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EFFECTS OF INCLUSION OF Ca^{2+}, Mg^{2+}, EDTA OR EGTA DURING THE PREPARATION OF ERYTHROCYTE GHOSTS BY HYPOTONIC HAEMOLYSIS

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SUMMARY

1. The effects of Ca^{2+}, Mg^{2+}, EDTA and ethanedioxy-bis(ethylamine)tetraacetic acid (EGTA) upon the composition and permeability of human erythrocyte ghosts have been studied.

2. By the use of two representative types of ghost preparation as controls it was possible to distinguish between effects due to the metal ions and effects due to ionic strength.

3. Ca^{2+} and Mg^{2+} increase the level of retention of intracellular proteins and reduce the permeability of the membrane preparations. EGTA and EDTA have the opposite effects. EGTA is as effective as EDTA suggesting that Ca^{2+} is more important than Mg^{2+} in these phenomena, but other experiments demonstrate that Mg^{2+} can largely substitute for Ca^{2+}.

4. Low ionic strength has deleterious effects upon the morphological integrity of the ghosts. These appear to accompany the loss of non-haemoglobin protein from the membranes. The presence of Ca^{2+} and Mg^{2+} protects against these effects of low ionic strength.

INTRODUCTION

Our previous studies[1], together with those of others[2–4], have demonstrated that ionic strength has a profound influence upon the properties of isolated erythrocyte membrane preparations. This work has now been extended to examine the role of divalent metal ions in some of these phenomena.

The effects of the presence of Ca^{2+} and Mg^{2+} and chelating agents [EDTA and ethanedioxy-bis(ethylamine)tetraacetic acid (EGTA)], during the preparation of erythrocyte ghosts by hypotonic haemolysis, have been studied at two osmolarities

Abbreviation. EGTA, ethanedioxy-bis(ethylamine)tetraacetic acid

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at which the properties of the control ghosts show fundamental differences 2. These osmolarities were (a) 40 imosM; the ghosts are relatively impermeable to small molecules, retain appreciable levels of intracellular enzymes and have essentially latent membrane ATPase activities. (b) 5 imosM, the ghosts are highly permeable to small molecules, the intracellular enzymes are much reduced or absent, and latency of ATPase activities is not observed

MATERIALS AND METHODS

Chemicals

ATP, acetylthiocholine iodide, L-leucyl-β-naphthylamide hydrochloride, p-nitrophenyl phosphate and haemoglobin were obtained from Sigma Chemical Co, London. 62C47 (1,5-bis(4-trimethylammonium phenyl)pentan-3-one diiodide), a specific acetylcholinesterase inhibitor, was a gift of Dr H. T. Openshaw. The Wellcome Research Laboratories, Kent. Ethanedioxy-bis(ethylamine)tetraacetic acid (EGTA) was obtained from B.D H. Ltd, Poole, Dorset. All other chemicals used were of the highest grade commercially available, including diaminoethanetetraacetic acid (EDTA).

[^{14}C]Sucrose from the Radiochemical Centre Amersham was the gift of Dr D. J Candy.

Enzymic and chemical estimations

These were as described in a previous paper[1].

Phase contrast microscopy

The preparations were examined with a Zeiss Photomicroscope. The membrane samples were diluted in the appropriate solution and examined at a magnification of 1250 $\times$.

Preparation of ghosts

The method used was essentially that described previously[1], but modified by the inclusion of metal ions or chelating agents in the haemolysing and washing solutions.

