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ENHANCEMENT OF (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY OF HUMAN ERYTHROCYTE MEMBRANES BY HEMOLYSIS IN ISOSMOTIC IMIDAZOLE BUFFER

I. GENERAL PROPERTIES OF VARIOUSLY PREPARED MEMBRANES AND THE MECHANISM OF THE ISOSMOTIC IMIDAZOLE EFFECT *

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Summary

1. Membranes prepared from human erythrocytes hemolyzed in isosmotic (310 imosM) imidazole buffer, pH 7.4, show enhanced and stabilized (Ca^{2+} + Mg^{2+})-ATPase activity compared with membranes prepared from erythrocytes hemolyzed in hypotonic (20 imosM) phosphate or imidazole buffer, pH 7.4.

2. Exposure of intact erythrocytes or well-washed erythrocyte membranes to isosmotic imidazole does not cause enhanced (Ca^{2+} + Mg^{2+})-ATPase activity.

3. Exposure of erythrocyte membranes, in the presence of isosmotic imidazole, to the supernatant of erythrocyte hemolysis or to a partially purified endogenous (Ca^{2+} + Mg^{2+})-ATPase activator, promotes enhanced (Ca^{2+} + Mg^{2+})-ATPase activity. Under appropriate conditions, NaCl can be shown to substitute for imidazole. The results demonstrate that imidazole does not act directly on the erythrocyte membrane but rather by promoting interaction between an endogenous (Ca^{2+} + Mg^{2+})-ATPase activator and the erythrocyte membrane.

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* A preliminary report of some of these data was presented [1]. This work was taken from a dissertation presented by M.L. Farrance in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Abbreviations: 120, 1310, ideal milliosmolar imidazole at 20 and 310 imosM, respectively; P20, 20 imosM phosphate; HI-40, 40 mM-40 mM histidine/imidazole buffer.

Introduction

The use of hemoglobin-free erythrocyte membranes, sometimes referred to as ghosts, is common for investigation of plasma membrane properties. Any survey of the erythrocyte membrane literature reveals many methods for membrane isolation. It has been emphasized that membranes resulting from various methods differ in significant ways; from each other and from the native, intact membrane [2—5]. It appears that no single membrane preparation is best for all applications and that each preparative method must be carefully defined. The methods usually share some common steps: washing (for removal of buffy coat and plasma proteins), hemolysis, and further washing (for removal of hemoglobin). All of these may vary from the standpoint of pH, osmolarity, type of buffer, inclusion or exclusion of certain ions, and number of repetitions. Each of these factors may be important in the expression of the enzymatic properties of the final preparation. Other factors may also modify membrane-bound enzyme activity [6—13].

Omachi et al. [14] reported that erythrocytes hemolyze in "isotonic" imidazole buffers. It should be noted that osmolarity is not equivalent to tonicity although equivalence is often assumed or implied [1,14,15]. Isotonic refers to a solution that is physiologically isosmotic with some reference compartment, for example cytoplasm, whereas isosmotic merely refers to an equal number of osmotically active particles. Isotonic is clearly a more restrictive term than isosmotic. Thus, the hemolysis reported by Omachi et al. [14] reflects the response of erythrocytes to an isosmotic, but not isotonic, solution. Membranes were not prepared, and enzymatic activities were not measured by Omachi et al. [14], but Luthra et al. [15] reported greatly decreased (Ca^{2+} + Mg^{2+})-ATPase activity in membranes prepared from erythrocytes hemolyzed in "isotonic" imidazole buffer. After erythrocyte hemolysis in "isotonic" imidazole buffer, pH 7.4, at room temperature, membranes appeared fragmented and displayed "a very significant loss" of (Ca^{2+} + Mg^{2+})-ATPase activity. By contrast, we previously found enhanced and stabilized (Ca^{2+} + Mg^{2+})-ATPase activity after erythrocyte hemolysis in isosmotic imidazole buffer at 0—5° C [1]. This report deals with experiments performed in an attempt to resolve this difference, to examine the properties of 310 imosM imidazole membranes, and to elucidate the mechanism of the isosmotic imidazole effect.

