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STUDIES ON AN ACTIVATOR OF THE $(Ca^{2+}+Mg^{2+})$-ATPase OF HUMAN ERYTHROCYTE MEMBRANES

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SUMMARY

1. An activator of the $(Ca^{2+}+Mg^{2+})$-stimulated ATPase present in the human erythrocytes (membrane) has been isolated in soluble form from hemolysates of these cells. Partial purification has been achieved through use of carboxymethyl-Sephadex chromatography. The resulting activator fraction contained no hemoglobin and only 0.3 % of the total adenylate kinase activity of the cell.

2. Whereas the activator was released from erythrocytes subjected to hemolysis in 20 miosM buffer at pH 7.6 or at pH 5.8, only the membranes prepared at pH 7.6 were affected by it.

3. When $(Ca^{2+}+Mg^{2+})$-ATPase activity was measured by $^{32}P_i$ release from [γ-^{32}P]ATP, freeze-thawed erythrocytes, as well as membranes prepared at pH 5.8 and at pH 7.6, expressed lower values than noted by assay for total P_i release. When ADP instead of ATP was used as substrate, significant amounts of P_i were released by these erythrocyte preparations. Further study revealed (a) production of ATP and AMP from ADP with membranes and hemolysate alone, and (b) exchange of the γ- and β-position phosphate on [γ-^{32}P]ATP in the presence of membranes plus hemolysates. These observations established the presence of adenylate kinase activity in the (membrane-free) hemolysates and in membranes. It further supports the conclusion that P_i release from ADP by human erythrocytes (freeze-thawed) and by their isolated membranes is due to formation of ATP by adenylate kinase and hydrolysis of this generated ATP by $(Ca^{2+}+Mg^{2+})$-ATPase.

4. The following points were also established: (a) absence of an ADPase in human erythrocytes; (b) the $(Ca^{2+}+Mg^{2+})$-ATPase activator enhanced cleavage only of the γ-position of ATP and (c) the $(Ca^{2+}+Mg^{2+})$-ATPase activator is neither adenylate kinase nor hemoglobin.

INTRODUCTION

The occurrence of very low levels of Ca^{2+} in human erythrocytes has been reported [1, 2]. The mechanism by which these low levels are maintained in this cell has been attributed to an ATP-dependent Ca^{2+}-ATPase [3, 4]. Presumably, this pump

is manifested in isolated membranes as a $(Ca^{2+}+Mg^{2+})$-stimulated ATPase [3, 4].

In a study designed in part to explore the role of this calcium-activated ATPase [5] in human erythrocyte membrane, it was reported that a significant portion of the enzymatic activity was lost during membrane preparation, particularly at pH 7.6. No detectable activity was recovered in the membrane-free hemolysate. In ensuing investigations of the cause of this disparity, a low molecular weight soluble protein activator for the calcium-stimulated ATPase was identified in the hemolysate. During the course of this study, Bond and Clough [6] reported the presence in membrane-free hemolysates of a soluble protein, which activated the release of P_i from ATP by stimulating the $(Ca^{2+}+Mg^{2+})$-ATPase activity. However, there was some uncertainty about the source of enhanced P_i. It was conceivable that ADP (a product of ATPase) could be a source of enhanced phosphate release, probably due to the activation of ADPase, if the latter is present in red cells. Thus studies were undertaken to clarify this point. Our current results confirmed the observations of Bond and Clough [6] and also provide further insight into certain characteristics of $(Ca^{2+}+Mg^{2+})$-ATPase activator and the type of enzymatic activity stimulated.

MATERIALS

Sephadex G-75 and carboxymethyl-Sephadex C_{50} were purchased from Pharmacia Fine Chemicals (Piscataway, New Jersey). Precoated thin-layer chromatography sheets of poly(ethyleneimine) cellulose F (20 cm × 20 cm) for nucleotide separation were obtained from EM Laboratories (Darmstadt, Germany). ^{14}C-labeled radioactive substrates (ADP and ATP) and aquasol scintillant were from New England Nuclear, while ^{32}P-labeled substrates were prepared by method of Glynn and Chappel [7]. Chloroform and methanol were purchased from Mallinckrodt Chemical Works (St. Louis, Mo.). Nonradioactive substrates (ATP, ADP and AMP), muscle hexokinase, lactate dehydrogenase, pyruvate kinase, NADH, phosphoenolpyruvic acid, Tris and other chemicals were products of Sigma Chemical Company (St. Louis, Mo.).

METHODS

Preparation of hemolysate and membranes. Human blood was withdrawn from the anticubital veins of healthy donors and collected in heparinized tubes. Plasma and buffy coat were removed after centrifugation of this mixture at 4000 rev./min in Sorvall RC-2B using SW34 rotor. Unless otherwise stated, the packed cells were washed 3 times with 0.172 M Tris · HCl, pH 7.6. A modification of the method of Hanahan et al. [5] was used to prepare hemoglobin-free membranes. In a typical preparation, 2 ml of a 50 % suspension of erythrocytes in 0.172 M Tris · HCl, pH 7.6, was hemolyzed with 28 ml of distilled water and centrifuged at 27 000 × *g* for 30 min. The supernatant (hemolysate) was saved and, if necessary, stored in frozen state. The pellet was washed 3 times with 20 miosM Tris · HCl buffer, pH 7.6, to obtain hemoglobin-free membranes. The latter were stored at 4 °C in 20 miosM Tris · HCl buffer, pH 7.6, and used within 24 h. Membranes were freeze-thawed three times just prior to incubation for ATPase assay. When membranes were prepared at pH 5.8, a similar procedure of hemolysis and washing as described above was employed, except that the buffer used was Tris/maleate, pH 5.8.