Packed human erythrocytes (O^+) were washed twice with 4 vol. of chilled isotonic sodium bicarbonate buffer, pH 7.4. The washed cells were resuspended in isotonic bicarbonate buffer to give a cell:buffer ratio of 1·1. Immediately after washing, 5 ml of the cell suspension were lysed for 30 min with 25 ml of the appropriate haemolysing solutions containing divalent metal ions (as chloride salts) or chelating agents (adjusted to pH 7.4 with Tris–HCl) at the appropriate concentrations. The ghosts were sedimented by centrifugation at 20000 $\times$ g_{av} for 15 min, resuspended to 30 ml with the appropriate haemolysing solution and washed until the supernatants were haemoglobin-free. The desired osmolarity of the haemolysing solution within each series was maintained by the inclusion of compensating amounts of bicarbonate or Tris–HCl buffers at pH 7 4 as indicated

After preparation the ghosts were stored in closed containers at 0 °C, in the solutions indicated in the legends to the appropriate figures

Expression of results

Due to the differences found in protein composition of the various preparations, all estimations are related to the phospholipid content of the samples, which is regarded as a better measure of membrane than is protein content.

RESULTS

Effects of divalent metal ions or chelating agents on various parameters of ghosts prepared at 40 imosM

Table I compares the chemical and enzymological composition of human erythrocyte ghosts prepared at 40 imosM in the presence of no (control) or 1 mM concentrations of Ca^{2+}, Mg^{2+}, EGTA as EDTA as appropriate. At the concentration and pH used, EGTA is essentially specific for Ca^{2+}, whereas the specificity of EDTA is much wider.

TABLE I

THE EFFECTS OF THE PRESENCE OF DIVALENT METAL IONS OR CHELATING AGENTS DURING HYPOTONIC HAEMOLYSIS ON THE CHEMICAL AND ENZYMOLOGICAL COMPOSITION OF HUMAN ERYTHROCYTE GHOSTS, PREPARED AT AN OSMOLARITY OF 40 imosM

The ghosts were prepared as in the Materials and Methods section and the final pellets were then washed once and resuspended in isotonic Tris–HCl buffer, pH 7.4. The figures quoted are from a representative experiment. Other experiments which are less complete gave comparable results. Enzymic activities are expressed as nmole of product/h per μmole phospholipid phosphorus, with the exception of cholinesterase, which is expressed as μmole product/h per μmole phospholipid phosphorus. Haemoglobin and non-haemoglobin protein are expressed as mg per μmole phospholipid phosphorus.

	40 imosM bicarbonate buffer incorporating				
	Buffer alone (Control)	*Ca^{2+} (1 mM)*	*Mg^{2+} (1 mM)*	*EDTA (1 mM)*	*EGTA (1 mM)*
Haemoglobin	0.30	1.50	1.75	0.05	0.05
Non-haemoglobin protein	1.40	1.55	1.45	1.30	1.30
Naphthylamidase	300	740	280	15	10
Acid *p*-nitrophenylphosphatase	390	800	410	380	390
Acetylcholinesterase	100	93	96	95	100
(Mg^{2+})-ATPase	120	60	65	290	320
(Na^{+}–K^{+})-ATPase	110	35	75	390	430
(Ca^{2+}–Mg^{2+})-ATPase	40	30	45	650	650

Inclusion of divalent metal ions during preparation markedly increased the retention of haemoglobin, naphthylamidase and acid phosphatase. In the type of ghosts represented by the control, these three components have been shown to be entrapped within the ghosts, and to be released in soluble form and in parallel fashion by a variety of lytic procedures[1]. Mg^{2+}, though slightly increasing haemoglobin retention, had no effect upon the retention of naphthylamidase or acid phosphatase.

Ghosts prepared in the presence of divalent metal ions were less permeable than the controls, as evidenced by ATPase values and [^{14}C]sucrose exclusion experiments (Fig. 1). Disruption of the permeability barrier demonstrated highly latent ATPase values.

