be possible to show that strophanthidin is not indispensable to the inhibition of the Na^+-dependent component; that is, in muscles treated only with ethacrynic acid, no decrease in efflux follows the elimination of external Na^+. When muscles treated only with ethacrynic acid in Na^+ Ringer were transferred to Na^+-free Ringer *plus* ethacrynic acid, a large stimulation of the efflux, lasting for at least 60 min, was observed.

In other experiments using the technique of KEYNES AND STEINHARDT[3], we measured the effects of ethacrynic acid on the Na^+ influx into strophanthidin-treated muscles. Ethacrynic acid reduced influx to 0.62 ± 0.6 ($n = 6$) times the resting level. In the same muscles the efflux was reduced from 0.59 to 0.18 ± 0.05 times the resting level.

In summary, we found that ethacrynic acid affects the two components of Na^+ efflux from frog skeletal muscle differently, stimulating the strophanthidin-sensitive, external K^+-dependent component, and inhibiting the external Na^+-dependent, strophanthidin-insensitive component. More interesting, perhaps, are the results of the uptake experiments indicating that in contrast to the erythrocyte—where the inhibition of efflux caused by ethacrynic acid is not associated with a depression of Na^+ influx[4]—the Na^+-dependent component of Na^+ efflux in skeletal muscle may involve an exchange of Na^+ for Na^+.

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Effects of ATP and Ca^{2+} on a K^+-activated phosphatase from red blood cell membranes

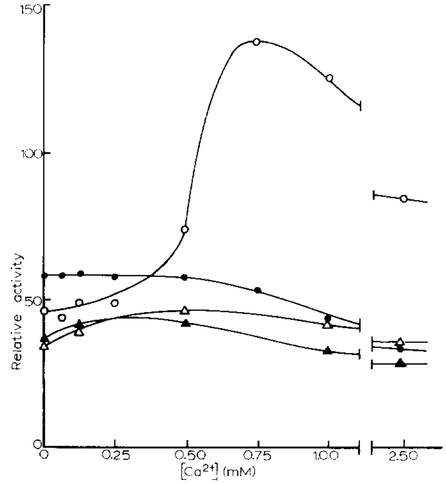
In a previous communication[1] we showed that ATP at low concentrations increases the hydrolysis rate of *p*-nitrophenyl phosphate by a K^+-activated phosphatase present in fragmented red blood cell membranes. Since this observation is at variance with the inhibitory action of ATP on K^+-sensitive phosphatases from other tissues[2–4], a more detailed study of the phenomenon seemed worthwhile. In this communication we wish to report our findings on the role that Ca^{2+} plays in the interaction of the enzyme with the nucleotide.

As previously reported[1], fragmented membranes were prepared by freezing and thawing "hemoglobin-free" human red blood cell ghosts prepared by successive washes in hypotonic Tris–HCl solutions.

Fig. 1 shows the results of an experiment in which the hydrolysis rate was estimated as a function of increasing concentrations of calcium in the presence of 0.5 mM Mg-EGTA*. Preliminary experiments demonstrated that in the absence of added calcium, no further effects can be observed by increasing the concentrations of Mg-EGTA from 0.5 up to 3.0 mM. From the curves plotted in Fig. 1 it is clear that: (1) In the presence of Mg-EGTA, the effect of ATP is reversed and the hydrolysis rate drops below the control value. (2) As the concentration of Ca^{2+} is increased, the activity in the presence of ATP and K^+ gradually rises, reaching a maximum between 0.5 and 0.7 mM Ca^{2+}. (3) Further increases in Ca^{2+} concentration lead to a slow decline in the activity under any condition. With no calcium added, Mg-EGTA has little effect in the absence of ATP and K^+.

These results suggest that enzyme activation in the presence of ATP and K^+ requires the presence of an EGTA-chelatable substance. The reappearance of the activation by addition of Ca^{2+} strongly points to this cation as the activating substance. Small amounts of Ca^{2+} in the incubation mixture would therefore account for the activating effect of ATP in the absence of Mg-EGTA[1].

Since ATP, apart from increasing the rate of hydrolysis, also changes the K_m of the phosphatase for the substrate and for K^+ (ref. 1), it seemed worthwhile to examine the action of ATP on the apparent affinities of the enzyme when Ca^{2+} is absent.



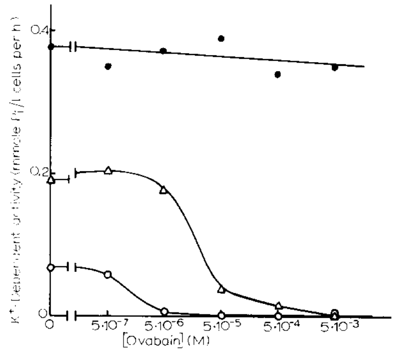


Fig. 1. p-Nitrophenyl phosphatase activity as a function of calcium concentration. The "K^+-free medium" contained: 5.0 mM $MgCl_2$, 6.3 mM Tris–p-nitrophenyl phosphate, 0.5 mM Mg-EGTA and 160 mM Tris–HCl (pH 7.8 at 37°). In the "K^+ medium", 100 mM KCl replaced an equivalent amount of Tris–HCl. Mg-EGTA was titrated to pH 7.8 with Tris base. ATP (0.25 mM) was added as Tris salt together with equimolar amounts of $MgCl_2$. The quantity of fragmented ghosts in the reaction mixture was that which gave an hematocrit of 15 % calculated on the original volume of the cells. The incubation was carried out at 37° for 30 min. Values plotted are normalized to total activity in the presence of ATP with no Mg-EGTA = 100. ○—○, K^+ + ATP; ●—●, K^+; △—△, K^+-free + ATP; ▲—▲, K^+-free.