Partial purification of $(Ca^{2+}+Mg^{2+})$*-ATPase activator.* In a typical preparation, 4 ml of 50 % suspension of erythrocytes in 0.172 M Tris · HCl, pH 7.6 were mixed with 60 ml of distilled water. After centrifugation at 27 000 × *g* for 30 min, 45 ml membrane-free hemolysate (representing 1.4 ml packed erythrocytes) was dialyzed for 18 h at 4 °C against 20 mM Tris/maleate, pH 6.8, and loaded onto a 100-ml bed volume column (25 cm × 2 cm) of carboxymethyl-Sephadex C_{50} equilibrated in 20 mM Tris/maleate buffer, pH 6.8 at room temperature. Subsequent passage of the same buffer through the column resulted in the recovery of the activator in the first 40 ml of eluate. No hemoglobin was detected and less than 1 % of the applied adenylate kinase activity was recovered in this fraction with 100 % recovery of the $(Ca^{2+}+Mg^{2+})$-ATPase activator. Continued elution of the column with increased pH and salt concentration lead to the removal of hemoglobin, adenylate kinase and other proteins. Protein content was determined by the method of Lowry et al. [8].

Adenylate kinase assay. This assay was similar to that described by Parcy et al. [9], except that Tris · HCl was used instead of a phosphate buffer and the reaction was initiated by addition of AMP rather than $MgCl_2$.

$(Ca^{2+}+Mg^{2+})$*-ATPase assay.* The ATPase assay used here was essentially the same as that described by Hanahan et al. [5], except that the histidine/imidazole buffer was replaced by a Tris · HCl buffer. In some experiments, ADP was used as a substrate instead of ATP. The reaction was carried out at 37 °C in a total volume of 1.5 ml. The reaction was terminated by the addition and thorough mixing of 2 vols of chloroform/methanol, 2 : 1 (v/v) instead of using 10 % trichloroacetic acid. Use of chloroform/methanol lead to reduction in the acidic hydrolysis of ATP. Upon centrifugation, all water-soluble compounds were recovered in the upper phase and a precipitate of denatured proteins was observed at the interface. An aliquot of the upper phase was analyzed for P_i essentially by the method of Fiske and SubbaRow [10], using perchloric acid instead of sulfuric acid. Methanol from the upper phase did not interfere in phosphate analysis. In most instances, activity is expressed as μmol of P_i released by 1 ml of packed cell equivalent membrane.

Separation of nucleotides and radioactivity analysis. In some experiments where radioactive nucleotide substrates were used, poly (ethyleneimine) cellulose thin-layer chromatography [11] in 2 M sodium acetate, pH 5.5, allowed the separation of reaction products (ATP, ADP, AMP, IMP+P_i). The spots visualized under ultraviolet light were removed by scraping and mixed with 1 ml of 1 M HCl and 15 ml of Aquasol (a scintillant) and radioactivity assayed using Beckman Model 2 liquid scintillation counter.

In experiments where only $^{32}P_i$ was measured, the reaction was stopped by addition of 10 % trichloroacetic acid instead of chloroform/methanol and centrifuged. An aliquot of the clear supernatant was treated with Norit to remove nucleotides and centrifuged. The $^{32}P_i$ recovered in the soluble supernatant obtained by centrifugation was counted for radioactivity.

RESULTS

A. General observations

It had been reported earlier [5] and subsequently repeatedly observed in this laboratory that a significant portion of $(Ca^{2+}+Mg^{2+})$-ATPase activity was lost when

hemoglobin-free human erythrocyte ghosts were prepared by lysis and subsequent washings in low osmolarity medium at pH 7.6. No ATPase activity was detected in the (membrane-free) hemolysate and washings. However, it was possible to increase the activity of the remaining membrane ATPase if this hemolysate was added to the

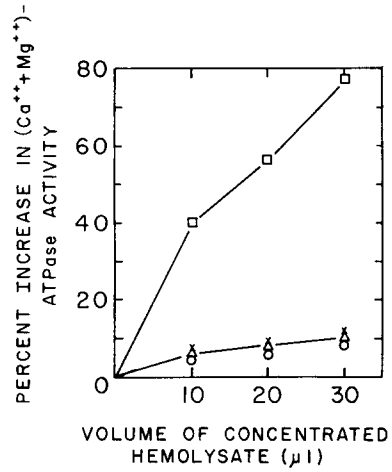

Fig. 1. Occurrence of ($Ca^{2+}+Mg^{2+}$)-ATPase activator in various hypotonic washes obtained during membrane preparation. 1 ml (packed) human erythrocytes was exposed 4 times in succession to 30 ml hypotonic media and the cell-free wash recovered and concentrated to 5 ml. Increasing amounts of these concentrates were reacted with 0.1 ml of 33 % packed-cell equivalent pH 7.6 membranes at 44 °C for 2 h in the ($Ca^{2+}+Mg^{2+}$)-ATPase assay medium. The percentage increase in P_i release was plotted against the volume of concentrated hemolysate added (□, 1st wash; ×, 2nd wash; △, 3rd wash; ○, 4th wash).