Acetylcholinesterase activities in Ca^{2+} or Mg^{2+} prepared ghosts were similar to those of the controls and to chelator-prepared ghosts. This would be expected since the enzyme is located on the outer membrane surface and so permeability limitations do not occur (see ref. 1)

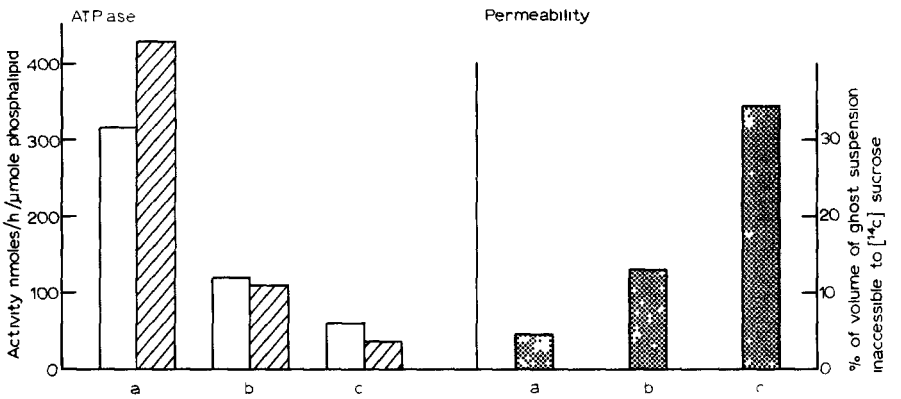

Fig 1 Relationship of ATPase activities to permeability of ghosts Ghosts were prepared as described in Materials and Methods at 40 imosM, and resuspended in a minimal volume of the appropriate preparation buffer All preparations were adjusted to the same concentration of phospholipid phosphorus (2 25 μmoles/ml) ATPase activities were measured as described in Materials and Methods To 3 ml of ghost suspension at 0 °C, 0 1 ml of carrier-free [^{14}C]sucrose (6 1 · 10^{-1} μCi/ml, 1 75 · 10^{-2} mg/ml) was added The suspension was mixed and allowed to stand for 5 min at 0 °C, and then centrifuged for 15 min at 10000 × g_{av} Aliquots of the supernatants were removed and added to scintillator for radioactivity assessment From these measurements the volume inaccessible to [^{14}C]sucrose was calculated Open column, (Mg^{2+})-ATPase Hatched column, (Na^+–K^+)-ATPase Solid column, space impermeable to [^{14}C]sucrose (a) ghosts prepared in the presence of 1 mM EGTA, (b) control, (c) ghosts prepared in the presence of 1 mM Ca^{2+}

Non-haemoglobin protein levels in ghosts prepared with Ca^{2+} or Mg^{2+} were marginally increased above the control, whereas levels in chelator-prepared ghosts were marginally lower.

Inclusion of chelators during preparation produced ghosts with very much lower levels of haemoglobin and naphthylamidase than the controls*, and high and non-latent ATPase activities These ghost preparations were permeable to [^{14}C]sucrose (Fig. 1), indicating that the high ATPase activities in chelator-prepared ghosts reflected the penetration of substrate to the internal hydrolysis site.

Freshly prepared ghosts appeared intact by phase contrast microscopy and were rounded or spherical (Fig. 2a). No breakdown was evident in the presence of chelating agents (Fig. 2c) Ghosts prepared in the presence of Ca^{2+} (Fig. 2b) or Mg^{2+} appeared more "rigid" (*i.e* did not change their shape on collision) than the controls, whereas ghosts prepared in the presence of chelators appeared more "flexible" (*i.e.* underwent shape changes on collision) than the controls.

The properties of ghosts prepared at this osmolarity in the presence of chelator

* The behaviour of acid *p*-nitrophenylphosphatase, which was retained under conditions in which other intracellular proteins were lost appeared anomalous The retained enzyme appeared to be identical in properties (K_m and activation energy) to the leached enzyme. Its size (approx 70000 mol wt) is comparable to that of haemoglobin (68000) and naphthylamidase (approx 95000) This suggests that its retention is due to a specific adsorption rather than an entrapment based on relative molecular size

suggest that such ghosts would be a highly suitable preparation for many studies. They are haemoglobin-free, highly permeable, and in addition several other features have been preserved: intact morphology, all the cholinesterase activity (and phospholipid, cholesterol and sialic acid, unpublished observations) of intact erythrocytes,

