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ERYTHROCYTE MEMBRANE-BOUND ENZYMES
ATPase, PHOSPHATASE AND ADENYLATE KINASE IN HUMAN, BOVINE AND PORCINE ERYTHROCYTES

MICHAEL HELLER* AND DONALD J. HANAHAN

Department of Biochemistry, College of Medicine, The University of Arizona, Tucson, Ariz. 85724 (U.S.A.)

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SUMMARY

1. The activities of three enzymes, ATPase (ATP phosphohydrolase, EC 3.6.1.3), *p*-nitrophenyl phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) and adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) were determined in erythrocyte membranes of three mammalian species (human, porcine and bovine).

2. The (Na^+-K^+)-activated ATPase and K^+-activated *p*-nitrophenyl phosphatase are present in the membranes of human and porcine erythrocyte but not in those of the bovine species.

3. Treatment of the membranes of human erythrocyte with high concentrations of NaI in the presence of ATP, solubilized part of the ouabain insensitive ATPase and most of the residual adenylate kinase. The (Na^+-K^+)-activated ATPase remains insoluble, but its recovery is only about 34 %. The K^+-activated *p*-nitrophenyl phosphatase also remains insoluble, but is fully recovered in the sediment.

4. An ATPase activated by Ca^{2+} in the concentration range of 0.30–0.6 mM, is present in human, but absent in bovine, erythrocyte membranes. This enzyme is not inhibited by ouabain.

5. Ca^{2+} inhibits the K^+-activated *p*-nitrophenyl phosphatase of the human erythrocyte membrane in the concentration range of 0.4–0.8 mM. ATP or ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid reverses the inhibition.

INTRODUCTION

Studies in this laboratory have been concerned with biochemical characterization of the plasma membranes of mammalian erythrocytes. One direction to these investigations has been to elucidate the chemical nature of the lipids in these membranes while another has been to explore the characteristics of the enzymes and proteins present therein. In order to provide a basis for comparison of membranes from different species, two enzyme systems, namely acetylcholine esterase and the

* Present address: Department of Biochemistry, Hebrew University, Hadassah Medical School, Jerusalem, Israel.

ATPases *plus* *p*-nitrophenyl phosphatase have been investigated in some detail. The latter enzyme system forms the subject matter of the current presentation, whereas results with the former system will be reported elsewhere[1].

In this study we have measured the ATPase and *p*-nitrophenyl phosphatase activities in erythrocyte membranes of three mammalian species: human, bovine and porcine. An attempt was made to separate the ouabain-sensitive and -insensitive ATPases of human erythrocytes by extraction with concentrated NaI by the method of NAKAO *et al.*[2]. The effects of this treatment on activity of these ATPases and of the ouabain-sensitive phosphatase and residually membrane-bound adenylate kinase have been compared. The influence of Ca^{2+} on human and bovine erythrocyte membrane ATPase and human erythrocyte *p*-nitrophenyl phosphatase has been investigated.

MATERIALS AND METHODS

Bovine and porcine blood were obtained fresh from adult animals and clotting was prevented by use of an acid–citrate–dextrose, USP solution (0.8 g citric acid, 2.2 g sodium citrate, and 2.45 g dextrose per 100 ml solution). Outdated human blood in sterile bags containing acid–citrate–dextrose was kindly donated by the Red Cross Blood Bank (Tucson, Arizona). The erythrocyte membranes were prepared according to DODGE *et al.*[3] using hypotonic Tris–HCl buffer (pH 7.5). In addition, bovine erythrocyte ghosts were prepared in the presence of 5 mM Ca^{2+} or Mg^{2+} according to BURGER *et al.*[4].