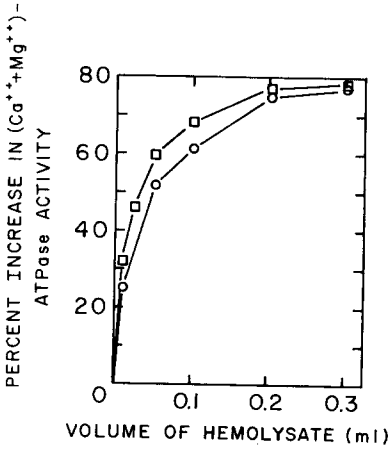

Fig. 2. Effect of dialysis on ($Ca^{2+}+Mg^{2+}$)-ATPase activator. 1 ml of the first (concentrated) hypotonic wash (see Fig. 1) was dialyzed against 3 l of water at 4 °C for 24 h. The sample volume increased to 1.4 ml. After proper dilution of a non-dialyzed sample, the two preparations were reacted with 0.1 ml of 30 % packed-cell equivalent pH 7.6 membranes. The percentage increase in P_i release was plotted against concentration of dialyzed (○) and non-dialyzed (□) hemolysates. Other conditions of assay were the same as in Fig. 1.

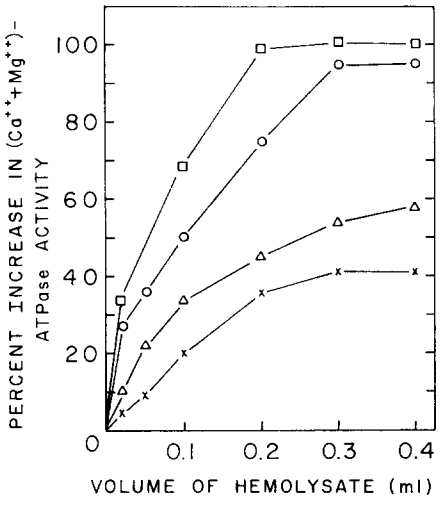

Fig. 3. Effect of temperature on (Ca^{2+} + Mg^{2+})-ATPase activator. The first (concentrated) hypotonic wash (see Fig. 1) was exposed to various temperatures (□, 0 °C; ○, 44 °C; △, 59 °C; ×, 73 °C) for 2 h and then added to the (Ca^{2+} + Mg^{2+})-ATPase assay medium. The percentage increase in P_i release was plotted against concentration of heat-treated hemolysate. Other conditions of assay were the same as in Fig. 1.

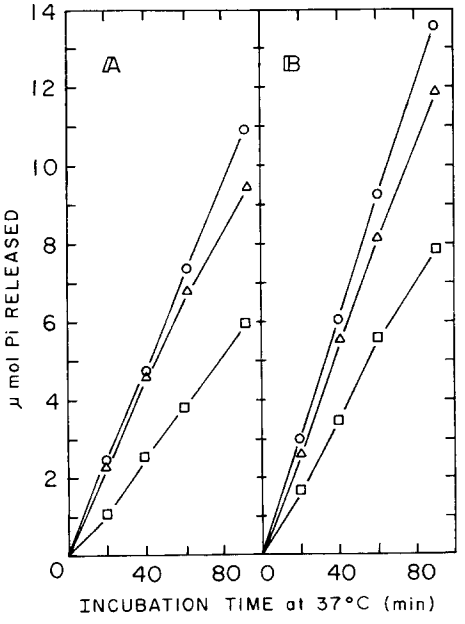

Fig. 4. Comparison of membrane (Ca^{2+} + Mg^{2+})-ATPase and corresponding activator obtained by two different procedures. pH 7.6 membranes and activator were prepared by (A) our procedure and by (B) the procedure of Bond and Clough [6]. Thus, 0.2 ml of 40 % packed cell equivalent membranes obtained by the two procedures were reacted with ATP in the presence of 0.1 ml membrane-free hemolysate (see Methods) obtained either by Bond and Clough's procedure (△) or by the present procedure (○), with a control where no activator was added (□). Reaction was stopped at specified time intervals and P_i formation was measured as described in Methods.

assay mixture. The $(Ca^{2+}+Mg^{2+})$-ATPase activity of the membranes was maximally stimulated by hemolysate obtained during the first hypotonic wash (Fig. 1). This established that loss of $(Ca^{2+}+Mg^{2+})$-ATPase activator occurred on hemolysis and not on further washing of hemolysed erythrocytes. Consequently, in subsequent experiments, the supernatant from first hypotonic wash only was used as source of activator. On further characterization of the activator it was found to be nondialyzable (Fig. 2), relatively heat-stable (Fig. 3) and non-extractable with organic solvent. A chloroform/methanol extract of the activator preparation had no demonstrable effect on the ATPase activity. Bond and Clough [6] demonstrated the presence of a soluble protein factor which stimulated $(Ca^{2+}+Mg^{2+})$-ATPase activity of hemoglobin-free human erythrocyte membranes. Since these investigators used EDTA during membrane preparation, a comparison of their hemolysate and membranes was made with membranes and hemolysate prepared by our procedure. While the latter membranes had slightly lower $(Ca^{2+}+Mg^{2+})$-ATPase activity levels compared to the Bond and Clough preparations, the hemolysate obtained from both procedures was capable of stimulating membranes obtained by the two procedures (Fig. 4). Thus, in essence, we confirm the results of Bond and Clough [6].

It was also observed in our laboratory [5] that expression of $(Ca^{2+}+Mg^{2+})$-ATPase activity was higher in membranes prepared at pH 5.8 instead of pH 7.6. However, in the present studies the osmolarity of the hypotonic buffer used in the first wash was 20 miosM instead of 40 miosM as previously reported [5]. It was necessary to confirm our previous observation on the difference between pH 5.8 and 7.6 membrane, under present experimental conditions. Results on the ATPase

TABLE I

RETENTION OF $(Ca^{2+}+Mg^{2+})$-ATPase ACTIVATOR ON MEMBRANES PREPARED IN TRIS/MALEATE BUFFER pH 5.8

Membranes at pH 7.6 were prepared as described in Methods, the first wash supernatant was designated as "7.6 hemolysate". Membranes at pH 5.8 were prepared from 3 ml packed erythrocytes as described in Methods and further washed (4 times) with 20 miosM Tris · HCl buffer pH 7.6 using 90 ml in each wash. The supernatants obtained after each wash after centrifugation at 27 000 × *g* for 30 min were combined and concentrated to a volume of 4.5 ml. This concentrated supernatant was designated as "5.8 wash". The effect of 0.25 ml of either "7.6 hemolysate" or "5.8 wash" on $(Ca^{2+}+Mg^{2+})$-ATPase of 0.1 ml of 22 % packed cell equivalent membrane preparations in a total volume of 0.75 ml ATPase assay medium at 37 °C was studied.