Materials

Imidazole (Grade I), L-histidine-HCl · H_2O, Na_2ATP (Sigma Grade), and Na_2-ADP (Grade I) were purchased from Sigma. All other chemicals were reagent grade. Glass-distilled, deionized water was used for all solutions.

Methods

Outdated packed human erythrocytes were obtained from the local blood bank. All preparation steps were performed at 0—5° C, except as noted. Cells were initially centrifuged at 5000 × *g* for 10 min in a SS-34 fixed-angle rotor of a RC-5 Sorvall refrigerated centrifuge. The packed cells were washed 3 times

with 155 mM NaCl, removing any remaining buffy coat at each step. Following the general procedure of Dodge et al. [16], erythrocytes were hemolyzed in one of three buffers (pH 7.4): 20 ideal milliosmolar (imosM) imidazole (I20), 20 imosM phosphate (P20), or 310 imosM imidazole (I310). Calculation of osmolarity was done using the Henderson-Hasselbalch equation assuming a pK_a value for imidazole of 7.05 [17] (using an Advanced osmometer, measured osmolarity of I310 was 290 mosM). The hemolysis buffer was added rapidly and vigorously to a measured volume of packed, washed erythrocytes with a ratio of buffer : cells of 14 : 1. The resulting hemolysate was mixed thoroughly and centrifuged at $48000 \times g$ for 20 min. The pellet of packed ghosts was washed 4 times with I20 (pH 7.4) if hemolyzed in imidazole (I20 or I310), and 4 times with P20 if hemolyzed in P20. After each wash and centrifugation, the supernatant fluid was aspirated, and the tube was rotated to allow the loosely packed membranes to slide off the small button of unlysed cells and/or debris; the button was then aspirated. P20 membranes were washed 4 times with 40 mM-40 mM histidine/imidazole buffer (HI-40), pH 7.1; these washes were required to reduce the inorganic phosphate content of the P20 membranes to a negligible level. I20 and I310 membranes were usually washed only once with HI-40 (washing 4 times had no effect on ATPase activity). An equal volume of HI-40 buffer was added to the membranes, and the resulting suspension was stored in the refrigerator on ice. Protein content of the membrane suspension was determined by the method of Lowry et al. [18] using bovine serum albumin as the standard. If, during the course of preparation, membranes were treated with hemolysate, etc., the membranes were washed at least twice (or as many times as necessary to remove any traces of hemoglobin) before storage.

The ATPase incubation medium contained (in a final volume of 1 or 2 ml) 0.01—0.10 ml membrane suspension, 3 mM ATP (Na_2ATP, neutralized to pH 7.1), 18 mM-18 mM histidine-imidazole buffer (pH 7.1), 3 mM $MgCl_2$, 80 mM NaCl, 15 mM KCl, and 0.1 mM ouabain. $CaCl_2$ (0.1 mM) was added to appropriate tubes for determination of $(Ca^{2+} + Mg^{2+})$-ATPase, which was taken as "extra" ATP splitting induced by Ca^{2+} addition in the presence of Mg^{2+} and ouabain. Tubes without Ca^{2+} gave Mg^{2+}-ATPase activity and also served as a blank to correct for nonenzymatic breakdown of ATP and for the presence of inorganic phosphate in the membrane preparation. All assay tubes (in duplicate) were incubated at 37°C in a shaking water bath for one hour. The reaction was started by addition of substrate and stopped by the addition of 1/2 the incubation medium volume of ice-cold 1.5 M perchloric acid. After thorough mixing and centrifugation, 0.5 or 1 ml of supernatant was analyzed for inorganic phosphate using the method of Fiske and SubbaRow [19]. Results are expressed as μmol of inorganic phosphate released per mg membrane protein per h.