The membranes from cow and pig red cells were suspended in 10 mM Tris–HCl buffer (pH 7.5), whereas the human membrane also contained 1 mM EDTA. The protein concentrations were in the range 3–4 mg/ml. Human erythrocyte membranes were treated with 2 M NaI as outlined by NAKAO *et al.*[2]. The ghosts in suspension, which appeared normal under the phase contrast microscope, were frozen to $-70°$ (in dry ice in acetone) then thawed at 40° (water bath) 3 times. 10 ml of the suspension of the ghosts were mixed with an equal volume of a solution containing 4 M NaI, 10 mM $MgCl_2$, 5 mM EDTA, 10 mM ATP, 10 mM dithiothreitol at pH 8. The mixture was stirred gently for 30 min in a water–ice bath and this is referred to as the "2 M NaI-treated membranes" (see Table I). Water was then added to lower the concentration of the NaI to 0.8 M, and the suspension was mixed again for 30 min. This mixture is referred to as the "0.8 M NaI-treated membranes". The supernatant obtained following centrifugation at 20000 × *g* for 30 min is designated "sup I". The sediment was washed 3 times with 5 mM EDTA at pH 8, and is designated "Pct III". Before assaying for enzymatic activities, the various fractions were dialyzed overnight against 100 vol. of 1 mM EDTA, 1 mM dithiothreitol in 10 mM Tris–HCl buffer (pH 7.5). All the operations were done at 4° unless otherwise stated. The fractions were stored at $-30°$ for several weeks without appreciable loss of the enzymatic activities.

Enzymatic assays

ATPase and adenylate kinase. The assay of these two activities, which involves the conversion of ATP to ADP, is performed in presence of an auxiliary enzyme system containing pyruvate kinase and lactate dehydrogenase. This has permitted the measurement of ADP formation through the observation of NADH oxidation at

340 nm. The reaction mixtures had a final volume of 2.5 ml, using a thermostatted Gilford 2000 recording spectrophotometer. (a) Total ATPase was assayed at pH 7.4, 37°, in the presence of Na^+ and K^+ with the following components at their final concentrations: 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 133 mM NaCl, 14 mM KCl, 3.63 units/ml lactate dehydrogenase (lactate: NAD Oxidoreductase, EC 1.1.1.27); 2.4 units/ml of pyruvate kinase (ATP–pyruvate phosphotransferase, EC 2.7.1.40), 0.5 mM NADH, 2.5 mM ATP, and 0.4–1 munit of ATPase (or 0.25–1 mg membrane protein). (b) Ouabain-insensitive ATPase was assayed as described above except that 0.1–0.5 mM ouabain was added to the reaction mixture before the initiation of the reaction. Alternatively, the reaction was performed as described for "total ATPase", the rate of the reaction was recorded, then ouabain was added, and the new rate was recorded again. The activity of the ouabain-sensitive ATPase was calculated by subtracting the rate of (b) from that of (a).

Adenylate kinase was assayed immediately after the rate of the ATPase reaction was recorded (as described for the ATPase) by the addition of AMP to a final concentration of 0.2 mM. Adenylate kinase converts 1 mole of the AMP 1 mole of ATP to 2 moles of ADP, which is observed as the oxidation of 2 moles of NADH.

1 unit of ATPase is defined as the amount of enzyme which catalyzes the oxidation of 1 μmole NADH per h by the auxiliary enzyme system, as determined from the decrease in absorbance at 340 nm. Each μmole of NADH oxidized, represents the formation of 1 μmole of ADP from ATP. The molar extinction coefficient of NADH at 340 nm was taken as $6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$. The specific activity of the ATPase is expressed as units/mg protein. The definitions of the activity and specific activity of the adenylate kinase are similar to those of ATPase, except that the values of the μmoles of NADH which were oxidized are divided by 2 due to the fact that each mole of AMP and ATP produce 2 moles of ADP. The activity of adenylate kinase was computed from the absorbance values obtained after the addition of AMP to the reaction mixture *minus* the absorbance values obtained before the addition of AMP.

Phosphatase (p-nitrophenyl phosphatase). The phosphatase activity was measured as the rate of hydrolysis of *p*-nitrophenyl phosphate at 418 nm in the Gilford recording spectrophotometer. (a) Total phosphatase was measured in a final volume of 2.5 ml at 37° containing 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 5 mM *p*-nitrophenyl phosphate, 10–15 mM KCl and 0.2 mg membrane protein. (b) Ouabain-insensitive phosphatase was assayed as above except that 0.1–0.5 mM ouabain was present or was added during the run.

