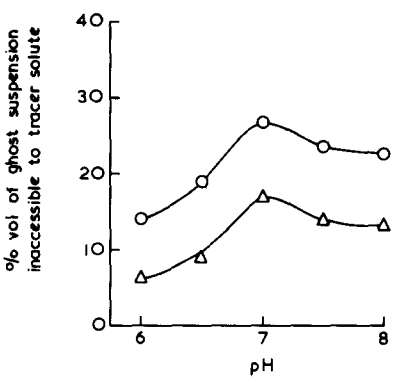
ecules such as [^{3}H]inositol and [^{14}C]choline appeared to be most effective when ghosts were prepared and incubated at pH 7.0 (Fig. 3). Under physiological ionic conditions, acidic pH appeared to be less favourable for the recovery of the permeability barrier of the ghosts than alkaline pH (Fig. 3).

Effects of divalent cations and their chelators on permeability

The isoionic medium was modified by including EGTA or EDTA (1 mM) and also by changing the levels of calcium (up to 10 mM). Free calcium levels $\leqslant 10$ μM were achieved by using appropriate amounts of $CaCl_2$ buffered with 1 mM EGTA [20]. The pH of these media was maintained at 7.0. Haemoglobin-free ghosts were prepared whilst maintaining a particular level of calcium constant throughout the preparative procedure.

Haemoglobin retention by the ghosts after one lysis passed through a maximum around a calcium concentration of 1—10 μM (Table IV). Examinations of the ghosts by phase contrast microscopy showed that haemolysis of the cells was complete at all levels of Ca^{2+}. Complete removal of haemoglobin was found to be facilitated in the presence of extremely high calcium levels (e.g. 10 mM). Ghosts prepared in the ionic medium containing 1 mM EGTA were the most difficult to free of residual haemoglobin (Table IV).

Freshly isolated, haemoglobin-free ghosts prepared at various calcium concentrations within the range mentioned excluded dextran, as judged by phase contrast microscopy. At calcium concentrations above 1 mM, the ghosts became distorted in the presence of 2% dextran. As can be seen from Fig. 4 the extent of recovery of impermeability of the ghosts to [^{3}H]inositol and to [^{14}C]choline also passed through a maximum at around 10 μM Ca^{2+}. Mg^{2+} was much less effective than Ca^{2+} in preserving the resealing capacity of the ghosts (Fig. 4).



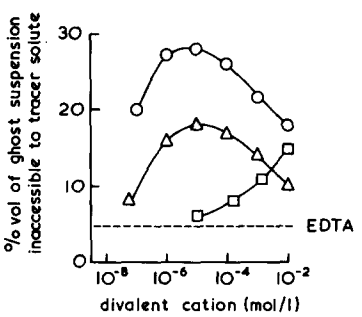


Fig. 3. Effects of pH on resealing of haemoglobin-free, isoionic ghosts. A specified pH was maintained throughout the preparative procedure. Other experimental conditions are as in Table III. All the preparations were incubated at 37°C for 1 h before measuring exclusion of [^{3}H]inositol (○), and of [^{14}C]choline (△) as described in Fig. 1.

Fig. 4. Effects of divalent cations (Ca^{2+} and Mg^{2+}) on resealing of haemoglobin-free, isoionic ghosts. A specified concentration of Ca^{2+} or of Mg^{2+} at pH 7.0 was maintained throughout the preparative procedure. Control ghosts were made in the isoionic medium containing 1 mM EDTA or EGTA as appropriate. In the case of Mg^{2+}, 0.1 mM EGTA was used to chelate endogenous Ca^{2+} in the medium. Further experimental details are as in Table IV. All the preparations were incubated at 37°C for 1 h before measuring exclusion of [^{3}H]inositol (○, with Ca^{2+}; □, with Mg^{2+}) and of [^{14}C]choline (△, with Ca^{2+}).