Type of membrane preparation	Type of hemolysate added	μmol P_i released/h per ml packed cell equivalent membranes
pH 7.6 membranes	None	8.8
pH 7.6 membranes	7.6 hemolysate	19.1
pH 7.6 membranes	5.8 wash	24.3
pH 5.8 membranes	None	14.0
pH 5.8 membranes	7.6 hemolysate	16.0
pH 5.8 membranes rewashed (×4) with 20 miosM Tris · HCl pH 7.6	None	12.3
pH 5.8 membranes rewashed (×4) with 20 miosM Tris · HCl pH 7.6	7.6 hemolysate	15.1
pH 5.8 membranes rewashed (×4) with 20 miosM Tris · HCl pH 7.6	5.8 wash	20.8

activity (Table I) of membranes prepared as described here confirm our earlier observations that membranes prepared at pH 5.8 exhibit higher ($Ca^{2+}+Mg^{2+}$)-ATPase activity than membranes prepared at pH 7.6.

In an extension of the above studies, it was observed that the initial hemolysate obtained upon hypotonic lysis at pH 5.8, when adjusted to pH 7.6, was capable of stimulating pH 7.6 membranes. On the other hand, the initial hemolysate from the pH 7.6 or 5.8 treatment had a minimal effect on pH 5.8 membrane (Table I and Fig. 6). Furthermore, it was noted that ($Ca^{2+}+Mg^{2+}$)-ATPase activator was retained on pH 5.8 membranes which could be partially released when these membranes were washed 4 times with 20 miosM Tris · HCl buffer pH 7.6. This washing was capable of stimulating pH 7.6 membrane ($Ca^{2+}+Mg^{2+}$)-ATPase.

B. Specificity of ($Ca^{2+}+Mg^{2+}$)-ATPase activity

The next step was to establish that the phosphate release due to the hemolysate was contributed by cleavage of the γ-position phosphate of ATP. A simple experiment was conducted in which membranes prepared either at pH 7.6 or at pH 5.8 were reacted with [γ-^{32}P]ATP. ($Ca^{2+}+Mg^{2+}$)-ATPase activity was measured both by radioactivity determination and by total P_i analysis (see Methods). Results obtained by radioactivity determination were found to be lower than those obtained by P_i analysis in pH 7.6 membranes, without added hemolysate. However the magnitude of difference was much greater in the case of pH 5.8 membranes (Fig. 5). Furthermore, no stimulation of $^{32}P_i$ release was observed with increasing concentration of hemolysate in either pH 7.6 or pH 5.8 membranes. Confirmation of these observations was obtained in another experiment in which it was found that freeze-thawed erythrocytes

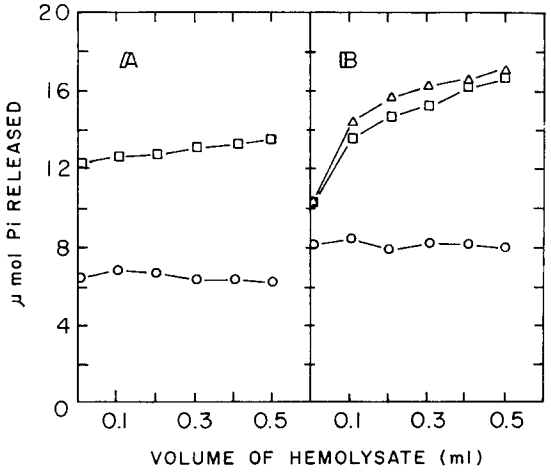

Fig. 5. Effect of hemolysate on pH 7.6 and pH 5.8 membrane ($Ca^{2+}+Mg^{2+}$)-ATPase activities using [γ-^{32}P]ATP as substrate. 0.2 ml of 47 % packed cell equivalent membranes prepared at pH 5.8 (A) and at pH 7.6 (B) were reacted with [γ-^{32}P]ATP in presence of various volumes of hemolysates (see Methods) obtained by erythrocyte lysis at pH 5.8 (□) and at pH 7.6 (△). P_i release was measured both by phosphorus analysis (□, △) and by $^{32}P_i$ radioactivity determination (○). Further details provided in Methods.

TABLE II

$(Ca^{2+}+Mg^{2+})$-ATPase ACTIVITY OF HUMAN ERYTHROCYTE MEASURED BY TOTAL P_i AND $^{32}P_i$ RELEASE

Human erythrocytes washed either in 0.172 M Tris · HCl, pH 7.6, or in 0.31 M histidine, pH 7.5*, were freeze-thawed 3 times before ATPase assay, in which [γ-^{32}P]ATP was present (further details in the text). It was noticed repeatedly that erythrocytes washed with 0.31 M histidine, pH 7.5, express more $(Ca^{2+}+Mg^{2+})$-ATPase activity as compared to the cells washed in 0.172 M Tris · HCl, pH 7.6. The explanation for this is not clear at this time.