Endogenous $(Ca^{2+} + Mg^{2+})$-ATPase activator was partially purified by the method of Luthra et al. [7]. The sole modification of that method was in the use of imidazole buffers for hemolysis. In experiments in which activator was added to the incubation medium (0.2 ml activator fraction/1 ml total incubation volume), an equal amount of 20 mM Tris/maleate buffer, pH 6.8 at 25°C was added to the control.

In all assays, conditions were such that less than 15% of total substrate was

utilized during any incubation period. Enzymatic activity was linear with time and proportional to the amount of membrane protein added. Membranes were usually assayed on the day after preparation but were assayed on days 0, 1, 4, 7, 10 and 20 for the stability study. Data were analyzed for statistical significance by Student's paired or unpaired *t*-test as appropriate.

Results

All final membrane preparations were creamy white (free of gross hemoglobin contamination) and were of a smooth consistency. Imidazole membranes consistently packed more densely during centrifugation than phosphate membranes. Membranes prepared from erythrocytes hemolyzed in I310, I20, or P20 had significantly different (Ca^{2+} + Mg^{2+})-ATPase activities. The (Ca^{2+} + Mg^{2+})-ATPase activity of I310 membranes was markedly enhanced compared to that of I20 or P20 membranes (Table I).

If membrane proteins, other than (Ca^{2+} + Mg^{2+})-ATPase, were selectively eluted from erythrocyte membranes during hemolysis in I310, the specific activity of the (Ca^{2+} + Mg^{2+})-ATPase of I310 membranes would appear to be higher. Such a loss of protein sufficient to cause the large increase in (Ca^{2+} + Mg^{2+})-ATPase activity is unlikely, however, since the yield (± S.E.) of protein/ml erythrocytes was the same: 2.89 ± 0.16 mg/ml, 2.91 ± 0.15 mg/ml, and 2.86 ± 0.21 mg/ml for I310, P20, and I20 membranes, respectively.

Hemolysis at 25°C, instead of the usual 0—5°C, slightly decreased the (Ca^{2+} + Mg^{2+})-ATPase activity of I310 and P20 membranes (Table II). Nevertheless, the (Ca^{2+} + Mg^{2+})-ATPase activity of I310 membranes from erythrocytes hemolyzed at 25°C was higher than comparable values from the literature [20]. Storage at 0—5°C had little effect on the (Ca^{2+} + Mg^{2+})-ATPase activity of I310 membranes with no significant change being apparent for at least 7 days, whereas P20 membranes were stable only through the first day. However, either freeze-thawing or storage at −20°C for 10 days abolished the (Ca^{2+} + Mg^{2+})-ATPase activity of I310 membranes.

Experiments were designed to examine possible changes in (Ca^{2+} + Mg^{2+})-ATPase activity due to exposure of low activity membranes to I310. After erythrocytes were hemolyzed in I20 or P20, I310 was systematically substituted

TABLE I

ERYTHROCYTE MEMBRANE (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY: INFLUENCE OF HEMOLYSIS BUFFER

Erythrocytes were hemolyzed in I310, I20, or P20 (pH 7.4) and membranes were prepared and assayed according to Methods. ATPase activity is expressed as μmol PO_4^{3-} · mg^{-1} protein · h^{-1}, mean ± S.E.

Type of membrane	*n*	(Ca^{2+} + Mg^{2+})-ATPase activity
I310	87	3.25 ± 0.07 *,**
I20	46	0.76 ± 0.04 *
P20	29	0.50 ± 0.03

* Significantly greater than P20 membrane value, $P < 0.01$.
** Significantly greater than I20 membrane value, $P < 0.01$.

TABLE II

ERYTHROCYTE MEMBRANE (Ca^{2+} + Mg^{2+})-ATPase: INFLUENCE OF TEMPERATURE OF HEMOLYSIS

Membranes were prepared from erythrocytes hemolyzed in I310 or P20 at different temperatures and assayed as in Methods. ATPase activity is expressed as μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$, mean ± S.E.

