BBA 76101

KINETICS OF (Na^+,K^+)-ATPase OF HUMAN ERYTHROCYTE MEMBRANES

I. ACTIVATION BY Na^+ AND K^+

H W PETER AND H U WOLF

Institut fur Biochemie der Universitat Mainz, J -J -Becher-Weg 28, BRD 65 Mainz (Germany)

(Received May 31st, 1972)

SUMMARY

The results of kinetic investigations on the ouabain-sensitive (Na^+, K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) of human erythrocyte membranes suggest the following sequence of reactions in the simultaneous presence of Na^+ and K^+:

1. The initial step is the binding of 1 K^+ per active centre.

2. The formation of an enzyme–K^+ complex is a prerequisite for the binding of 2 Na^+ and $MgATP^{2-}$ in a random reaction. Thus, the intermediates $Na_2{}^+E K^+$, EK^+–$MgATP^{2-}$ and $Na_2{}^+EK^+$–$MgATP^{2-}$ can be formed.

3 Only the $Na_2{}^+EK^+$–$MgATP^{2-}$ intermediate decomposes into the phosphorylated intermediate $Na_2{}^+EK^+$–P_i and $MgADP^-$.

4. The final step of the reaction is the splitting of $Na_2{}^+EK^+$–P_i into $Na_2{}^+EK^+ + P_i$.

INTRODUCTION

It is generally accepted that the enzymatic hydrolysis of ATP by (Na^+, K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) proceeds *via* a phosphorylated intermediate[1–5]. As shown by the label technique, the native enzyme reacts with MgATP in the absence of K^+ and the presence of Na^+ to form a phosphorylated complex, which splits into the end products only after the addition of K^+ (ref. 6):

$$E + [^{32}P]ATP \xrightarrow{^{22}Na^+} (E \sim {}^{32}P)^{22}Na^+ + ADP$$

$$(E \sim {}^{32}P)^{22}Na^+ \xrightarrow{K^+} E + {}^{32}P_i + {}^{22}Na^+ + K^+$$

Recently, Skou and Hilberg have questioned[7], however, whether this mechanism is valid under physiological conditions, inasmuch as in the physiological environment K^+ is as well present during the initial step. Therefore, we have reinvestigated the activation mechanism of (Na^+, K^+)-ATPase with respect to the simultaneous presence of Na^+ and K^+.

MATERIALS AND METHODS

Chemicals

ATP (disodium salt) was obtained from Boehringer und Söhne, Mannheim, sucrose was from Schuchardt AG, München, and all other chemicals were reagent grade from Merck, Darmstadt. N_2–CO_2 (95:5, v/v) and purified N_2 was obtained from Linde AG, Kostheim.

Preparation of the enzyme

In order to avoid any uncontrolled and possibly irreversible oxidation of the enzyme by O_2, water saturated with purified N_2 was used in all processes of the enzyme preparation, which was carried out at 2–4 °C.

Human blood (group O, Rh^+), which was stored at 4–6 °C for 3–5 days with citric acid–citrate–dextrose–stabilizer + adenine + guanosine (U.S.P. XVIII B cum AG, 100 ml stabilizer per 400 ml blood) was used as source for the enzyme. The erythrocytes spun down at 2–4 °C and 5000 × *g* for 30 min were resuspended in about 1 litre of physiological NaCl solution. The hemoglobin-bound oxygen was removed by subjecting the suspension to a stream of N_2–CO_2 (95:5, v/v) for about 1 h at 2–4 °C. Then the cell suspension was centrifugated for 30 min at 5000 × *g*, the supernatant as well as the leukocytes were carefully sucked off and discarded. This washing procedure was repeated twice.