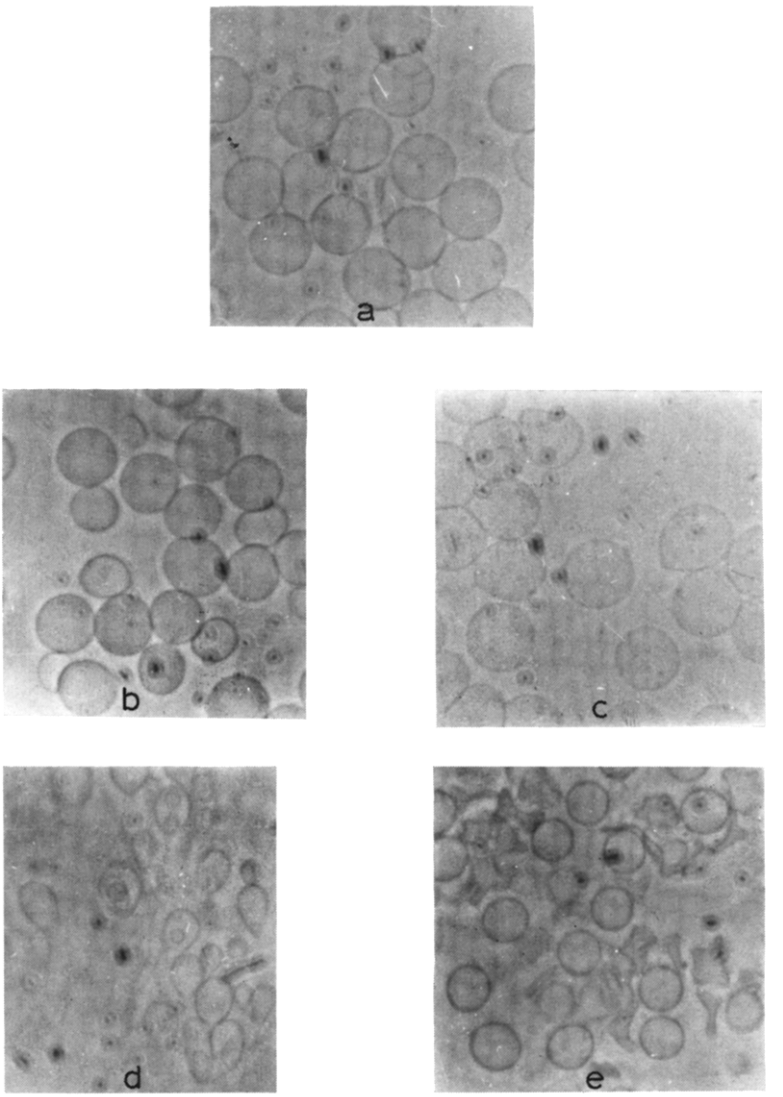

Fig 2 Effects of metal ions and chelators on the morphology of erythrocyte ghosts prepared at 40 mosM or 5 mosM Ghosts were prepared as described in Materials and Methods, resuspended in the appropriate buffer or preparation, and examined immediately by phase contrast microscopy Magnification approximately 2000 × The contrast in each photograph is approximately related to the haemoglobin content of each preparation. (a) Ghosts prepared in 40 mosM bicarbonate buffer (pH 7 4); (b) ghosts prepared in 40 mosM bicarbonate buffer containing 1 0 mM Ca^{2+}; (c) ghosts prepared in 40 mosM bicarbonate buffer containing 1 0 mM EDTA, (d) ghosts prepared in 5 mosM Tris–HCl buffer (pH 7 4), (e) ghosts prepared in 5 mosM Tris–HCl buffer containing 1.0 mM Ca^{2+}.

high ATPases (particularly (Ca^{2+}–Mg^{2+})-ATPase), together with appreciable amounts of non-haemoglobin protein. The intact morphology, together with non-haemoglobin protein levels comparable to the controls, suggests that extensive damage had not occurred. These ghosts are also capable of resealing under certain conditions (Bramley T.A , Coleman, R and Finean, J B., in preparation).