TABLE IV

EFFECTS OF Ca^{2+} ON HAEMOGLOBIN RELEASE FROM GHOSTS

A specified Ca^{2+} concentration at pH 7.1 (10 mM HEPES) was maintained throughout the preparative procedure. A Ca^{2+} concentration at 10 μM or below was achieved by using 1 mM Ca^{2+}-EGTA buffer (see the text). Other components of the medium were 130 mM KCl (allowance was made for high $[Ca^{2+}]$), 10 mM NaCl, and 2 mM $MgCl_2$ (or none). Glycol-induced lyses were effected under standard conditions as described [14]. Ghosts after first lysis were washed three times in 20 volumes of respective lysing media at 0°C. Lipid phosphorus and haemoglobin were measured as in Table III.

Ca^{2+} concentration	Ghost haemoglobin content (mg/μmol lipid phosphorus)		No. of lysis cycles required for final stage (0.025 mg haemoglobin/μmol lipid phosphorus)
	After first lysis	After fourth lysis	
1 mM EGTA	1.50	0.1	7
0.1 μM	1.45	0.1	7
1 μM	2.10	0.07	6
10 μM	1.95	0.055	5
0.1 mM	1.50	0.045	5
1 mM	1.38	0.04	5
10 mM	0.50	0.02	4

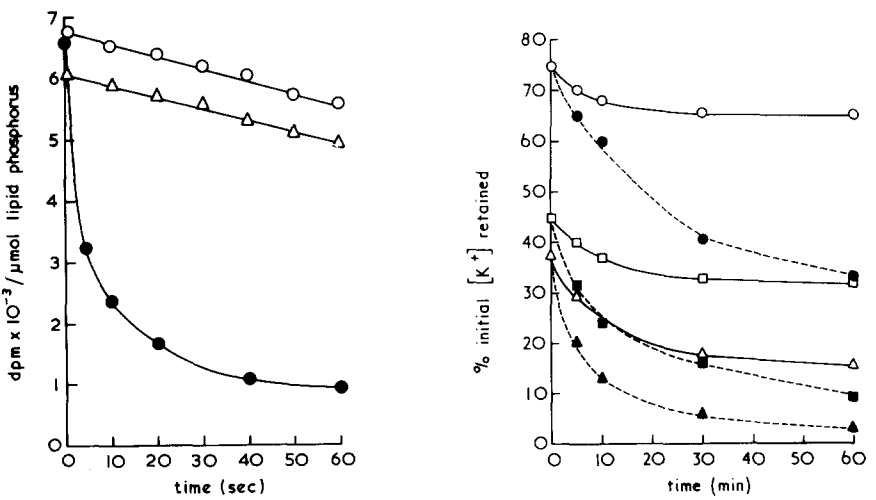


Fig. 5. Mediated permeation of 3-*O*-[^{14}C]methyl-D-glucose by haemoglobin-free, isoionic ghosts. Ghosts were loaded, during final lysis, with 3-*O*-[^{14}C]methyl-D-glucose or [^{3}H]inositol in the presence of 5 mM glucose or inositol as appropriate and incubated at 37°C for an hour. Packed ghosts were washed twice in 20 volumes of ice-cold isoionic medium containing 5 mM of respective sugar but without the radioactive tracer. Efflux was followed at 20°C as described in Materials and Methods. Efflux of [^{3}H]inositol (△) with or without $HgCl_2$ (0.7%) and efflux of 3-*O*-[^{14}C]methyl-D-glucose without (●) or with (○) $HgCl_2$ (0.7%).

Fig. 6. Effects of number of loading and lysis cycles and of divalent cation chelators (EGTA and EDTA) on permeability to K^+ of isoionic ghosts (pH 7.0). Ghosts at first (○,●), third (□,■) and fifth (△,▲) stages were incubated at 37°C for 1 h in the isoionic medium containing either 2 mM chelator (———, ○, □, △) or none (- - - - -, ●, ■, ▲). Addition of the chelator was made under ice-cold conditions. Packed ghost suspension (2.5 μmol phospholipid/ml) was suspended in 70 times its volumes of 140 mM NaCl, 10 mM HEPES, pH 7.0, and K^+ efflux measured at 20°C as described in Materials and Methods. Note that the isoionic medium contained 10 μM Ca^{2+} and 2 mM Mg^{2+}. See also Table V.

TABLE V

RATE CONSTANTS OF K^+ EFFLUX FROM ISOIONIC GHOSTS: EFFECTS OF Ca^{2+} AND EGTA

Values were calculated from the data in Fig. 6.