The ouabain sensitive phosphatase activity was estimated by subtracting the value of (b) from that of (a). The reaction was started by the addition of the substrate, and the increase of absorbance at 418 nm was continuously recorded. At pH 7.5 it was observed that non-enzymatic hydrolysis of the substrate was negligible. The molar extinction coefficient at 418 nm of the product, *p*-nitrophenol, at pH 7.5 is $11.5 \cdot 10^3\ M^{-1} \cdot cm^{-1}$. One unit of enzyme is defined as that amount of enzyme required to liberate 1 μmole of *p*-nitrophenol per h at 37° under the conditions specified above. Specific activity is defined as units/mg of protein.

All reactions described here had rates linear with respect to time for at least 15 min and with respect to membrane protein concentration in the ranges mentioned above. Protein concentrations were determined by the method of LOWRY *et al.*[5] with serum albumin as a standard.

Reagents

Ouabain (strophanthin G), *p*-nitrophenyl phosphate, *p*-nitrophenol, dithiothreitol and pyruvate were purchased from Calbiochem, ATP (Tris salt) Trizma base, ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), and EDTA were Sigma products. Lactate dehydrogenase (360 units/mg), pyruvate kinase (150 units/mg), adenylate kinase (360 units/mg), phosphoenolpyruvate (tricyclohexyl ammonium salt), NADH (disodium salt), AMP and ADP (free acids) were purchased from Boehringer Mannheim Corp.

RESULTS

The continuous spectrophotometric assay of ATPase and adenylate kinase

Both of the enzymes, ATPase and adenylate kinase, catalyze reactions in which ADP is formed:

$$ATP + H_2O \rightarrow ADP + P_i \qquad \text{(ATPase)} \qquad (1)$$

$$ATP + AMP \rightleftharpoons 2\ ADP \qquad \text{(adenylate kinase)} \qquad (2)$$

These reactions may be coupled to an auxiliary enzyme system such as:

$$ADP + \text{phosphoenolpyruvate} \rightleftharpoons \text{pyruvate} + ATP \qquad \text{(pyruvate kinase)} \qquad (3)$$

$$\text{Pyruvate} + NADH \rightleftharpoons \text{lactate} + NAD^+ \qquad \text{(lactate dehydrogenase)} \qquad (4)$$

A theoretical treatment of the kinetics of the problem of coupling auxiliary enzymes to a reaction and the practical considerations for implementing the results of the kinetic analysis were presented by McCLURE[6]. Lactate dehydrogenase from rabbit muscle is not affected by any of the components present in the reaction mixture. Despite the fact that pyruvate kinase is activated by K^+ and inhibited by Na^+ and Ca^{2+} (ref. 7), which are involved in the "pump" ATPase reaction, these ions have practically no effect on the pyruvate kinase reaction in either direction (neither activation nor inhibition) when the auxiliary enzymes are present in large excess. SCHONER *et al.*[8], in assaying the activity of brain ATPase, found the same results by the spectrophotometric determination of ADP and the colorimetric determination of Pi. SCHWARTZ *et al.*[9], also obtained a correspondence of the two methods with heart ATPase.

The spectrophotometric assay method has the advantage of measuring activity at constant concentrations of ATP due to the ATP-regenerating system constantly removing the inhibitory ADP and maintaining steady-state conditions.

In the present study the adenylate kinase was not affected by the various components present in the reaction mixture (Na^+, K^+, Mg^{2+} and ouabain). High levels of adenylate kinase might have been expected to compete for ADP with the auxiliary enzyme system. However, since the auxiliary enzyme system was several hundred-fold in excess, the adenylate kinase did not interfere.