Subject	μmol P_i released/ml packed cells per 2 h at 44 °C		
	Total P_i	$^{32}P_i$	$100\times{}^{32}P_i$/total P_i
P.S.	32.8	21.1	64
B.B.	35.5	23.0	65
P.S.*	43.7	27.4	63
B.B.*	45.9	31.9	69

also show 40 % less $^{32}P_i$ release as compared to total P_i release (Table II) when [γ-^{32}P]ATP was used as a substrate.

The observed difference in $^{32}P_i$ and total P_i release may be due to (a) further hydrolysis of ADP (product of ATPase), (b) decreased specific activity of γ-position ^{32}P-phosphate on ATP attributable to adenylate kinase activity, or (c) both (a) and (b). These possibilities were explored as described below.

In the first set of experiments where ADP was used as a substrate it was found

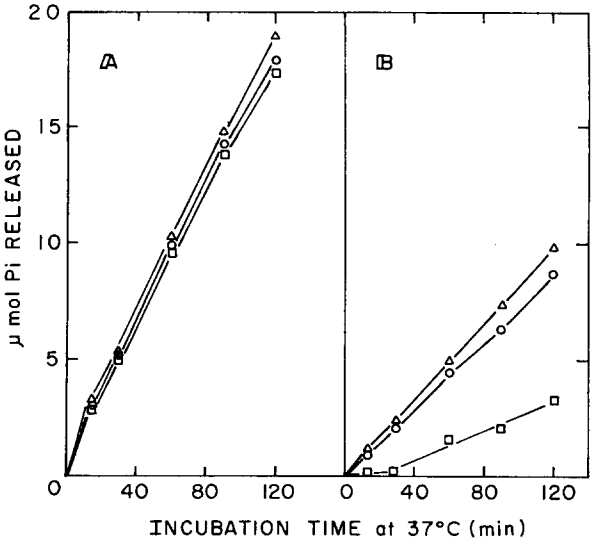

Fig. 6. Effect of hemolysate on pH 7.6 and pH 5.8 membranes $(Ca^{2+}+Mg^{2+})$-ATPase activities using ADP as substrate. 0.2 ml of 40 % packed cell equivalent membranes prepared at pH 5.8 (A) and pH 7.6 (B) were reacted with ADP (2 mM) in presence of varying volumes of the hemolysate (see Methods) obtained from a pH 7.6 lysis (□, none; ○, 0.05 ml, △, 0.1 ml). P_i release was measured at various time intervals.

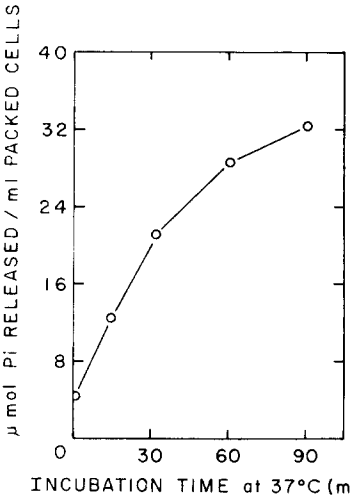

Fig. 7. Release of P_i from ADP by human erythrocytes. 0.2 ml of 49 % suspension of freeze-thawed (3 times) erythrocytes in 0.172 M Tris · HCl, pH 7.6 were reacted with ADP (2 mM) and rate of P_i release as a function of incubation time at 37 °C is plotted.

that pH 7.6 membranes could produce P_i from ADP but to a lesser extent as compared to ATP (Fig. 6). However on addition of membrane-free hemolysate to the reaction mixture, a substantial increase in P_i release from ADP (Fig. 6) was obtained. On the other hand, pH 5.8 showed a 4-fold higher release of P_i from ADP as compared to pH 7.6 and no additional stimulation of P_i release was achieved when hemolysate was added in the reaction mixture. Similarly, freeze-thawed erythrocytes also showed P_i release from ADP (Fig. 7). It was noted that the release of P_i from ADP by total erythrocytes (freeze-thawed) was still higher than any of the reconstituted systems of membranes and hemolysate. The hemolysate alone did not cause any release of P_i from ADP. It was also found that no P_i release was obtained when AMP was used as a substrate. This ruled out the effect of a nonspecific phosphatase.

In the second set of experiments, pH 7.6 membranes were reacted with [γ-^{32}P]-ATP and hemolysate in presence and absence of Ca^{2+} [5]. Subsequent to a 90 min incubation period, the reaction was terminated and an aliquot of the reaction mixture was mixed with hexokinase and glucose. The products before and after hexokinase treatment were separated by thin-layer chromatography (see Methods). The distribution of ^{32}P counts on the β- and γ-position phosphate of the nucleotides was calculated from counts in the glucose 6-phosphate and ADP spot. The data presented in Table III. show that there was an exchange of γ-position phosphate and β-position phosphate of ATP which caused a decreased specific activity of γ-position ^{32}P of ATP. It was interesting to note that such an exchange occurred in the absence of Ca^{2+} (Table III) This exchange, however, was minimal in the presence of membranes alone (in absence of hemolysate). Such a reaction could be explained only by the reversible action of adenylate kinase. Evidence of the presence of adenylate kinase activity under present conditions of assay were also provided by the production of [^{14}C]ATP and [^{14}C]-AMP from [^{14}C]ADP when reacted with hemolysate alone (Fig. 8). Adenylate kinase activities were quite comparable in hemolysates obtained by our procedure and by the

TABLE III

RELATIVE DISTRIBUTION OF ^{32}P RADIOACTIVITY IN γ- AND β-POSITION PHOSPHATES OF ATP UPON INCUBATION OF MEMBRANES WITH [γ-^{32}P]ATP IN ATPase MEDIUM WITH AND WITHOUT Ca^{2+} AND HEMOLYSATE

Membranes prepared at pH 7.6 were incubated at 37 °C in ATPase medium with or without Ca^{2+}, in absence and presence of hemolysate using [γ-^{32}P]ATP as a substrate. The reaction was terminated by chloroform/methanol (see Methods). An aliquot from the upper phase was further reacted with glucose and hexokinase (see Methods). Aliquots obtained (i) before and (ii) after hexokinase treatment were subjected to thin-layer chromatography to separate various reaction products and radioactivity in these were measured as detailed in methods.