120 ml packed erythrocytes were lysed at pH 7.6–7.8 and 2–4 °C in a hemolyzing solution containing about 10 mM NaCl, 10 mM KCl, 20 mM Tris–HCl, 20 mM sucrose, 1 mM EDTA and 2 mM $MgCl_2$ (final concentrations). The final volume was 1200 ml. After centrifugation at 22000 × *g* for 30 min (MSE High Speed 18, 6 × 250 ml rotor) the supernatant and the small button of unhemolysed erythrocytes were discarded. During the following washing procedures of the resulting ghosts the osmolarity of the original hemolysis solution (≈ 100 mosM) was lowered to about 10 mosM by using a solution with the following constituents: 1 mM NaCl, 1 mM KCl, 2 mM Tris buffer (pH 7 7) and 2 mM sucrose. The yield was about 120 ml suspension of nearly colourless ghosts. After the addition of 120 ml 1 M sucrose the suspension was frozen and then stored at −20 °C.

The enzyme preparation showed a (Na^+, K^+)-ATPase activity of 1.2 munits/mg protein and a (Mg^{2+}, Ca^{2+})-ATPase activity of 2.4 munits/mg protein. This enzyme preparation was used to perform the experiments on the Na^+ activation. In the experiments on the K^+-activation, however, the preparation procedure was slightly modified, which did not result in a change of the kinetic parameters of the enzyme:

The second washings of the membrane fragments were carried out at room temperature and in the presence of 0.008 % deoxycholate. The next washings were performed at 2–4 °C again and without any further addition of deoxycholate. This preparation showed an (Na^+, K^+)-ATPase of 6.8 munits/mg protein and a (Mg^{2+}, Ca^{2+})-ATPase activity of 6.4 munits/mg protein.

The hemoglobin content of both ghost preparations was <0.1 % of the original content (determined according to the method of Rimington[8], modified by Arnold[9].

The protein contents were determined by a micro-Kjeldahl method. Previously the membrane-bound phospholipids have been removed by methanol–chloroform (1:1, v/v)[10].

Determination of enzyme activity

The conditions for activity measurements are reported in Results. The test solution contained 10 mM Tris, 1 mM Mg^{2+} and 1 mM MgEDTA The test volume was 10.0 ml throughout, the temperature 30 °C. The enzyme activity was determined essentially as described in a preceding paper[11].

The activity of (Na^+, K^+)-ATPase in the context of this paper is defined as the difference between analogous test samples in the absence and the presence of 0.1 mM ouabain, by which (Na^+, K^+)-ATPase is completely inhibited, whereas the (Mg^{2+}, Ca^{2+})-ATPase remains unaffected.

For the evaluation of the $MgATP^{2-}$ concentration $K = 0.215$ mM was used as dissociation constant of the $MgATP^{2-}$ complex (*cf.* ref. 12), this value being valid under the conditions of the present experiments.

Derivation of the reaction rate equations

The derivation of the rate equations is performed on the basis of a rapid equilibrium reaction according to models presented by Botts and Morales[13], Laidler[14], and Ohlenbusch[15], and to the method reported by Cleland[16].

Definitions

The symbols used in this paper are defined as follows:

P_i = inorganic phosphate

E = free enzyme

$[E]_t$ = total enzyme concentration

S = substrate = $MgATP^{2-}$

k = reaction rate constant

K = equilibrium constant

k_1 = rate constant of the reaction

$$Na_2{}^+EK^+\text{–}MgATP^{2-} \rightarrow Na_2{}^+EK^+\text{–}P_i + MgADP^-$$

k_2 = rate constant of the reaction

$$Na_2{}^+EK^+\text{–}P_i \rightarrow Na_2{}^+EK^+ + P_i$$

$k_{-K^+}/k_{K^+} = K_{K^+}$ = equilibrium constant of the reaction

$$E + K^+ \rightleftharpoons EK^+$$

$k_{-Na^+}/k_{Na^+} = K_{Na^+}$ = equilibrium constant of the reaction

$$EK^+ + 2\,Na^+ \rightleftharpoons Na_2EK^+$$

$\bar{k}_{-Na^+}/\bar{k}_{Na^+} = \bar{K}_{Na^+}$ = equilibrium constant of the reaction

$$EK^+\text{–}MgATP^{2-} + 2\,Na^+ \rightleftharpoons Na_2{}^+EK^+\text{–}MgATP^{2-}$$