Treatment of human erythrocyte membranes with NaI

Membranes prepared from human erythrocytes and suspended in isotonic Tris–HCl buffer appeared to be round upon examination using the phase contrast microscope. Occasionally, some crenated forms were observed. These forms were observed whether the membranes were prepared from freshly drawn or outdated

TABLE I

ATPase, ADENYLATE KINASE AND *p*-NITROPHENYL PHOSPHATASE IN HUMAN ERYTHROCYTE MEMBRANES

Preparation of membranes and the NaI treatment are described in the text. n.d. or n.c. means "not determined" or "not calculated". A unit of activity is defined as 1 μmole ADP formed per h. Specific activity is defined as units/mg protein. The total units (*e.g.* 20.8 in ghost "total ATPase") refer to the activity of a membrane preparation containing 38.6 mg protein.

Fraction	*Expt.*	*Total ATPase*		*Ouabain-insensitive ATPase*		*(Na^+-K^+)-activated ATPase*		*Adenylate kinase*		*p-Nitrophenyl phosphatase*		
		Units	*Specific activity*	*Units*	*Specific activity*	*Units*	*Specific activity*	*Units*	*Specific activity*	*Units*	*Total*	*Ouabain-insensitive*
Ghost (intact)	A	20.8	0.54	11.25	0.29	9.55	0.25	13.8	0.36			
Ghost (frozen and thawed)	A	18.8	0.49	12.8	0.33	6.00	0.16	14.2	0.37	9.63	0.25	0.15
	A	22.7	0.59	12.4	0.32	10.2	0.27	25.2	0.66			
	B	33.2	0.86	19.0	0.49	14.3	0.38	12.85	0.33			
	B	40.8	1.05	22.3	0.55	19.2	0.47	9.65	0.25			
2 M NaI-treated membranes	A	9.9	0.26	9.9	n.c.	n.d.	n.d.	6.22	0.16			
	B	5.2	0.13	5.2	0.13	n.d.	n.d.	16.0	0.41			
0.8 M NaI-treated membranes	A	5.2	0.13	5.2	0.13	n.d.	n.d.	10.4	0.27			
Sup I	A	3.39	n.c.	3.3	n.c.			6.0	n.c.			
	B	4.15	0.21	3.95	0.19	n.c.	0.01					
Pct III	A	5.0	n.c.	2.68	n.c.	2.36		0.4	—	10.5	0.54	0.24
	B	12.5	0.685	6.24	0.35	6.05	0.33	—	—			
	B	12.7	0.697	8.3	0.46	6.25	0.33	0.2	0.025			
	B	14.5	0.800	10.65	0.58							

blood. When the membranes were suspended in hypotonic Tris–HCl buffer, their diameter increased and the crenated extensions disappeared, indicating that they still behaved as osmometers. Membranes subjected to freezing and thawing became fragmented, but the fragments still revealed an organized structure under the phase contrast microscope, whereas "Pct III" did not.

The ATPase activity of the human erythrocyte membrane as shown in Table I is quite low, compared to the activity of membranes from other tissues[10]. The values shown in Table I agree with the values reported by others[10,11]. Membranes treated with NaI as described in MATERIALS AND METHODS were assayed for activities of the three enzymes. Depressed total ATPase and no ouabain-sensitive ATPase were observed in the presence of NaI in the 2 M NaI, 0.8 M NaI and Supernatant I fractions. However, after three washes with 5 mM EDTA at pH 8, 40 % of the total ATPase activity, nearly half of which was ouabain-sensitive, was observed in the precipitate ("Pct III"). This represents no net purification or alteration of the ATPase activities of the erythrocyte. All phosphatase activity was recovered in "Pct III" with approx. 2-fold purification. Adenylate kinase was recovered almost entirely in the "Supernatant I" fraction.