Type of membrane	*n*	Temperature of hemolysis (°C)	(Ca^{2+} + Mg^{2+})-ATPase activity
I310	2	0— 5	3.45 ± 0.12 *
I310	2	20—25	2.74 ± 0.01 *
P20	2	0— 5	0.28 ± 0.03
P20	2	20—25	0.16 ± 0.01

* Significantly greater than P20 (0—5°C), $P << 0.0005$.

for one of the four hypotonic washes. Resultant membranes, as well as control I20/P20 and I310 membranes, were assayed for (Ca^{2+} + Mg^{2+})-ATPase activity. I310 washing caused enhancement of (Ca^{2+} + Mg^{2+})-ATPase activity if, and only if, I310 was substituted for the first hypotonic wash after hemolysis. This suggested that either there was something present at the time of the first wash or that the erythrocyte membrane changed during the course of washing so it could no longer respond to the I310. Further work confirmed the former suggestion. Erythrocytes, hemolyzed in I20, were washed several times with I20 and once with the supernatant of an I20 or I310 hemolysis. In each case, enough concentrated imidazole was added, if necessary, to make the final concentration about I310 (hemolysate/ I310). These membranes were then washed as if they had just been hemolyzed. The (Ca^{2+} + Mg^{2+})-ATPase activity of I20 membranes treated with hemolysate/I310 (2.46 ± 0.20) was significantly greater ($P << 0.0005$) than untreated I20 membranes (0.71 ± 0.07) and slightly, but significantly, less ($P < 0.01$) than I310 membranes (2.76 ± 0.25; values expressed as μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$, mean ± S.E.). Supernatants of both I20 and I310 hemolysis gave the same results.

From the above results, it appeared that something in the supernatant of hemolysis interacted (during preparation) with erythrocyte membranes in the presence of I310 and that this interaction resulted in an increase in the (Ca^{2+} + Mg^{2+})-ATPase activity of the resultant membranes. Bond and Clough [6] had reported the presence of an endogenous erythrocyte (Ca^{2+} + Mg^{2+})-ATPase activator in the membrane-free hemolysate of erythrocytes. However, they had assessed the activity of the hemolysate by adding it to already prepared membranes as the time of the ATPase assay. To see whether our results could possibly be due to an effect of this activator during preparation, we examined the influence of the supernatant of hemolysis in the ATPase assay. The addition of 0.2 ml supernatant of I310 or I20 hemolysis to a 1.0 ml total volume of incubation mixture produced enhanced (Ca^{2+} + Mg^{2+})-ATPase activity in I20 membranes, but not I310 membranes. The hemolysate had no intrinsic ATPase activity, and imidazole per se added to the incubation medium had no effect on the (Ca^{2+} + Mg^{2+})-ATPase activity of I20 membranes.

The finding that hemolysate increased (Ca^{2+} + Mg^{2+})-ATPase activity of I20 but not I310 membranes was at least suggestive that the I310 effect might be

TABLE III

ERYTHROCYTE MEMBRANE (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY: INFLUENCE OF ADDITION OF ACTIVATOR FRACTION TO THE ATPase INCUBATION MEDIUM

I20 and I310 membranes were prepared and assayed according to Methods with the addition of either 0.2 ml buffer or 0.2 ml activator fraction, as defined in Methods, in 1.0 ml total volume. ATPase activity is expressed as μmol PO_4^{3-} · mg^{-1} protein · h^{-1}, mean ± S.E.

Type of membrane (in ATPase assay)	n	Addition to incubation medium	(Ca^{2+} + Mg^{2+})-ATPase activity
I20	25	0.2 ml 20 mM Tris/maleate buffer, pH 6.8	0.58 ± 0.03
I310	3	0.2 ml 20 mM Tris/maleate buffer, pH 6.8	2.01 ± 0.09
I20	25	0.2 ml activator fraction	2.34 ± 0.13 *
I310	3	0.2 ml activator fraction	2.12 ± 0.19

* Significantly greater than addition of buffer to same type of membrane, $P << 0.0005$.

due to an effect of "activator" during preparation. In pursuing this, we noted that Luthra et al. [7] had reported partial purification of the activator. Using the method of Luthra et al. [7] (see Methods), we obtained fractions which, when added to the incubation medium, significantly ($P << 0.0005$) increased (Ca^{2+} + Mg^{2+})-ATPase of I20 membranes, but not I310 membranes (Table III). Fractions that contained activator activity ("activator fraction") also contained a small amount of hemoglobin. In agreement with Bond and Clough [6] the possibility that hemoglobin is the activator was ruled out.