$k_{-S}/k_S = K_S$ = equilibrium constant of the reaction

$$Na_2{}^+EK^+ + MgATP^{2-} \rightleftharpoons Na_2{}^+EK^+\text{–}MgATP^{2-}$$

$\bar{k}_{-S}/\bar{k}_S = \bar{K}_S$ = equilibrium constant of the reaction

$$EK^+ + MgATP^{2-} \rightleftharpoons EK^+\text{–}MgATP^{2-}$$

v = reaction rate at any concentration of Na^+, K^+ and $MgATP^{2-}$

$k_2[E]_t = V$ = reaction rate at infinite concentrations of Na^+, K^+ and $MgATP^{2-}$

RESULTS

The results of the present kinetic investigation are shown in Figs 1–4. A reaction scheme of the interaction of Na^+ and K^+, and the substrate with the enzyme is derived as follows:

As shown by Hexum *et al.*[17], $MgATP^{2-}$ is the true substrate of the (Na^+, K^+)-ATPase, in accordance with the results obtained from the Mg^{2+}-ATPase[12,18] and the Ca^{2+}-ATPase[11]. Hence, in the Lineweaver-Burk plots (Figs 1 and 3) $1/v$ is plotted *versus* $1/[MgATP^{2-}]$.

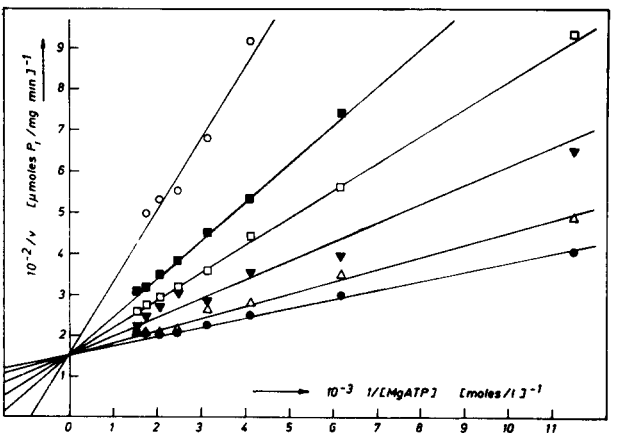


Fig. 1 Dependence of $1/v$ on $1/[MgATP^{2-}]$ at different K^+ concentrations. $[Na^+] = 86$ mM, pH 7.5 $[K^+]$: ○, 0.5 mM; ■, 1 mM, □, 1.65 mM; ▼, 3.0 mM; △, 5.0 mM; ●, 10.0 mM.

Fig. 1, the plot of $1/v$ *versus* $1/[MgATP^{2-}]$ at variable K^+ concentrations and at a constant Na^+ concentration (86 mM), exhibits one single intersection point on the ordinate. Kinetic analysis of this phenomenon on the basis of a rapid equilibrium reaction leads to the conclusion that K^+, as an essential activator of the (Na^+, K^+)-ATPase, is bound by the enzyme before the active enzyme–substrate–complex is formed. The linearity of the $1/v$ *versus* $1/[K^+]$ plot (Fig. 2) indicates that 1 K^+ is bound per active site of the (Na^+, K^+)-ATPase. Thus, the first step of the reaction sequence is

$$E + K^+ \underset{k_{-K^+}}{\overset{k_{K^+}}{\rightleftharpoons}} EK^+$$

Variation of the Na^+ concentration at a constant K^+ concentration (8.6 mM) yields the results presented in Figs 3 and 4. In contrast to Fig. 1, in Fig. 3 the joint intersection point of all straight lines does not occur at the ordinate, but in the second quadrant of the coordinate system. This indicates that there is no ordered sequence in the reaction between EK^+ and Na^+, and the substrate, respectively. From Fig. 4 it

