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THE STEADY-STATE KINETIC MECHANISM OF ATP HYDROLYSIS CATALYZED BY MEMBRANE-BOUND ($Na^+ + K^+$)-ATPase FROM OX BRAIN

IV. RATE CONSTANT DETERMINATION

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The expressions for the kinetic constants corresponding to the steady state model for hydrolysis of ATP catalyzed by ($Na^+ + K^+$)-ATPase proposed recently are analyzed with the object of determining the rate constants. The theoretical background for the necessary procedures is described. The results of this analysis are: (1) A small class (four) of rate constants are determined directly by the previously published values of the kinetic constants. (2) For a somewhat larger class of rate constants upper and lower bounds may be established. For several rate constants the upper and lower bounds differ by less than a factor 1.6 (for the '($Na^+ + K^+$)-enzyme', i.e. the enzyme activity with K^+ and millimolar substrate concentration) and 1.2 (for the 'Na^+-enzyme', i.e. the activity at micromolar substrate concentrations). (3) Experiments on inhibition by K^+ of the Na^+-enzyme at various Mg^{2+} concentrations are reported and analyzed. With the additional assumption that the rate constants governing the addition to ATP of Mg^{2+} is independent of whether or not ATP is bound to an enzyme molecule, a set of consistent values for all the 23 rate constants in the mechanism may be obtained. (4) The values of some rate constants lend further support to the contention discussed in a previous paper that the enzyme hydrolyzes ATP along two kinetically distinct pathways, depending on the presence of K^+ and on the concentration of substrate, without the necessity of having more than one active substrate site per enzyme unit at any time. (5) The results show that while the two enzyme forms, the 'Na^+-enzyme' E_1 and the 'K^+-enzyme' E_2K, add substrate with (second order) rate constants of the same order of magnitude (differing only by a factor of four in favor of the former), the rate constants for the reverse processes differ by a factor of 100, being largest for the K^+-enzyme. This is the main reason for the large difference in the Michaelis constants for the two forms reported previously. (6) Compatibility of the model with the well-known rapid dephosphorylation of the phosphorylated enzyme in the presence of K^+ requires the presence, at non-zero steady state concentration, of an enzyme-potassium-phosphate intermediate, which is acid labile and is therefore not detected as a phosphorylated enzyme using conventional methods.

Introduction

The work described here is a continuation of previously published work [1–3] (these papers will henceforth be referred to as I, II and III, respectively) on the steady-state kinetic mechanism of ($Na^+ + K^+$)-ATPase (adenosine-5′-triphosphatase, EC 3.6.1.3). We shall be concerned with the evaluation of the rate constants in all the steps of the mechanism proposed in III.

For simple mechanisms it is sometimes possible to determine the rate constants by simple algebraic manipulation of the equations defining the experimentally determined kinetic constants. For more

complex mechanisms, such as the one in question, this is not directly possible because the number of independent equations is less than the number of unknown rate constants. This situation may be remedied if a sufficient number of rate constants can be determined (or estimated) directly by other means.

Clearly a knowledge, or at least an estimate, of the rate constants in a mechanism provides a deeper insight into its properties than does a knowledge of the kinetic constants alone. In particular, it is a necessary prerequisite for a study of the energetics, i.e. the free energy changes involved in individual steps, and the concentrations of intermediates at steady state under a variety of conditions.

In this paper we examine the expressions given previously for the kinetic constants in terms of rate constants. These expressions, together with the conditions imposed by detailed balance at equilibrium, may be manipulated to show that: (i) a small class of rate constants are directly determined by the values of the kinetic parameters; (ii) for a somewhat larger class, upper and lower bounds, differing by less than a factor of two, may be established, and (iii) the existence (at appreciable steady state concentration) of an enzyme intermediate, not directly kinetically distinguishable by steady state kinetic methods, may be established. Furthermore, the analysis of the detailed kinetic experiments on the K^+-inhibition (at $[Na^+] = 150$ mM) of the Na^+-enzyme (i.e. the enzyme activity with substrate concentrations in the micromolar range) presented, provides estimates of most of the remaining rate constants. Finally, if in addition to the above an intuitively reasonable assumption concerning the interaction of Mg^{2+} with the enzyme is made, all the rate constants in the mechanism may be determined. From a discussion of these results emerges a strenghtening of the evidence pointing towards the existence of two completely distinct hydrolysis pathways for the $(Na^+ + K^+)$-ATPase without the necessity of having more than one active substrate site on an enzyme unit.