Effects of divalent metal ions or chelating agents on various parameters of ghosts prepared at 5 imosM

Table II compares the chemical and enzymological composition of human erythrocyte ghosts prepared at 5 imosM in the presence of no (control) or 0.33 mM and 1 mM concentrations of Ca^{2+}, Mg^{2+}, EGTA or EDTA as appropriate. Data obtained at two concentrations are included in this table to indicate the sharpness of the phenomena observed.

Inclusion of divalent metal ions during preparation progressively increased the retention of haemoglobin (Table II and Fig. 3a) naphthylamidase and acid phosphatase (Table II). This retention is due to entrapment of these proteins rather than surface adsorption, since ghosts prepared at 1 mM divalent metal ion released these proteins on lowering the divalent metal ion content (at 5 imosM) or on sonication; treatment with higher ionic strength did not.

Ghosts prepared in the presence of divalent metal ions were less permeable than the controls, as evidenced by [^{14}C]sucrose exclusion experiments (Table II).

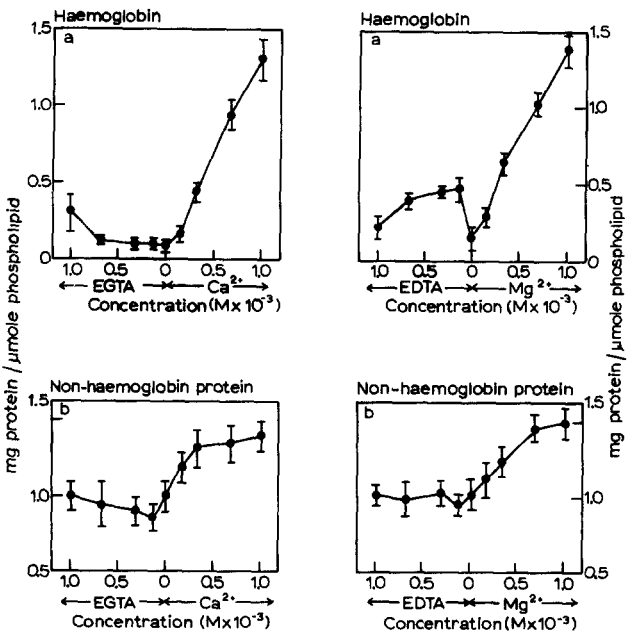

Fig 3 Effects of metal ions and chelators on haemoglobin and non-haemoglobin protein content of erythrocyte ghosts prepared at 5 imosM Ghosts were prepared as described in the Materials and Methods section and resuspended and stored in the appropriate buffer of preparation Each point represents the mean of three separate experiments; the bars represent the standard deviation The left hand series of graphs describe experiments performed with ghosts prepared in the presence of various concentrations of EGTA or Ca^{2+} as indicated, the right hand series of graphs related to various concentrations of EDTA or Mg^{2+} (a) haemoglobin, (b) non-haemoglobin protein

TABLE II

THE EFFECTS OF THE PRESENCE OF DIVALENT METAL IONS OR CHELATING AGENTS DURING HYPOTONIC HAEMOLYSIS ON THE CHEMICAL AND ENZYMOLOGICAL COMPOSITION OF HUMAN ERYTHROCYTE GHOSTS, PREPARED AT AN OSMOLARITY OF 5 mosM

The ghosts were prepared as in the Materials and Methods section and the final pellets were resuspended and stored in the appropriate buffer of preparation (or isotonic bicarbonate for ATPase determination) The figures quoted are the means of three separate experiments except for [^{14}C]sucrose exclusion (1 experiment) Expression of results as in Table I The [^{14}C]sucrose exclusion experiment was carried out and expressed as in Fig 1