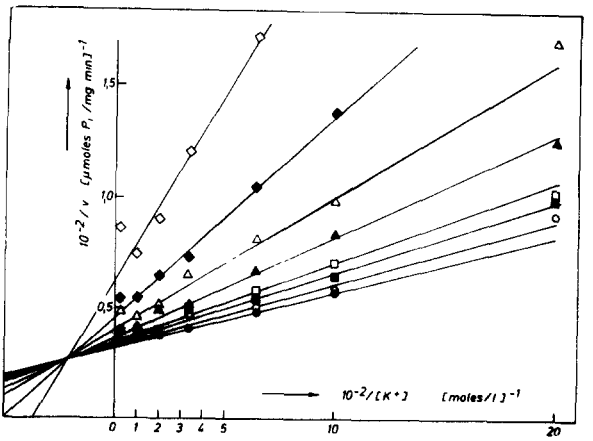


Fig 2 Dependence of $1/v$ on $1/[K^+]$ at different $MgATP^{2-}$ concentrations $[Na^+] = 86$ mM, pH 7 5 $[MgATP^{2-}]$· ◇, 0 087 mM, ◆, 0 162 mM, △, 0 244 mM, ▲, 0 319 mM, □, 0 409 mM, ■, 0 495 mM, ○, 0 578 mM; ●, 0 662 mM

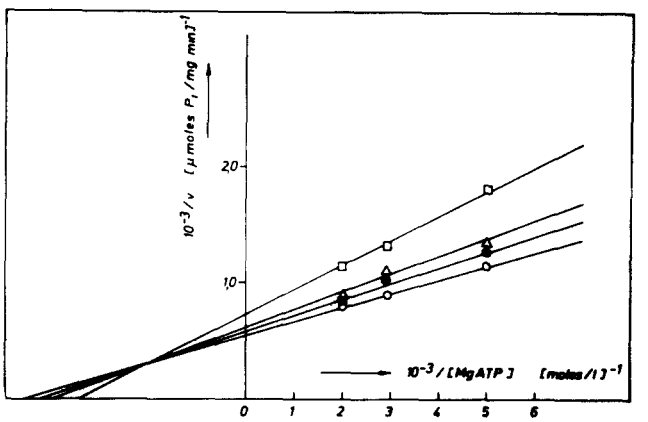


Fig. 3 Dependence of $1/v$ on $1/[MgATP^{2-}]$ at different Na^+ concentrations $[K^+] = 8\,6$ mM, pH 7.5 $[Na^+]$· □, 40 mM, △, 60 mM, ●, 80 mM; ○, 100 mM.

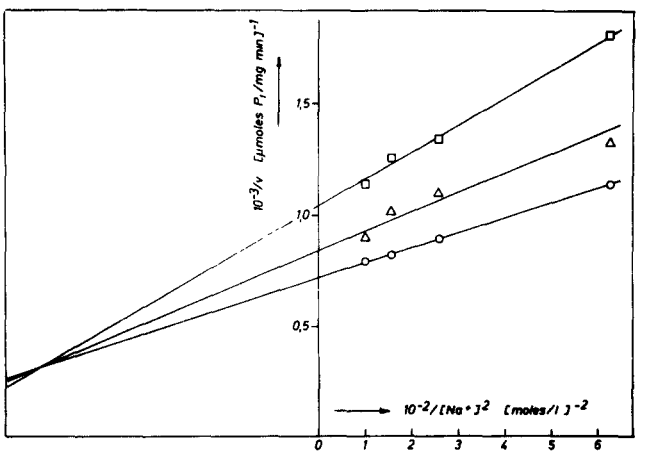
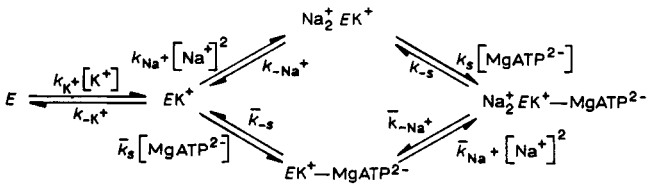
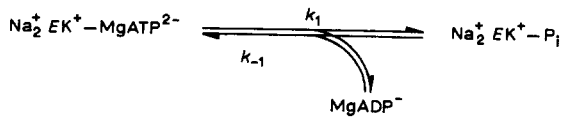