	Incubation time for ATPase reaction (min)	% ^{32}P counts in various spots on poly(ethyleneimine) cellulose thin-layer chromatograms					Relative ^{32}P radioactivity on γ- and β-position phosphates of ATP	
		Glc-6-*P*	IMP+P_i	AMP	ADP	ATP	γ	β
1. Membranes in ATPase medium without Ca^{2+}								
(a) in absence of hemolysate								
(i)	0	1.2	2.3	1.1	0.3	95.1	93.9	0.5
(ii)	0	92.8	4.4	1.3	0.8	0.7		
(i)	90	0.9	25.3	4.1	1.9	67.8	64.7	2.1
(ii)	90	66.2	23.8	5.0	4.0	0.9		
(b) in presence of hemolysate								
(i)	0	0.6	1.4	0.9	0.3	96.7	95.4	0.5
(ii)	0	91.6	5.0	1.7	0.8	0.9		
(i)	90	0.5	19.8	2.6	5.8	71.3	33.9	37.1
(ii)	90	37.5	15.2	3.5	42.8	0.8		
2. Membranes in ATPase medium with Ca^{2+}								
(a) in absence of hemolysate								
(i)	0	0.6	1.3	1.0	0.3	96.8	95.6	0.5
(ii)	0	91.6	5.2	1.7	0.8	0.7		
(i)	90	0.5	10.6	2.3	0.7	86.0	84.4	0.5
(ii)	90	84.3	10.9	2.6	1.2	0.9		
(b) in presence of hemolysate								
(i)	0	0.6	1.4	0.9	0.3	96.7	95.4	0.5
(ii)	0	91.6	5.0	1.7	0.8	0.9		
(i)	90	0.5	9.4	2.0	3.9	84.2	38.0	45.0
(ii)	90	40.0	7.8	2.4	48.9	0.9		

method of Bond and Clough [6]. Similarly the turnover of [^{14}C]ATP was found to be much higher in pH 5.8 membranes as compared to the pH 7.6 membranes (in absence of hemolysate) (Fig. 9). This suggested the greater retention of adenylate kinase on pH 5.8 membrane as compared to pH 7.6 membranes.

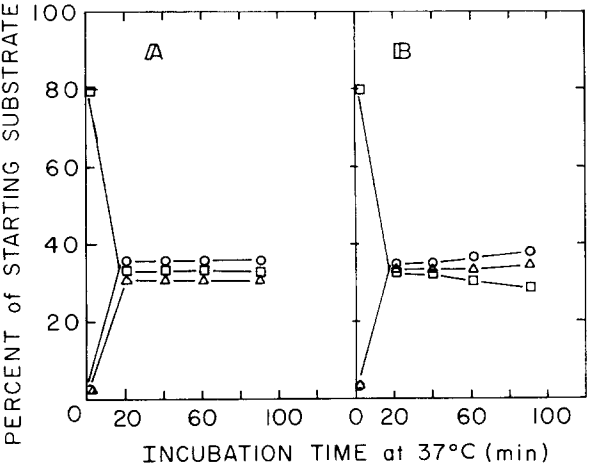

Fig. 8. Production of AMP and ATP from ADP by membrane-free hemolysate prepared by two different procedures. 0.2 ml of membrane free hemolysates obtained by the currently described method (A) and by the procedure of Bond and Clough (B) were reacted with [^{14}C]ADP (2 mM). The percent ^{14}C radioactivity distribution in various reaction products (○, AMP-IMP; △, ATP; □, ADP) at different time intervals is plotted. Further details on separation of reaction products and radioactivity measurement are given in Methods.

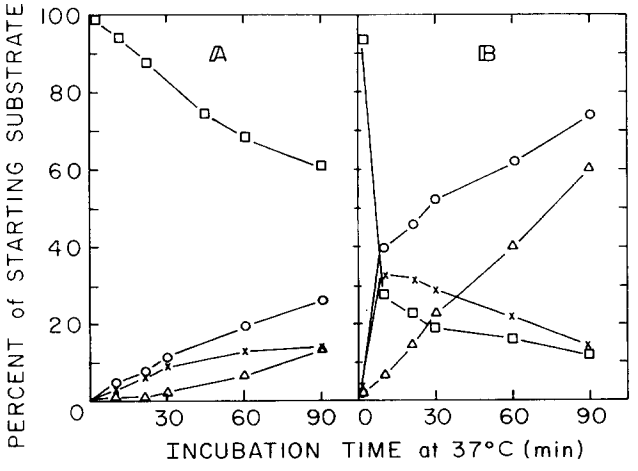

Fig. 9. Rate of turnover of [^{14}C]-ATP produced from [^{14}C]-ADP by pH 7.6 and pH 5.8 membranes. pH 7.6 (A) and pH 5.8 (B) membranes were reacted with [^{14}C]ADP (2 mM) in (Ca^{2+} + Mg^{2+})-ATPase medium. Percentage distribution of ^{14}C radioactivity in various reaction products (AMP + IMP, ○; ATP, ×; ADP, □) at different time intervals of incubation is plotted. P_i release (expressed as % of total ADP) was calculated by subtracting the % of ^{14}C radioactivity in ATP from % of ^{14}C radioactivity in AMP (△).