Enzyme activities in membrane from human, porcine and bovine erythrocytes

The specific activities of the (Na^+-K^+)-activated ATPase, *p*-nitrophenyl phosphatase and adenylate kinase in the membranes of human, porcine and bovine erythrocytes were compared. Table II shows that although the porcine erythrocyte

TABLE II

ATPase, *p*-NITROPHENYL PHOSPHATASE AND ADENYLATE KINASE IN HUMAN, BOVINE AND PORCINE ERYTHROCYTE MEMBRANES

Preparation of ghosts, by hemolysis of the erythrocytes, in the absence or presence of divalent cations, and the assay conditions, are given in the MATERIALS AND METHODS. Activity of *p*-nitrophenyl phosphatase was assayed in the presence of 5 mM $MgCl_2$ and 10–15 mM KCl. Ouabain was added at final concentrations of 0.4 mM to bovine erythrocyte membranes and 0.2 mM to human and porcine erythrocyte membranes, respectively.

Conditions	*Specific activity (units/mg protein)*				
	ATPase		*p-Nitrophenyl phosphatase*		*Adenylate kinase*
	Total	*Ouabain-insensitive*	*Total*	*Ouabain-insensitive*	
Bovine					
Tris buffer*	0.35	0.35	0.016	0.015	0.80
Tris buffer* + 5 mM Ca^{2+}	0.35	0.34	0.017	0.015	0.50
Tris buffer* + 5 mM Mg^{2+}	0.35	0.33	0.017	0.015	0.85
Porcine					
Tris buffer*	3.95	2.30	0.063	0.038	0.24
Human					
Tris buffer*	0.70	0.42	0.251	0.147	0.40

* 10 mM Tris–HCl buffer (pH 7.4).

membrane ATPase is 4–5 times more active than those of human, it has only 1/4 of the ouabain-sensitive or -insensitive phosphatase activity. The two enzymes from these species showed a ouabain-sensitive component comprising 40 % of the total activity. On the other hand, bovine erythrocyte membranes prepared either in the absence or the presence of divalent cations had lower ATPase and phosphatase activities than pig or human. Both activities of the bovine membranes had no ouabain-sensitivity which correlates with the absence of the monovalent cation "pump mechanism" in these high sodium cells[22].

Adenylate kinase activity was detected in the membranes of the erythrocytes of all three species tested. If the ratios of the adenylate kinase and total ATPase (activities given in Table II) are compared, then the pig has the lowest, and the cow the highest value.

Influence of cations, chelating agents and ouabain on membrane ATPase and p-nitrophenyl phosphatase activity

Tables III and IV show the effects of Ca^{2+} on the erythrocyte membrane

TABLE III

ATPase ACTIVITY IN "PCT III". EFFECT OF Na^+, K^+, Ca^{2+}, EDTA, OUABAIN

The assay conditions are given in MATERIALS AND METHODS. "Pct III" is the particulate fraction obtained from human erythrocyte membranes following NaI treatment as described in the text.

Expt. No.	*Additions (mM)**					*Specific activity (units/mg protein)*
	Na^+	*K^+*	*Ca^{2+}*	*Ouabain*	*EDTA*	
1	—	—	—	—	—	0.62
	133	14	—	—	—	0.86
	133	14	—	0.20	—	0.52
	133	14	0.50	0.20	—	1.09
	133	14	1.00	0.20	—	0.84
	133	14	1.00	0.20	0.92	1.05
2	—	—	0.38	—	—	0.47
	—	—	0.75	—	—	0.32
	—	—	0.75	—	0.90	0.45
	124	13	0.38	—	—	0.68
	124	13	0.75	—	—	0.43
	124	13	0.75	—	0.90	0.59
	124	13	0.38	0.19	—	0.60
	124	13	0.75	0.19	—	0.33
	124	13	0.75	0.19	0.90	0.51
3	—	—	—	—	—	0.33
	—	—	—	0.23	—	0.33
	—	—	0.59	—	—	0.56
	—	—	0.59	0.23	—	0.52
	133	14	—	—	—	0.52
	133	14	—	0.23	—	0.38
	133	14	0.59	—	—	0.64
	133	14	0.67	—	—	0.61
	133	14	0.59	0.23	—	0.49
	133	14	0.67	0.23	—	0.56

* Final conc., millimolar.