Fig. 4. Dependence of $1/v$ on $1/[Na^+]^2$ at different $MgATP^{2-}$ concentrations. $[K^+] = 8\,6$ mM, pH 7 5 $[MgATP^{2-}]$. □, 0 2 mM, △, 0 33 mM; ○, 0 48 mM

is evident, that at least 2 Na^+ are bound per active center to form the active enzyme–substrate complex. Thus, the complete reaction scheme for the formation of this complex has to be written as follows:



The decay of the active enzyme–substrate complex proceeds via the well documented phosphorylated intermediate[1–5]. In our reaction scheme the formation of this intermediate has to be written as



As shown by Fahn *et al.*[19–21] and by Siegel and Albers[22], this reaction is reversible, representing the well-known ATP–ADP exchange reaction. However, since the initial velocity of the enzyme reaction is measured in the present investigation, $[MgADP^-] \approx 0$ and hence the backward reaction can be neglected under these conditions.

The last step of the overall reaction is the decay of the phosphorylated intermediate:

$$Na_2{}^+EK^+\text{–}P_i \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} Na_2{}^+EK^+ + P_i$$

This reaction is also reversible[23,24]. However, on account of the reasons mentioned above, the backward reaction is also negligible since $[P_i] \approx 0$ at the beginning of the enzyme reaction.

The derivation of the reaction rate equation fitting the reaction scheme discussed above is performed as indicated in Materials and Methods. As pointed out by Ohlenbusch[15], a complete thermodynamic equilibrium is present between E, EK^+, EK^+–$MgATP^{2-}$, $Na_2{}^+EK^+$, and $Na_2{}^+EK^+$–$MgATP^{2-}$, if

$$k_1 \ll k_{-s} \tag{1}$$

This assumption seems to be reasonable on account of the types of the two respective reactions. In addition, in the case of an equilibrium, the general nonlinear steady state equations can be simplified yielding linear rate equations[14]. The linearity of Figs 1–4 provides the justification for the assumption of a rapid equilibrium type of reaction.

In the case of an equilibrium between EK^+, EK^+–$MgATP^{2-}$, $Na_2{}^+EK^+$, and $Na_2{}^+EK^+$–$MgATP^{2-}$

$$K_{Na^+}K_s = \bar{K}_{Na^+}\bar{K}_s \tag{2}$$

Because of Eqn 2, in a linear rate equation only three of the four constants appear explicitly[16], while the fourth (in the present case K_{Na^+}) is determined by the three others.

The complete reaction scheme discussed above yields the following rate equation, presented in the reciprocal form:

$$\frac{1}{v} = \frac{1 + \frac{k_2}{k_1} + \frac{\bar{K}_s}{K_s}\frac{k_2}{k_1}\frac{\bar{K}_{Na^+}}{[Na^+]^2}}{V} + \frac{\left[1 + \left(1 + \frac{K_{K^+}}{[K^+]}\right)\frac{K_{Na^+}}{[Na^+]^2}\right]\frac{k_2}{k_1}\bar{K}_s}{V} \cdot \frac{1}{[MgATP^{2-}]} \quad (3)$$

From the rate equation (3) the characteristics of the plots 1–4 appear as follows:

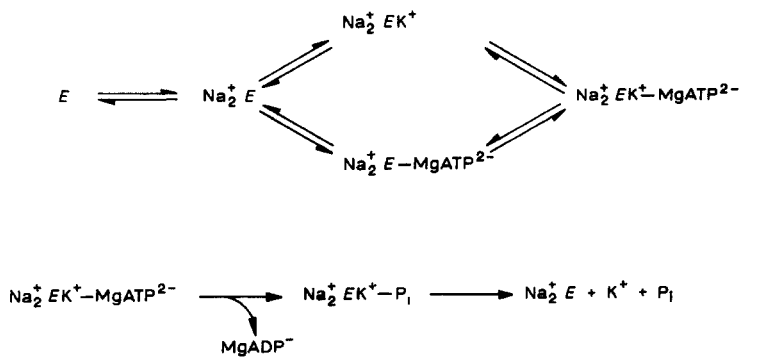
(1) $1/v$–$1/[S]$ plot at constant Na^+ and variable K^+ concentrations (Fig. 1): Dependence of the slopes on the reciprocal K^+ concentration. Intercept between all straight lines at one point at the ordinate.

(2) $1/v$–$1/[K^+]$ plot at constant Na^+ and variable substrate concentrations (Fig. 2): Dependence of the intercepts and slopes on the substrate concentration. Intercept between all straight lines at one point in the second quadrant.

(3) $1/v$–$1/[S]$ plot at constant K^+ and variable Na^+ concentrations (Fig. 3): Dependence of the intercepts and the slopes on the reciprocal Na^+ concentration. Intercept between all straight lines in the second quadrant.

(4) $1/v$–$1/[Na^+]^2$ plot at constant K^+ and variable substrate concentrations (Fig. 4): Dependence of the intercepts and the slopes of the straight lines on the substrate concentration. Intercept between all straight lines at one point in the second quadrant.

It should be emphasized that no other reaction rate equation derived from any other reaction scheme involving Na^+, K^+ and $MgATP^{2-}$, is able to fulfill the characteristics of the four figures presented in this paper. *E.g.* the reaction sequence



yields a reaction rate equation which differs from Eqn 2 significantly with respect to the $[Na^+]$ and $[K^+]$ dependence of the intercept of the straight lines with the ordinate. Since Eqn 2 is at variance with Figs 1 and 3, the last mentioned reaction scheme cannot be true.

Since the reaction rate equation (2) is rather complicated, in all cases the coordinates of the intercepts between the straight lines represent apparent constants.

For this reason, no efforts were made to evaluate numerical values of true equilibrium or reaction rate constants.

DISCUSSION

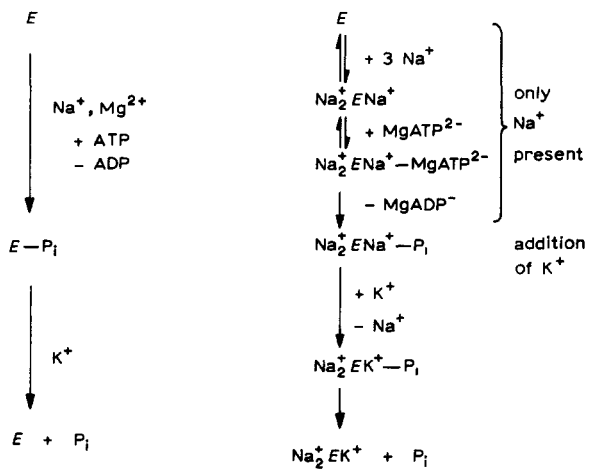
The (Na^+, K^+)-ATPase mechanism presented here contains the binding of K^+ to a K^+-specific site of the free enzyme as initial step. This ion remains connected with the enzyme during all following reaction steps.

In the following two reaction steps 2 Na^+ and the substrate $MgATP^{2-}$ are bound in a random reaction to form the active $Na_2^+EK^+$–$MgATP^{2-}$ complex. The existence of an enzyme–substrate complex containing unmodified ATP has been shown by Shamoo and Brodsky[25]. Thus, in the simultaneous presence of Na^+ and K^+, both ions are used to enable the enzyme to bind the substrate $MgATP^{2-}$. This finding is in good agreement with results obtained recently by Shamoo and Brodsky[26].