TABLE IV

PURIFICATION OF $(Ca^{2+}+Mg^{2+})$-ATPase ACTIVATOR AND ADENYLATE KINASE ACTIVITY FROM HEMOLYSATE BY CARBOXYMETHYL-SEPHADEX COLUMN CHROMATOGRAPHY

Eluting type: a = 20 mM Tris/maleate buffer, pH 6.8; b = 40 mM Tris/maleate buffer, pH 7.0; c = 40 mM Tris/maleate buffer, pH 7.1; d = 50 mM Tris · HCl buffer, pH 8.2; e = 90 mM KCl in 50 mM Tris · HCl buffer, pH 8.2.

Fraction	Eluting type	Solvent volume (ml)	Total protein (mg)	$(Ca^{2+}+Mg^{2+})$-ATPase activator†		Adenylate kinase activity		% Recovery		Fold purification	
				Total units	Units/mg protein	Total units*	Units/mg protein	$(Ca^{2+}+Mg^{2+})$-ATPase activator	Adenylate kinase	$(Ca^{2+}+Mg^{2+})$-ATPase activator	Adenylate kinase
Crude hemolysate	a	45	626.0	1384.0	2.21	379.0	0.605	—	—	0	—
1	a	83	27.4	1660.0	60.6	1.16	0.042	120 %	0.31	27.5	—
2	a	37	4.2	0	0	0.90	0.214	0	0.27	—	—
3	b	41	5.8	0	0	0.586	0.101	0	0.15	—	—
4**	c	135	135.0	0	0	384.0	2.84	0	101.3	—	4.7
5**	d	95	517.0	0	0	0	0	0	0	—	—
6	e	126	2.5	0	0	0	0	0	0	—	—

† One unit is equivalent to 40 % increase in $(Ca^{2+}+Mg^{2+})$-ATPase activity of pH 7.6 membranes using ATP as substrate.

* One unit represents μmol ADP formed/min at 22 °C.

** Hemoglobin present.

C. Purification studies

The above experiments, which were designed in part to demonstrate the presence of ADPase, are undoubtedly complicated in interpretation, because of the presence of adenylate kinase. Filtration of whole hemolysate on Sephadex G-75 did not give good separation of $(Ca^{2+}+Mg^{2+})$-ATPase activator from adenylate kinase and hemoglobin. The elution profile on this media was provocative, however, in that $(Ca^{2+}+Mg^{2+})$-ATPase activator was eluted at a volume indicative of a 50 000–60 000 molecular weight protein and appeared to separate from the bulk of adenylate kinase activity. However when the hemolysate was subjected to carboxymethyl-Sephadex column chromatography at pH 6.8, the $(Ca^{2+}+Mg^{2+})$-ATPase activator was recovered essentially in the first column volume, was free of hemoglobin and contained $< 1\%$ of the applied adenylate kinase activity. This fraction, when reacted with pH 7.6 membranes caused a reduction in P_i release from ADP without affecting the stimulation of P_i release from ATP (Fig. 10). In fact the number of units of $(Ca^{2+}+Mg^{2+})$-ATPase activator recovered was 1.2 times more than that of the crude load hemolysate (Table IV) when calculated from initial rates of activation (Fig. 10). This suggested that carboxymethyl-Sephadex chromatography might have removed some inhibitor of the $(Ca^{2+}+Mg^{2+})$-ATPase activator. This partially purified activator still showed four bands by gel electrophoresis (Fig. 11).

Even though nearly all adenylate kinase activity was removed in the hemolysate, some P_i release from ADP was still obtained on hemolysate addition to pH 7.6 membranes (Fig. 10). It seemed likely that this activity could be attributed to the combined action of any remaining adenylate kinase and $(Ca^{2+}+Mg^{2+})$-ATPase.

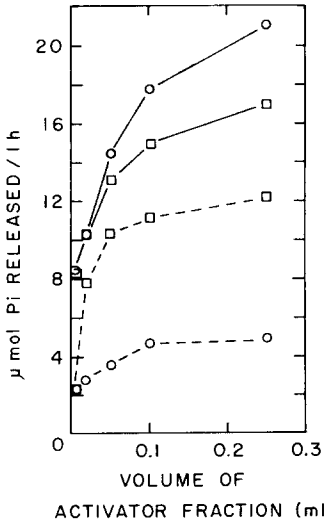

Fig. 10. Preferential activation of P_i release from ATP by a partially purified $(Ca^{2+}+Mg^{2+})$-ATPase activator in presence of membranes. 0.1 ml of 24.5 % packed-cell equivalent membranes prepared at pH 7.6 were reacted with ATP (—) and ADP (– – –) and varying volumes of crude activator fraction (hemolysate prior to chromatography) (□) and partially purified activator (○) obtained from carboxymethyl-Sephadex column (see Table IV) in total reaction volume of 0.75 ml. Results were plotted after proper dilution correction of ATP activity obtained in the case of partially purified activator.

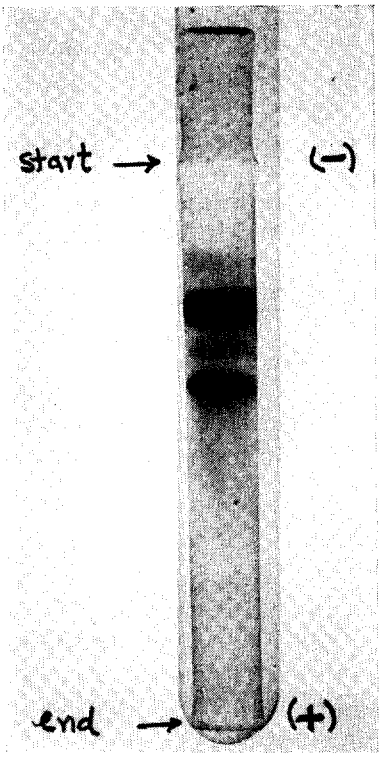

Fig. 11. Disc gel electrophoresis pattern of partially purified ($Ca^{2+}+Mg^{2+}$)-ATPase activator. A sample of 27 μg protein of a partially purified ($Ca^{2+}+Mg^{2+}$)-ATPase activator was separated by the method of Ornstein and Davis [12] with subsequent staining and destaining according to Fairbanks et al. [13].