	5 mosM Tris–HCl buffer incorporating								
	Buffer alone (Control)	*Ca^{2+} (0 33 mM)*	*Ca^{2+} (1 mM)*	*Mg^{2+} (0 33 mM)*	*Mg^{2+} (1 mM)*	*EDTA (0 33 mM)*	*EDTA (1 mM)*	*EGTA (0 33 mM)*	*EGTA (1 mM)*
Haemoglobin	0 1	0.45	1 32	0 66	1 40	0 46	0 22	0 10	0 32
Non-haemoglobin protein	0 92	1 22	1 40	1 25	1 50	1 00	1 00	0 90	1 00
Naphthylamidase	15	80	210	75	185	10	15	10	15
Acid *p*-nitrophenylphosphatase	60	135	280	135	275	120	155	165	155
Acetylcholinesterase	70	96	94	96	90	68	70	60	70
(Mg^{2+})-ATPase	250	300	310	290	320	350	350	350	350
(Na^{+}–K^{+})-ATPase	240	180	120	240	230	270	300	170	220
(Ca^{2+}–Mg^{2+})-ATPase	140	130	60	460	240	640	800	720	1000
[^{14}C]Sucrose exclusion	2 7	—	14 7	—	14 7	—	3 4	—	3·7

The effect of this permeability change upon ATPase activities is discussed later.

Ca^{2+}- or Mg^{2+}-prepared ghosts have high non-haemoglobin protein levels and their cholinesterase activities were comparable to the activity of intact erythrocytes. They appeared morphologically intact (Fig 2c), in contrast to the control preparation (Fig. 2d) which rapidly fragmented

Inclusion of chelators during preparation had no effect upon naphthylamidase retention, which remained minimal. A significant increase in haemoglobin retention was noted in ghosts prepared in EDTA-containing solutions (Fig 3a, Table II) amounting to 0.3–0.5 % of the haemoglobin of the intact erythrocyte This was not released by sonication, but was removed by increasing the ionic strength, suggesting adsorption to the membrane 2–3 % of the acid phosphatase of the original erythrocytes was retained by chelator-prepared ghosts This level was reduced by higher ionic strength (310 imosM), again suggesting adsorption It should be noted that a similar retention of this enzyme was observed for ghosts prepared at 40 imosM in the presence of chelators, conditions where haemoglobin retention was minimal. The soluble and adsorbed enzymes cannot be differentiated since their kinetics and energies of activation are identical (T A. Bramley, unpublished observations)

Ghosts prepared in the presence of chelators at this osmolarity are highly permeable to [^{14}C]sucrose, and demonstrate high ATPase levels which are non-latent (Table II). The measured permeability of the control ghosts is close to that of chelator prepared ghosts (Table II), and therefore all ATPase levels should be high. The low level of (Ca^{2+}–Mg^{2+})-ATPase (even after sonication) may be explained by an inactivation[1] (loss of thiol groups?) which occurs in the absence of either chelating agents or the divalent metal ions. Ghosts prepared with divalent metal ions exhibit low (Ca^{2+}–Mg^{2+})-ATPase levels. However, these low levels can be greatly increased by sonication, showing the original level is determined by latency rather than inactivation. Although [^{14}C]sucrose exclusion experiments (Table II) indicate that the permeabilities of Ca^{2+} ghosts and Mg^{2} ghosts are the same, the (Ca^{2+}–Mg^{2+})-ATPase levels indicate that Mg^{2+} is less effective than Ca^{2+}. This is partly confirmed by sonication experiments, which demonstrated the greater latency of (Ca^{2+}–Mg^{2+})-ATPase in Ca^{2+}-prepared ghosts as compared to Mg^{2+}-prepared ghosts. The presence of divalent metal ions during preparation had no effect upon the (Na^{+}–K^{+})- and Mg^{2+}-ATPase levels, probably due to the preincubation of the ghosts in the assay medium which contains low levels ($1 \cdot 10^{-4}$ M) of chelating agents incorporated into the assay to remove Ca^{2+} which interferes in both of these ATPase assays[16] This preincubation appears to partially to reverse the effects of divalent metal ions on ghost permeability at 5 imosM but not at 40 imosM (Table I).