Turning to the question of whether the activator could exert its effect during preparation, we performed experiments like those described above using activator fraction/I310 instead of hemolysate/I310. The results were essentially the same (Table IV). Exposure of low activity membranes to the activator fraction/I310 significantly ($P << 0.0005$) increased their (Ca^{2+} + Mg^{2+})-ATPase activity. However, exposure of I20 membranes to activator fraction alone (without I310) or to 20 mM Tris/maleate buffer, pH 6.8, (activator fraction buffer) alone had no effect on their subsequently determined (Ca^{2+} + Mg^{2+})-ATPase activity. These results demonstrate a resemblance between the effect of the activator on low activity membranes, during preparation on the one hand, and during incubation on the other hand. A resemblance to the influence of I310

TABLE IV

ERYTHROCYTE MEMBRANE (Ca^{2+} + Mg^{2+})-ATPase ACTIVITY: INFLUENCE OF TREATMENT DURING PREPARATION WITH ACTIVATOR FRACTION IN THE PRESENCE OF I310

I20 and I310 membranes were prepared, exposed to activator fraction (see Methods) in I310, and assayed for (Ca^{2+} + Mg^{2+})-ATPase activity according to Methods. Control I20 and I310 membranes are included for comparison. ATPase activity is expressed as μmol PO_4^{3-} · mg^{-1} protein · h^{-1}, mean ± S.E.

Type of membrane	n	(Ca^{2+} + Mg^{2+})-ATPase activity
I310	6	2.24 ± 0.21 *
I20	9	0.67 ± 0.08
I20, treated with activator fraction/I310	8	1.93 ± 0.20 *
I310, treated with activator fraction/I310	2	2.95 ± 0.14 *

* Significantly greater than I20, $P << 0.0005$.

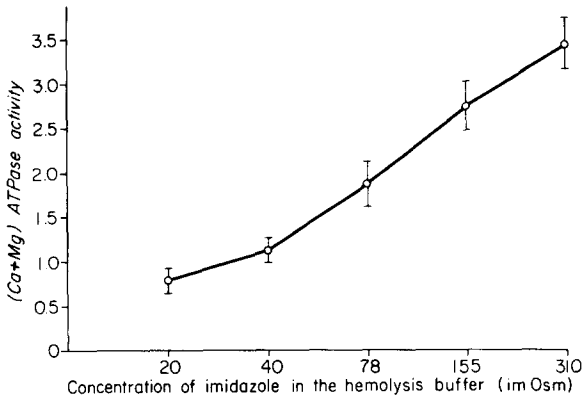

Fig. 1. The dependence of $(Ca^{2+} + Mg^{2+})$-ATPase activity of membranes on the imidazole concentration of the hemolysis buffer. Membranes were prepared from erythrocytes hemolyzed in varying concentrations of imidazole from I20 to I310 and were washed in I20. Membranes were assayed for $(Ca^{2+} + Mg^{2+})$-ATPase activity. All procedures are described completely in Methods. $(Ca^{2+} + Mg^{2+})$-ATPase activity is expressed as μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$, mean ± S.E., $n = 3$.

hemolysis was also apparent, except that I310 appeared to be required during hemolysis (or preparation) but not during incubation.