However, these results seem to be in contrast to reports of others[27–30], who suggest that Na^+ controls the formation of the phosphorylated intermediate, while K^+ controls its dissociation. This obvious discrepancy might be explained on the basis of different experimental conditions, which have been applied: in contrast to our experiments the enzymatic reaction was initiated in the absence of K^+ in most of the experiments done so far. Under these conditions, Na^+ might have occupied the specific site for K^+. Apparently, this "wrong activation" has no significant influence on the formation of the phosphorylated intermediate: Nagano *et al.*[31] could not detect any difference between the two intermediates, which were formed in the presence of Na^+ and $Na^+ + K^+$. In addition, this hypothesis is supported by Priestland and Whittam[32], who found that Na^+ and K^+ compete for the K^+-specific site of the erythrocyte membrane (Na^+, K^+)-ATPase.

The next step, the dissociation of the phosphorylated intermediate, is essentially dependent on the presence of K^+ (refs 27–30). This is not in contrast to our findings: K^+ is bound tightly to the enzyme at all stages of the reaction sequence.

Thus in the light of our findings, we should interprete the common summary reaction sequence (left-hand part of the following scheme) as follows (right-hand part):



According to this scheme, the formation of the phosphorylated intermediate, which is dependent on Na^+ and Mg^{2+}, proceeds via the following steps in the presence of Na^+ alone:

(1) Formation of an $Na_2{}^+ENa^+$ complex. In this complex Na^+ has occupied the K^+-specific site, characterized by the Na^+ symbol written to the right of "E".

(2) Formation of an $Na_2{}^+ENa^+$–$MgATP^{2-}$ complex by binding the substrate $MgATP^{2-}$.

(3) Formation of an $Na_2{}^+ENa^+$–P_i complex by releasing $MgADP^-$.

At this stage, no further reaction can proceed in the absence of K^+: The Na^+, which has been bound to the K^+-specific site, has to be substituted for K^+ to form the reactive $Na_2{}^+EK^+$–P_i. Only this intermediate is able to decompose to $Na_2{}^+EK^+ + P_i$.

The right hand part of the scheme mentioned above is characterized by the occurrence of two different phosphorylated intermediates, *i.e.* $Na_2{}^+ENa^+$–P_i and $Na_2{}^+EK^+$–P_i. This concept is in agreement with former results[2,33,34], which refer to the existence of two different phosphorylated enzymes, which differ with respect to the binding of K^+

Under these points of view, our results are not essentially in contrast to former ones[6,27–30]. They rather show that the enzyme reacts in different ways in the presence of Na^+, and of $Na^+ + K^+$, respectively.

The formation of an $Na_2{}^+EK^+$–P_i complex indicates that the (Na^+, K^+)-ATPase has three binding sites for monovalent ions including a specific site for K^+ in good agreement with the results of Middleton[35], who presents also a model with three binding sites, one of them however, being designated as a specific site for Na^+. In addition, Judah and Ahmed[36], Albers *et al.*[37], Järnefelt[38] and Ahmed *et al.*[39] propose a model for active Na^+ transport, in which the (Na^+, K^+)-ATPase combines with 3 Na^+ during intermediate formation and with 1 Na^+ and 2 K^+ during intermediate decomposition

ACKNOWLEDGEMENTS

The authors are grateful to Professor K. Dose for his interest and many helpful discussions concerning this paper.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft.