Ghosts prepared and stored at 5 imosM fragment[1,2] (Fig. 2d) and marked destruction of cholinesterase activity occurs[1] (Table II) Both phenomena were prevented by divalent metal ions, which conferred upon the ghosts a more regular shape (Fig. 2e) and greater "rigidity" Ghosts prepared in the presence of chelating agents at 5 imosM fragmented even more readily than the controls. Furthermore, the membranes of chelator-prepared ghosts appeared to be highly fluid and mobile, to fuse with neighbouring membranes, and to form vesicles within themselves. The mobility, fluidity and lack of contrast of chelator-prepared material made photographic recording of this material technically impossible using our equipment

We have previously shown that erythrocyte ghosts prepared in the range

5–15 imosM at pH 7.4 have specifically lost more non-haemoglobin protein than ghosts prepared at higher osmolarity[1]. The phenomenon was also demonstrated by the controls in the present series of experiments. Fig 3b demonstrates that the inclusion of EDTA had little effect upon the levels of non-haemoglobin protein at this osmolarity. The inclusion of divalent metal ions caused a progressive increase in non-haemoglobin protein retention, reaching a level which was comparable with the controls of higher osmolarity ghosts.

DISCUSSION

In other studies Ca^{2+} has been shown to increase the retention of various intracellular proteins when present during initial lysis[5,6] and during extensive washing in hypotonic media[2,4,7]. The effects of magnesium, when studied, have proved to be somewhat similar[7,8]. EDTA has been used in the preparation of ghosts by other workers[6,7,9–12] but the effects of inclusion of chelator have often been incidental rather than a primary objective. Since EGTA appears to have been as effective as EDTA in the present study, the involvement of Ca^{2+} would appear to be more important than Mg^{2+} in these phenomena; but Mg^{2+} is able to substitute to a large extent in the absence of Ca^{2+}.

Differing permeabilities, in relation to the presence of the divalent metal ions and chelators employed during preparation, can be seen from other work. The EDTA-prepared ghosts of Blomfield *et al.*[9] were highly permeable as indicated by their high ATPase levels. The EDTA-prepared ghosts of Palek *et al*[6] had a higher permeability to sucrose than controls, whereas Ca^{2+}-prepared "ghosts" had lower permeability Ca^{2+} and Mg^{2+} increased the latency of some intracellular enzymes, Ca^{2+} being more effective than Mg^{2+} (ref. 7).

Fragmentation of ghosts at low ionic strength has been observed previously in several studies in the absence[1,2,4], and presence[7,10] (contrast however Marchesi and Palade[12]) of chelating agents. The present studies show that fragmentation at low ionic strength is prevented by divalent metal ions, in agreement with the observations of others[4,7]. Moreover, although chelating agents appear to enhance this fragmentation, they are not primarily responsible for it, since ghosts prepared at higher osmolarities in the presence of EDTA do not fragment (see also ref. 7). Such ghosts, however, have been shown to be highly permeable. High permeability can be associated with fragmentation[7] but the preparation of morphologically whole ghosts (40 imosM:1 mM EDTA) which have a high permeability clearly shows that high permeability and fragmentation do not necessarily accompany each other.

However, the same results clearly show that low ionic strength is primarily responsible for fragmentation. One item of significance in this respect may be the difference in non-haem protein levels which are observed between 5 imosM:1 mM EDTA and 40 imosM:1 mM EDTA. This loss seems to be correlated more closely with fragmentation than any other phenomenon Sensitivity to fragmentation in our previous study[1] was also equated with the region in which a specific loss of non-haemoglobin protein occurred Incorporation of Ca^{2+} or Mg^{2+} during preparation not only preserves the morphological integrity of preparations but also prevents the loss of these proteins. In this context it may be noted that many of the extraction

conditions for "contractile" proteins from erythrocytes involve exposure of the ghosts to media of low ionic strength[13–15].

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