No. of loading and lysis cycles	Rate constants (h^{-1}) of K^+ efflux from ghosts incubated with	
	Ca^{2+} (10 μM)	EGTA (2 mM)
1	0.42	0.02
3	0.60	0.03
5	1.12	0.11

Permeability to 3-O-methyl-D-glucose

From Fig. 5 it is apparent than the efflux of 3-*O*-[^{14}C]methyl-D-glucose is much more rapid than that of [^{3}H]inositol. These effluxes were measured under equilibrium exchange conditions. At 5 mM level, 3-*O*-[^{14}C]methyl-D-glucose reached an equilibrium with the external medium with a half-time of approx. 3 s. This value agrees closely with that for intact cells [21]. The rapid flux of 3-*O*-[^{14}C]methyl-D-glucose could be completely inhibited with 0.7% $HgCl_2$ (Fig. 5), as expected if it was catalysed by the glucose carrier system of the membrane [21].

Permeability to K^+

Fig. 6 illustrates the extent of retention of K^+ by the ghosts at different stages of preparation. The ability of the ghosts to entrap K^+ appeared to be markedly reduced during the series of glycol-induced lyses. This loss of capacity for resealing to K^+ was most rapid during initial cycles of loading and lysis. The amount of K^+ retained by the haemoglobin-free, isoionic ghosts was only about 20% of the initial K^+ content whereas the ghosts after one lysis retained K^+ to the extent of 70%. Compared to the ghosts after one lysis, haemoglobin-free ghosts lost K^+ at a relatively faster rate (Fig. 6 and Table V).

Further studies of factors affecting K^+ permeability were therefore restricted to ghosts after one lysis. Maximum K^+ retention in such ghosts was achieved with 10—100 μM Ca^{2+} and pH 6—7.7 during lysis. Subsequent leakage of K^+ from the ghosts was minimised by addition of a chelator such as EDTA or

TABLE VI

EFFECTS ON K^+ RETENTION OF INCUBATION PERIOD IN PRESENCE OF A DIVALENT CATION CHELATOR

A chelator, at a concentration of 2 mM, was added under ice-cold conditions immediately after isolation of ghosts, or at different intervals during a 1 h period of incubation at 37°C. K^+ retention by the ghosts was measured as described in Fig. 6.

Time of incubation in chelator-free medium (min)	Percent initial K^+ retained at intervals (min)		
	30	60	180
0	75	72	70
15	64	55	31
30	63	54	30
60	65	54	31

EGTA (2 mM) at or immediately after haemolysis. Table VI shows the effects on the time-course of K^+ retention of the addition of a chelator to the ghost sample. Efflux of K^+ was reduced only when EGTA or EDTA was added to freshly isolated ghosts. These results suggest that resealing to Ca^{2+} occurred immediately on incubation at 37°C and that Ca^{2+} trapped within the ghosts stimulated the efflux of K^+.

Discussion

To remove the cytoplasmic contents from the erythrocyte, it is necessary to render the plasma membrane temporarily permeable to large molecules and to expose the cytoplasmic face of the membrane to a replacement medium. In our preparative procedure, we have sought to minimise damage to the membrane by effecting haemolysis in an ionic medium approximating that of the normal erythrocyte cytoplasm. The consequences of this preparative procedure in terms of membrane composition and morphology were discussed in an earlier publication [14].

The usefulness of a membrane preparation for studies of physiological properties of the plasma membrane is largely dependent on the extent to which the normal permeability characteristics of the membrane of the intact cell can be restored following haemolysis. Using glycol-induced osmotic haemolysis under isoionic conditions all the haemoglobin can be removed and substantial resealing achieved: impermeability of these haemoglobin-free isoionic ghosts to macromolecules approaches that of the intact cells (Tables I and II) and impermeability to passive permeation of smaller molecules such as inositol or choline is largely restored (Figs. 1—6). In this respect, these preparations are superior to haemoglobin-free ghosts prepared by lysis at low ionic strengths [1—12] and are comparable to resealed "pink" ghosts prepared by lysis in buffers with osmotic strength around 40 mosM and then incubated at 37°C in an isoionic medium [3]. Permeability to K^+ is greater in haemoglobin-free isoionic ghosts than it is in the resealed hypoionic "pink" ghosts but ghosts after one glycol lysis under isoionic conditions are comparable in this respect to the hypoionic "pink" ghosts and contain much less haemoglobin (Table IV). Carrier-mediated glucose permeation survives repeated glycol lysis relatively well and in this respect the ghosts compare very favourably with intact cells (Fig. 5), as do ghosts prepared by hypoionic haemolysis [21].