Either the situations were not analogous or imidazole itself was not the critical moiety. To help answer this, erythrocytes were exposed to a series of imidazole buffers at pH 7.4, and the resulting membranes were washed in I20, pH 7.4. At 310 imosM and lower concentrations of imidazole, all erythrocytes hemolyzed. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of subsequently obtained membranes varied directly with the imidazole concentration of the hemolysis buffer with a half-maximal effect at about 105 imosM imidazole (Fig. 1).

Results from several other experiments make it unlikely that the I310 effect is due to a direct effect of imidazole and are compatible with the idea that imidazole promotes interaction of the endogenous $(Ca^{2+} + Mg^{2+})$-ATPase activator with the membrane. First, washed erythrocytes were treated with solutions isosmotic with respect to both imidazole and NaCl, pH 6.05—8.05. Such solutions do not cause hemolysis of erythrocytes. Thus, erythrocytes could be exposed to I310 without hemolysis. Since the pK_a of imidazole is 7.05 [17], the pH range from 6.05 to 8.05 produced a change in the ratio of non-protonated : protonated imidazole molecules from 0.1 at pH 6.05 to 10 at pH 8.05. After the above treatment, the erythrocytes were washed in 0.9% NaCl. They were then homolyzed in I20 and membranes were prepared. In no case was the $(Ca^{2+} + Mg^{2+})$-ATPase activity of these membranes significantly different from that of untreated I20 membranes (not shown). Control experiments showed no changes due to pH alone. Second, well washed I20 membranes washed or stored in I310 did not have enhanced $(Ca^{2+} + Mg^{2+})$-ATPase activity. Obviously, imidazole does not act through a direct effect on the erythrocyte membrane, and hemolysis must occur (or have occurred) for the activation phenomenon to take place.

It seemed possible that the ionic strength of the hemolysis medium is important for the imidazole effect. Erythrocytes were first hemolyzed in I20 and then, without washing, enough concentrated NaCl was added to make the ionic

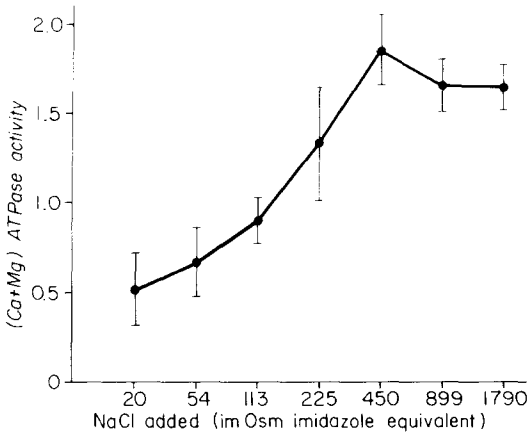

Fig. 2. Dependence of $(Ca^{2+} + Mg^{2+})$-ATPase activity of I20 membranes on NaCl added to the I20 hemolysate. Erythrocytes were hemolyzed in I20, varying amounts of NaCl were then added the hemolysate, membrane preparation was completed, and the $(Ca^{2+} + Mg^{2+})$-ATPase activity of the membranes was determined. All procedures are completely described in Methods. The concentration of NaCl added to the I20 hemolysate is expressed as its equivalent of imosM imidazole to facilitate comparison with results in Fig. 1. $(Ca^{2+} + Mg^{2+})$-ATPase activity is expressed as μmol $PO_4^{3-} \cdot mg^{-1}$ protein $\cdot h^{-1}$, mean ± S.E., $n = 3$.

strength equivalent to various concentrations of imidazole. The $(Ca^{2+} + Mg^{2+})$-ATPase activity of the resultant membranes showed enhancement proportional to the NaCl concentration with a peak effect at a concentration of NaCl (0.62%) equivalent to 450 imosM imidazole (Fig. 2). It should be noted that such NaCl solutions [21] would not serve as hemolysis buffers because no erythrocytes would hemolyze in 0.62% NaCl and only 25% would hemolyze in 0.42% NaCl (equivalent to I310).