REFERENCES

1 J C Skou, *Biochim Biophys Acta*, 42 (1960) 6
2 R. L. Post, A K Sen and A S Rosenthal, *J Biol. Chem.*, 240 (1965) 1437.
3 K Ahmed and J D. Judah, *Biochim Biophys Acta*, 93 (1964) 603
4 K. Nagano, N Mizuno, M Fujita, Y Tashima and M Nakao, *Biochim Biophys. Acta*, 143 (1967) 243
5 D A. Hems and R. Rodnight, *Biochem. J*, 101 (1966) 516
6 J. S Charnock, A S. Rosenthal and R L Post, *Aust J. Exp Biol*, 41 (1963) 675.
7 J C Skou and C Hilberg, *Biochim Biophys Acta*, 185 (1969) 198.
8 C Rimington, *Br Med J*, 1 (1942) 177
9 A. Arnold, Diplomarbeit, Mainz, 1970
10 C F Reed, S N. Swisher, C. V Marinetti and J Eden, *Lab. Clin. Med.*, 56 (1960) 281.
11 H U. Wolf, *Biochim Biophys Acta*, 266 (1972) 361
12 H U Wolf and L Adolph, *Eur J Biochem*, 8 (1969) 68.

13 J Botts and M. Morales, *Trans Faraday Soc*, 49 (1953) 696.
14 K J. Laidler, *Trans. Faraday Soc*, 52 (1956) 1374
15 H-D. Ohlenbusch, *Die Kinetik der Wirkung von Effektoren auf stationare Fermentsysteme*, Springer-Verlag, Berlin, 1962
16 W W. Cleland, in P D. Boyer, *The Enzymes, Kinetics and Mechanism*, Vol. II, Academic Press, New York, 2nd edn, 1970, p 1
17 T Hexum, F. E. Samson, Jr and R. H. Himes, *Biochim Biophys Acta*, 212 (1970) 322.
18 J G Nørby, *Abstr. 6th FEBS Meet, Madrid, 1969*, p. 234
19 S Fahn, G J Koval and R W Albers, *J Biol Chem*, 241 (1966) 1882.
20 S. Fahn, M R. Hurley, G J. Koval and R W Albers, *J Biol Chem.*, 241 (1966) 1890.
21 S Fahn, G. J Koval and R W. Albers, *J Biol. Chem.*, 243 (1968) 1993
22 G. J Siegel and R. W Albers, *J. Biol. Chem*, 242 (1967) 4972
23 P J. Garrahan and I. M Glynn, *J Physiol. London*, 192 (1967) 237
24 I M Glynn and V L Lew, *J Physiol London*, 207 (1970) 393
25 A E Shamoo and W A Brodsky, *Biochim. Biophys. Acta*, 241 (1971) 846.
26 A E Shamoo and W A. Brodsky, *Biochim Biophys Acta*, 255 (1972) 220.
27 T Kanazawa, M Saito and Y. Tonomura, *J Biochem. Tokyo*, 61 (1967) 555.
28 H Bader, R L Post and G H Bond, *Biochim Biophys Acta*, 150 (1968) 41
29 A L. Green and C. B Taylor, *Biochem Biophys Res. Commun.*, 14 (1964) 118
30 J. S. Charnock and H. A Potter, *Arch Biochem Biophys*, 134 (1969) 42.
31 K. Nagano, N Mizuno, M Fujita, Y Tashima, T. Nakao and M Nakao, *Biochim. Biophys. Acta*, 143 (1967) 239.
32 R N Priestland and R Whittam, *Biochem J*, 109 (1968) 369
33 A K. Sen, T. Tobin and R L Post, *J. Biol Chem*, 244 (1969) 6596
34 T Akera and T. M. Brody, *J Pharmacol. Exp. Ther.*, 176 (1971) 545
35 H. W. Middleton, *Arch Biochem Biophys*, 136 (1970) 280
36 J D Judah and K Ahmed, *Biological Rev. Cambridge*, 39 (1964) 160.
37 R. W Albers, S. Fahn and G J. Koval, *Proc. Natl. Acad. Sci U.S.*, 50 (1963) 474.
38 J Jaernefelt, *Biochim. Biophys. Acta*, 59 (1962) 643.
39 K Ahmed, J. D Judah and P. G Scholefield, *Biochim. Biophys. Acta*, 120 (1966) 351.