TABLE V

EFFECT OF HEXOKINASE, GLUCOSE AND ACTIVATOR ON P_i RELEASE FROM ATP AND ADP BY pH 7.6 MEMBRANES

Membranes were reacted with ATP or ADP in the presence (8 units/ml incubation medium) or absence of hexokinase and glucose (2 mM) with and without partially purified ($Ca^{2+}+Mg^{2+}$)-ATPase factor at 37 °C for 60 min.

Reaction mixture	μmol P_i released/ml packed cell equivalent membrane	
	from ATP	from ADP
pH 7.6 membranes	6.05	1.02
pH 7.6 membranes plus partially purified ($Ca^{2+}+Mg^{2+}$)-ATPase activator	17.42	3.64
pH 7.6 membranes plus hexokinase and glucose	0.82	0.204
pH 7.6 membranes plus hexokinase, glucose and partially purified ($Ca^{2+}+Mg^{2+}$-ATPase activator	3.63	0.17

In order to completely rule out the presence of ADPase in these erythrocyte membranes, an experiment was conducted in which an excess of hexokinase and glucose was added to the reaction mixture so that the pool of ATP formed from ADP by membrane adenylate kinase would be used faster (and preferentially) by hexokinase to produce glucose 6-phosphate and thus reduce any P_i release by action of ATPase. The results of this experimental approach are summarized in Table V and decisively show that there is no P_i release from ADP if hexokinase and glucose are present in the reaction mixture. Hexokinase and glucose when added separately did not affect P_i release from either ADP or ATP.

In another experiment the possibility of the following reaction:

$$ATP \rightarrow AMP + PP_i \rightarrow 2\ P_i$$

enhancing release of P_i from ATP in the presence of $(Ca^{2+} + Mg^{2+})$-ATPase activator was eliminated, since sodium pyrophosphate was not hydrolysed either by membranes or by a partially purified $(Ca^{2+} + Mg^{2+})$-ATPase activator alone or in the presence of both.

DISCUSSION

The results of this investigation essentially confirm the observations of Bond and Clough [6] and show stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity of human erythrocyte membranes by a soluble protein obtained from membrane-free hemolysates of these cells. It was further shown that this stimulation was attributable to an enhanced cleavage of the γ-position phosphate on substrate ATP. This activator did not possess any ATPase or adenylate kinase activity as such. Though inorganic phosphate was released when ADP was used as a substrate, the presence of an ADPase as such in these membranes was eliminated since the P_i release could be related entirely to the combined action of the $(Ca^{2+} + Mg^{2+})$-ATPase and adenylate kinase.

Further study on this stimulatory activity revealed that only 12 % of the cell's activator could produce maximal stimulation of the $(Ca^{2+} + Mg^{2+})$-ATPase activity found in the same amount of cells. Thus, it is conceivable that membranes, such as those prepared at pH 5.8, can retain this factor in saturable amounts, whereas this is not the case for membranes prepared at pH 7.6 in low osmolarity buffers. Partial purification of the soluble activator revealed a protein in the molecular weight range of 50 000–60 000 with no detectable enzymatic activity. This preparation could be stored at −25 °C for at least one month without loss in stimulatory capacity.

The physiological role of the $(Ca^{2+} + Mg^{2+})$-ATPase activator is not clear at this time. However, it is provocative to consider that this activator may be related to Ca^{2+} transport in erythrocytes which is associated with $(Ca^{2+} + Mg^{2+})$-ATPase [3, 6] or may be a part of a system of actomyosin-like fiber thought to maintain the flexibility and characteristic biconcave shape of this cell [14].

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REFERENCES

1 Harrison, D. G. and Long, C. (1968) J. Physiol. 199, 367–381
2 Long, C. and Mouat, B. (1971) Biochem. J. 123, 829–836
3 Schatzman, H. J. and Vincenzi, F. F. (1969) J. Physiol. Lond. 201, 369–395
4 Lee, K. S. and Shin, B. C. (1969) J. Gen. Physiol. 54, 713–729
5 Hanahan, D. J., Ekholm, J. and Hildenbrandt, G. (1973) Biochemistry 12, 1374–1387
6 Bond, G. H. and Clough, D. L. (1973) Biochim. Biophys. Acta 323, 592–599
7 Glynn, I. M. and Chappell, J. B. (1963) Biochem. J. 90, 147–149
8 Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
9 Parcy, J. R., Horenstein, J. M., Goins, L., Jones, D. and Laver, M. (1974) J. Biol. Chem. 249, 1874–1879
10 Fiske, H. and SubbaRow, J. (1925) J. Biol. Chem. 66, 375–400
11 Randerath, K. and Randerath, E. (1967) Methods in Enzymology XII, Part A (Grossman, L. and Moldave, K., eds) pp. 323–361, Academic Press, New York
12 Ornstein, L. and Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
13 Fairbanks, H., Steck, A. and Wallach, E. E. (1971) Biochemistry 10, 2606–2617
14 Rosenthal, A. S., Kregenow, F. M. and Moser, H. (1970) Biochim. Biophys. Acta 196, 254–262