Discussion

Hanahan [5] and Luthra et al. [15] reported that membranes from erythrocytes hemolyzed in "isotonic" imidazole buffer had a markedly lowered $(Ca^{2+} + Mg^{2+})$-ATPase activity associated with extensive membrane fragmentation. They concluded that imidazole had severely and directly damaged the erythrocyte plasma membrane. By contrast, the present results show that membranes from erythrocytes hemolyzed in isosmotic imidazole buffer, pH 7.4, have high $(Ca^{2+} + Mg^{2+})$-ATPase activity. Imidazole itself does not appear to directly damage the erythrocyte plasma membrane since no hemolysis is caused by the exposure of erythrocytes to: (1) a buffer "isotonic" with respect to imidazole and NaCl [14,15] or histidine [15] or (2) a buffer with imidazole concentrations of 620 or 1240 imosM. If imidazole were directly deleterious to plasma membranes, one would expect that the damage should occur upon exposure to such high imidazole concentrations. It is difficult to reconcile the present results with those of Hanahan [5] and Luthra et al. [15]. Only a small portion of the difference could be accounted for by a difference in the temperature of hemolysis. One possible difference may be the technique of freeze-thawing of the prepared membranes. Freeze-thawing or sonication is commonly used to expose "latent" ATPase activities [2,3,5—7,22,23]. If

freeze-thawing had been used by Luthra et al. [15], this could explain the low $(Ca^{2+} + Mg^{2+})$-ATPase activity and the fragmentation that they saw. We found that freeze-thawing results in a dramatic loss of activity of I310 membranes. We can only speculate on this point because these papers [5,15] did not give complete experimental details. Variation of $(Ca^{2+} + Mg^{2+})$-ATPase activities from various laboratories may rest on such details of experimental procedure and again emphasizes that the isolated erythrocyte membrane is merely an approximation of the native structure [5].

Our results show that the enhanced $(Ca^{2+} + Mg^{2+})$-ATPase activity of I310 membranes is not due to nonspecific causes such as protein loss from the membrane or to a direct effect of imidazole on the membrane. The I310 effect is caused by an ionic strength dependent effect of a previously reported [6,7] endogenous $(Ca^{2+} + Mg^{2+})$-ATPase activator on erythrocyte plasma membrane. Thus, 310 imosM imidazole, pH 7.4, has a dual effect: the non-protonated species can readily penetrate the erythrocyte membrane and cause osmotic hemolysis and the contribution of the protonated species to the ionic strength of the hemolysate is sufficient to promote an interaction between the activator and the membrane. It is this interaction which enhances the $(Ca^{2+} + Mg^{2+})$-ATPase activity. The enhancement of the $(Ca^{2+} + Mg^{2+})$-ATPase activity of erythrocyte membranes either by treatment of low activity membranes during preparation with hemolysate/I310 or activator/I310 or treatment of low activity membranes with hemolysate or activator in the incubation medium appears to be produced by the same mechanism which is active during erythrocyte hemolysis in I310: i.e., promotion of an ionic strength-dependent interaction of an endogenous activator with the membrane. Exposure of high activity (I310) membranes to such conditions does not significantly change their $(Ca^{2+} + Mg^{2+})$-ATPase activity. This suggests that once the membrane $(Ca^{2+} + Mg^{2+})$-ATPase has been activated, further exposure to activating conditions does not increase or decrease the degree of membrane $(Ca^{2+} + Mg^{2+})$-ATPase activation. The results presented in this paper do not show whether the activator merely changes the erythrocyte membrane during preparation (or incubation) or whether the activator combines with and remains on the membrane. This question is addressed in a subsequent communication [24].

We feel that the I310 membrane preparation can be useful to investigators both because of its ease of preparation and its high $(Ca^{2+} + Mg^{2+})$-ATPase activity. In addition, further knowledge about the activator of $(Ca^{2+} + Mg^{2+})$-ATPase and its role in cellular biology may be of importance in the investigation of certain diseases in which a defect of Ca^{2+} transport may be implicated (e.g., muscular dystrophy, cystic fibrosis, sickle cell anemia, hereditary spherocytosis [25]).

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