TABLE IV

ATPase IN BOVINE ERYTHROCYTE MEMBRANES. EFFECT OF Na^+, K^+, Ca^{2+}, OUABAIN AND EGTA

Assay conditions are given in the text. Bovine membranes were prepared by hemolysis of intact cells in 10 mM Tris–HCl buffer (pH 7.5).

Expt. No.	*Additions (mM)**					*Specific activity (units/mg protein)*
	Na^+	*K^+*	*Ouabain*	*Ca^{2+}*	*EGTA*	
1	—	—	—	—	—	0.35
	—	—	0.40	—	—	0.30
	104	10	—	—	—	0.38
	104	10	0.40	—	—	0.30
	—	—	0.40	0.40	—	0.27
	—	—	0.40	0.80	—	0.26
	—	—	0.40	0.80	1.35	0.25
	104	10	0.40	0.40	—	0.29
	104	10	0.40	0.80	—	0.28
	104	10	0.40	0.80	1.35	0.26
2	—	—	—	—	—	0.33
	132	14	—	—	—	0.41
	132	14	0.20	—	—	0.33
	132	14	0.20	1.00	—	0.33

* Final conc., millimolar.

TABLE V

p-NITROPHENYL PHOSPHATASE IN HUMAN ERYTHROCYTE MEMBRANE. EFFECT OF Mg^{2+}, K^+, Na^+, AND OUABAIN

See Table II for details.

Expt. No.	*Additions (mM)**				*Specific activity (units/mg protein)*
	Mg^{2+}	*K^+*	*Na^+*	*Ouabain*	
A	1	—	—	—	0.14
	5	—	—	—	0.15
	10	—	—	—	0.14
B	5	—	—	—	0.16
	5	5	—	—	0.24
	5	20	—	—	0.26
	5	—	5	—	0.16
	5	—	10	—	0.16
	5	—	100	—	0.13
C	5	—	—	—	0.15
	5	20	—	—	0.28
	5	—	—	0.50	0.15
	5	20	—	0.50	0.15
Pct III	5	—	—	—	0.25
	5	10	—	—	0.56
	5	15	—	—	0.49
	5	15	—	0.50	0.25

* Final conc., millimolar.

ATPase of human and bovine blood. In Table III, the hydrolysis of ATP by human erythrocyte membranes is shown to be increased optimally in a restricted range of concentrations of Ca^{2+}. NaI-extracted human membrane residue ("Pct III") was used as the enzyme source. Ouabain appears to have no effect on this activation (Table III, Expt. 3). Inhibition of hydrolysis by higher concentrations of Ca^{2+} is reduced by addition of EDTA. Bovine erythrocyte membranes (Table IV) are observed to have a small amount of ouabain-sensitive ATPase activity, and are further inhibited only slightly by the addition of high Ca^{2+} concentrations.

The data in Table V show that human erythrocyte membrane phosphatase is activated by K^+, but inhibited by Na^+ in 100 mM concentration. The activating effect of K^+ was abolished by ouabain, but this compound has no inhibitory effect when used alone. These properties were also exhibited by the NaI-extracted residue "Pct III".

The reported inhibitory effects of Ca^{2+} on the ouabain-sensitive ATPase in human erythrocytes[13] are hard to defect because of the presence in the same preparation of the other enzyme which is Ca^{2+} activated (*cf.* Table IV). However, Ca^{2+} at the concentration range of 0.4–0.8 mM inhibited the K^+-activated *p*-nitrophenyl phosphate hydrolysis by the human erythrocyte membrane (Table VI). The inhibitory effect of Ca^{2+} was independent of K^+ concentration. The inhibition by Ca^{2+} was relieved by the addition of ATP or ATP + EGTA. It, therefore, seems that the inhibitory effect of Ca^{2+} on the K^+-activated phosphatase corroborates the observation

TABLE VI

p-NITROPHENYL PHOSPHATASE OF HUMAN ERYTHROCYTE MEMBRANE. EFFECT OF K^+, Ca^{2+}, ATP, EGTA AND OUABAIN

Assay conditions are given in MATERIALS AND METHODS and includes 5 mM $MgCl_2$ present.