Fig. 2. Effect of Ca^{2+} on ouabain sensitivity of K^+-dependent p-nitrophenyl phosphatase activity. △—△, control; ○—○, ATP + Mg-EGTA; ●—●, ATP + Mg-EGTA + 0.7 mM $CaCl_2$. Other conditions as described in Fig. 1.

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* Mg-EGTA, equimolar mixture of $MgCl_2$ and ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (Sigma Chem. Co.).

Results in Table I show that EGTA only abolishes the increase in the turnover rate, leaving unaltered or enhancing all the other effects of the nucleotide. These results suggest that in the absence of Ca^{2+}, ATP is still able to combine with the enzyme in a way that it is not essentially different from that seen when Ca^{2+} is present. This interpretation implies that Ca^{2+} and ATP combine on different sites, and that Ca^{2+} activation is due to a change induced by the nucleotide in the reactivity of the enzyme towards the cation. Table I also shows that inhibition by ATP in the absence of Ca^{2+} (Fig. 1) is due to an increase in the K_m of the enzyme for the substrate without change in the maximum velocity. This result is compatible with competitive inhibition by ATP^{2-4}, but seeing that the effects of the nucleotide on the apparent affinity for K^+ are maintained in the absence of Ca^{2+}, it becomes difficult to exclude the existence of separate sites for *p*-nitrophenyl phosphate and ATP. Comparison of the ratio of velocities in the absence and presence of optimal amounts of Ca^{2+} (Fig. 1) with the ratio of maximum velocities in Table I allows an estimation of the amount of Ca^{2+} likely to be present in the incubation media. Assuming that EGTA chelates equimolar amounts of Ca^{2+}, at most 0.170 mM Ca^{2+} would give the increase in turnover rate shown in Table I.

TABLE I

EFFECTS OF EGTA ON KINETIC PARAMETERS OF THE *p*-NITROPHENYL PHOSPHATASE

All the media contained 5.0 mM $MgCl_2$. The total molarity was maintained at 160 mM by adjusting the concentration of Tris–HCl. 100 mM KCl was used for "v_{max}" and "*p*-nitrophenyl phosphate" data and 6.3 mM *p*-nitrophenyl phosphate for "K^+" data. 0.25 mM ATP, 3.0 mM Mg-EGTA and 20 mM NaCl were added when indicated. Other conditions as described in Fig. 1. Values were obtained from Lineweaver–Burk plots. The "K_m" and "v_{max}" values in the presence of Na^+ were not measured because in the presence of 100 mM K^+, Na^+ has no effect[1].

Addition	v_{max} *(mmoles P_i/l cells per h)*	K_m *(mM)*	
		p-Nitrophenyl phosphate	K^+
None	1.84	14.7	12.5
ATP	3.76	21.4	19.5
ATP + Mg-EGTA	1.84	21.2	21.0
ATP + Na^+	—	—	4.7
ATP + Mg-EGTA + Na^+	—	—	2.5

Further experimental evidence that has to be taken into account to obtain more insight into the mechanism of action of ATP and Ca^{2+} concerns the effect of ouabain. Fig. 2 shows the result of an experiment in which the sensitivity of the enzyme towards the glycoside was measured in the presence and absence of Ca^{2+} and ATP. From the inspection of the curves it is clear that: (1) In the absence of Ca^{2+}, ATP strongly increases the ouabain sensitivity of the phosphatase that then approaches the value usually found for the ATPase[5,6]. (2) When optimal amounts of Ca^{2+} are present together with ATP, the system almost completely loses its ouabain sensitivity. In this respect it is economical to assume that both the increase in the K^+-dependent activity and the loss of its ouabain sensitivity arise from the same change induced on the phosphatase by Ca^{2+} when ATP is present.

In view of the close relationship claimed by most authors between the K$^+$-activated phosphatase and the cation transport system, it is intriguing that the (Na$^+$-K$^+$)-independent ATPase activity, whose meaning to the transport ATPase is not yet clear, is enhanced, and the ouabain-sensitive activity is inhibited by Ca^{2+} (ref. 6) in the same range of concentrations that affects the phosphatase.

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A spin-label study of erythrocyte membranes

In 1965, Stone *et al.*[1] introduced the technique of spin-labelling of polypeptides and proteins with paramagnetic nitroxide radicals and showed that structural and kinetic information can be obtained from the ESR spectrum of the spin label. Since then, quite a few applications of this technique have appeared in the literature[2–4]. In our laboratory, we have been interested in studying the physical properties of phospholipids[5] and the nature of molecular interactions in synthetic lipid–protein systems[6] and in biological membranes[7,8]. We have recently examined a series of spin-labelled proteins complexed with phospholipids in aqueous systems and in isooctane, by ESR spectroscopy[5]. The results obtained have given us clues about binding between proteins and phospholipids and have prompted us to extend the spin-label technique to natural membranes. In this communication we report preliminary results obtained from spin-labelled erythrocyte membranes examined by ESR spectroscopy.

Human erythrocyte ghosts were prepared from 1–2-week-old blood-bank blood (group A, Rh + ve) by the method of Dodge, Mitchell and Hanahan[9], and were freed from traces of haemoglobin by exposure to pH 8 at low ionic strength followed by desalting. The water-washed stroma suspension was adjusted to pH 7.4 by washing with 20 mosM sodium phosphate buffer. Protein was determined by the ninhydrin procedure of Moore and Stein[10] using crystalline bovine serum albumin as a reference standard.

The spin label, *N*-(1-oxyl-2,2,5,5-tetramethylpyrrolidinyl)maleimide (I) was