The ghosts prepared under conditions of physiological ionic strength recover impermeability to macromolecules spontanously, but the extent of resealing with respect to small molecules is significantly dependent on the concentrations of both Ca^{2+} and H^+ (or OH^-), passing through a maximum at concentrations of these ions close to those encountered in the cytoplasm (Figs. 3 and 4). In contrast, hypoionic ghosts behave unphysiologically in the sense that they can reseal only when incubated in the presence of Ca^{2+} concentrations far higher than those normally present within the cell [6,22]. It is possible that under conditions of low ionic strength, Ca^{2+} that is tightly bound in the membrane structure may be largely removed whereas under isoionic conditions this apparently important Ca^{2+} pool remains undisturbed (See Simons [31]). It seems likely that these ions at appropriate concentrations influence packing of mem-

brane components so as to bring the permeability channels or holes created during haemolysis down below a critical size. At higher concentrations ($\geqslant$1 mM Ca^{2+} in media of physiological ionic strength) which have been observed to inhibit resealing we have detected (unpublished) both elution of polyacrylamide gel band 4.1 [24] and changes in lipid composition.

The process of molecular reorganisation required for resealing of isoionic ghosts seems to be dependent both on appropriate Ca^{2+} and H^+ concentrations and on temperature during handling of the ghosts, with maximum resealing achieved by incubation at 37°C. Resealing of hypoionic ghosts to macromolecules [5], to small molecules [4] and to cations [3,23] is also improved by warming. Thus, the reorganisation of membrane components associated with resealing may require an appropriate fluidity of structure so that changes in molecular packing influenced by Ca^{2+} and H^+ can proceed unhindered.

The decreasing success of procedures for resealing to small molecules with increasing numbers of lyses remains a deficiency in the preparative technique which may be inherent in any procedure involving repeated membrane rupture and resealing. It does not appear to be explained by loss of any membrane components (see ref. 14) and one can only suggest that there is a progressive disorganisation of structure which becomes increasingly difficult to reverse with each additional lysis. Nevertheless, the achievement of substantial resealing to molecules as small as inositol in ghosts which are haemoglobin-free is an advance on previous procedures.

The permeability of any ghost preparation to K^+ is probably a combination of its inherent "leakiness" and the operation of a Ca^{2+}-controlled K^+-gating system in the membrane. The latter would be expected to be very sensitive to changes in internal Ca^{2+} concentrations during the preparative procedures. The best preparation with respect to passive permeability to K^+ is one made by lysing at 0°C in the presence of micromolar levels of Ca^{2+} and then incubating at 37°C in the presence of a chelator (EGTA, EDTA or ATP) rather than in a Ca^{2+}-containing medium (Fig. 6 and Tables V and VI). Thus, for substantial resealing to occur sufficient Ca^{2+} must be present at the instant of haemolysis but the Ca^{2+} level must then be reduced in order to maximise impermeability of the membrane to K^+. It would appear that there is an intracellular pool of Ca^{2+} that exercises critical control over permeability of the membrane to K^+, but which is held only lightly by the membrane structure and is readily removed during incubation of ghosts at 37°C in the presence of a chelator. Such an effect of intracellular Ca^{2+} on K^+ permeability has long been recognised in intact cells [25—28] and in resealed, hypoionic "pink" ghosts [29—31] and the availability of a haemoglobin-free isoionic ghost preparation may aid its further characterisation. The small Ca^{2+} pool which is presumed to remain after chelator incubation must be more tightly bound in the membrane structure and probably has a membrane-stabilising effect. The functions of these two pools are clearly distinguished by the new preparative procedure which, therefore, offers a favourable approach to their further study.

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