*Additions (mM)**					*Specific activity (units/mg protein)*
K^+	*Ouabain*	Ca^{2+}	*ATP*	*EGTA*	
—	—	—	—	—	0.10
—	0.6	—	—	—	0.13
—	0.6	0.4	—	—	0.09
—	0.6	0.8	—	—	0.09
—	0.6	0.8	0.4	—	0.11
—	0.6	0.8	0.4	0.55	0.12
11	—	—	—	—	0.22
11	—	0.4	—	—	0.14
11	—	0.8	—	—	0.11
11	—	0.8	0.4	—	0.16
11	—	0.8	0.4	0.55	0.18
54	—	—	—	—	0.19
54	0.6	—	—	—	0.15
54	0.6	0.4	—	—	0.10
54	0.6	0.8	—	—	0.08
54	0.6	0.8	0.4	—	0.20
54	0.6	0.8	0.4	0.55	0.18

* Final conc., millimolar.

made by DUNHAM AND GLYNN[18] and by EPSTEIN AND WHITTAM[13] on the inhibitory effect of Ca^{2+} on the (Na^+-K^+)-activated ATPase. The presence of a Ca^{2+}-activated *p*-nitrophenyl phosphatase in human erythrocyte membranes similar to a Ca^{2+}-activated ATPase was not detected.

DISCUSSION

The results presented here show the relationship between the K^+-activated phosphatase and the (Na^+-K^+)-activated ATPase in human, porcine and bovine erythrocytes. JUDAH *et al.*[14] were the first to describe the presence of K^+-activated phosphatase, which is ouabain sensitive, in human erythrocytes. GARRAHAN *et al.*[15] have investigated the kinetic behavior of this enzyme in more detail. The latter authors concluded that only one phosphatase is responsible for the hydrolysis of various phosphate compounds used to assay its activity. The reaction studied was activated by K^+ and inhibited by ouabain.

COLDMAN AND GOOD[12] and DUGGAN *et al.*[16] and others have compared the K^+/Na^+ molar ratio in the erythrocytes of various species, and from the data shown in these reports it may be inferred that the erythrocytes having a high K^+/Na^+ molar ratio contain (Na^+-K^+)-activated ATPase. This proposal is supported by data in this current paper. It could be argued that membranes obtained from bovine erythrocytes, using the procedure of DODGE *et al.*[3], do not represent the total membrane proteins due to fragmentation and losses during hemolysis and subsequent washings[4]. Therefore, it might be reasoned that the (Na^+-K^+)-activated ATPase could have been lost. However, bovine erythrocyte membrane, prepared by hemolysis in the presence of 5 mM Ca^{2+} or Mg^{2+} which preserve the morphological integrity of the membrane and the retention of lipid and acetylcholine esterase activity, have neither (Na^+-K^+)-activated ATPase nor K^+-activated phosphatase activities. This indicates the absence of the "cation pump" mechanism in the erythrocytes of the adult cow. It is not clear why some animals have erythrocytes with a high K^+/Na^+ molar ratio and consequently a "cation pump", while others lack it. It is not certain if this is an hereditary trait or a result of developmental alterations, since BREWER *et al.*[27], and HANAHAN AND EKHOLM[18] have shown that calf erythrocytes have a high K^+/Na^+ molar ratio and a (Na^+-K^+)-activated ATPase while adult cows have a low cation ratio and lack the latter enzyme.

Supporting evidence for the link between the (Na^+-K^+)-activated ATPase and K^+-activated phosphatase may be obtained from the results shown in Table I. An attempt was made to achieve a complete separation between the (Na^+-K^+)-activated ATPase and the (Na^+-K^+)-insensitive independent ATPase by using high concentrations of NaI according to NAKAO *et al.*[2]. The results indicate that both the (Na^+-K^+)-activated ATPase and K^+-activated phosphatase remained in the same particulate fraction ("Pct III"). On the other hand, part of the (Na^+-K^+)-independent ATPase was solubilized as was most of the adenylate kinase which was present in the isolated membranes.

Another relationship observed between the ATPase and the phosphatase is the effect of Ca^{2+}. DUNHAM AND GLYNN[19] and EPSTEIN AND WHITTAM[13] have reported on the inhibitory effect of Ca^{2+} on the activity of the human erythrocyte (Na^+-K^+)-activated ATPase. Due to the presence of a Ca^{2+}-activated ATPase in the erythrocyte

ghost preparations, which curtails the inhibitory effect of Ca^{2+} on the (Na^+-K^+)-activated ATPase, no attempts were made to check this effect here. However, we were unable to demonstrate the presence of a Ca^{2+}-activated phosphatase in human erythrocytes membranes. Ca^{2+} in the concentration range of 0.4–0.8 mM inhibited the K^+-stimulated phosphatase. Of interest, Ca^{2+} neither activate nor inhibit the ATPase activity of the bovine erythrocyte membrane (Table IV). This raises another question concerning the "pumping" mechanism operation in the erythrocyte, namely does the absence of a (Na^+-K^+)-activated ATPase K^+-activated phosphatase imply absence of Ca^{2+}-activated ATPase, and hence a complete lack of cation transport?

The presence of Ca^{2+}-activated phosphatase, which apparently displays reaction dependences different from that of the (Na^+-K^+)-activated ATPase, and its association with the Ca^{2+} transport mechanism of the human erythrocyte, has been reported by SCHATZMAN AND VINCENZI[20]. The process of Ca^{2+} transport and the Ca^{2+}-activated enzyme both operate in a manner which is independent of Na^+ and/or K^+, and also are not inhibited by cardiac glycosides or oligomycin.

ROSENTHAL *et al.*[21] described a "soluble" ATPase, extracted from human erythrocyte ghosts, which was activated mainly by Ca^{2+} and inhibited by Mg^{2+}. The activity was low, with approx. 0.012 moles ATP hydrolyzed per h per mg protein. The activity of the Ca^{2+}-activated enzyme in the membrane suspension of human erythrocytes as reported by HORTON *et al.*[22] was 0.87 moles P_i per h per mg protein and was found to be depressed in erythrocytes from cystic fibrosis patients (0.6 moles P_i per h per mg protein). The calculated activity in the present report varies from 0.2 to 0.55 mole ADP per h per mg protein, in different preparations of the insoluble fraction "Pct III". On the basis of these two different specific activities of soluble (*cf.* ref. 20) and insoluble enzymes, it is therefore suggested that two different Ca^{2+}-activated ATPases exist. The Ca^{2+} concentration required for optimal activity, was shown by HORTON *et al.*[22] to be near 0.3 mM, and we have found it to be between 0.3 and 0.6 mM.

The ATPase activity that is related to the cation transport across the plasma membrane has been shown to be a lipoprotein enzyme, and an integral part of the membrane structure[23]. Adenylate kinase activity measurements done by RONQUIST[24], on washed intact human erythrocytes and whole cell hemolyzates indicated that the membrane bound enzyme represents only 2 % of the total activity. It, therefore, appears that the adenylate kinase is not a "true" membrane bound enzyme, and only the efficiency of the washings will determine the degree of its removal (*cf.* ref. 25 for removal of glycolytic enzymes from human membranes). The assay of adenylate kinase was included in the present study for two reasons: (a) to show how much of a "soluble" enzyme could remain bound to the membrane in different preparations (Table II shows the variation in the specific activity of this enzyme in human, bovine and porcine membranes), and (b) to show that adenylate kinase, if too active, could have interfered in the assay of ADP formed in the assay from the other enzymes by competing with the pyruvate kinase for the same substrate. Inasmuch as its activity was low compared to the pyruvate kinase, its presence did not interfere with the assay.

The data presented here indicate the species differences in certain membrane enzymes, and stresses the close relation between the (Na^+-K^+)-activated ATPase and K^+-activated phosphatase.

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