Materials and Methods

The enzyme preparation and the assay procedures used here have been detailed in paper I [1] of this series.

Theoretical background

We showed in I that the simple model presented in Fig. 1 would provide an adequate description of the steady state kinetic experiments: for the Na^+-enzyme, if $E \equiv E_1$, the intermediate EM is omitted, and $Ex \equiv E_1P + E_2P$ (these latter showing spontaneous dephosphorylation with the same rate constant k_3), and for the $(Na^+ + K^+)$-enzyme (i.e. the enzyme activity in the presence of K^+ and millimolar substrate concentrations) if $E \equiv E_2K$ and EM ($= E_2KM$) is included.

In drawing the model in Fig. 1 it has been assumed that, for both enzymes, EMA, the enzyme-substrate complex is the same whether it is obtained directly by the addition to E of MA (= MgATP) or by adding first A ($= ATP_{free}$) and then M ($= Mg^{2+}_{free}$). This is the natural assumption to make, lacking evidence to the contrary. However, this identity imposes a useful condition for the rate constants.

Consider the model in Fig. 1 at equilibrium. Because of detailed balance the product of the (first order or pseudo-first order) rate constants in the forward and reverse direction of the cycle E–EA–EMA–E must be equal:

$$(k'_1 A)(k'_2 M)\, k_{-1} = (k_1 MA) \cdot k'_{-2} \cdot k'_{-1} \qquad (1)$$

$$(A \equiv [ATP_{free}];\ M \equiv [Mg^{2+}_{free}];\ MA \equiv [MgATP])\,.$$

At the same time we have, because of the chemical equilibrium $Mg^{2+} + ATP \rightleftharpoons MgATP$ that

$$\frac{A \cdot M}{MA} = K_T \qquad (2)$$

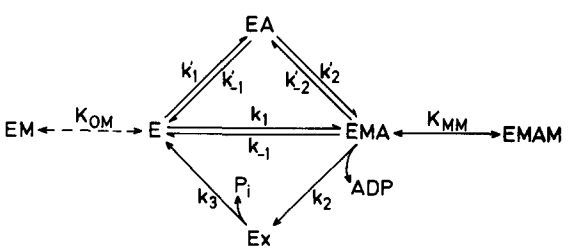

Fig. 1. The simple model from paper I (Ref. 1) compatible with the kinetic experiments for the Na^+-enzyme and for the $(Na^+ + K^+)$-enzyme. In the former case the intermediate EM is absent. Double-headed arrows indicate equilibrium.

From Eqn. 1 we then have

$$\frac{k'_{-1}}{k'_1} \cdot \frac{k'_{-2}}{k'_2} = K_T \cdot \frac{k_{-1}}{k_1} \qquad (3)$$

Note that all ratios in Eqn. 3 are dissociation constants:

$\frac{k'_{-1}}{k'_1}$ = dissociation constant for EA ⇌ E + A

$\frac{k'_{-2}}{k'_2}$ = dissociation constant for EAM ⇌ EA + M

$\frac{k_{-1}}{k_1}$ = dissociation constant for EMA ⇌ E + MA

These enzyme dissociation constants are thus connected with K_T, the MgATP dissociation constant, through Eqn. 3. This equation is satisfied under all conditions if $k'_{-1}/k'_1 = k_{-1}/k_1$ and hence $k'_{-2}/k'_2 = K_T$. In thermodynamic terms it means that the standard Gibbs free energies of ATP and MgATP are changed by the same amount when adding an enzyme molecule. This follows from the relation $\Delta G^0 = -RT\ln K_{eq}$, applied to the individual equilibria in the cycle. We shall refer to this assumption as the 'Gibbs energy assumption'.

In paper I we defined and determined the kinetic constants for the mechanism Fig. 1 for both enzyme forms. These definitions are reproduced in the left column of Table I. If we can estimate E_0, the total enzyme concentration, then, using Eqn. 3 and the Gibbs energy assumption, we can express all the rate constants in Fig. 1 in terms of V_1 ($\equiv V_{MA}/E_0$), the rate constant ratio r, and the kinetic constants, as shown in the right hand column of Table I. If r is known or can be estimated, all the rate constants in Fig. 1 may thus be determined.

TABLE I

DEFINITION OF KINETIC CONSTANTS FROM PAPERS I [1] AND III [3] (LEFT COLUMN) AND EXPRESSIONS FOR RATE CONSTANTS, USING DETAILED BALANCE CONDITION AND GIBBS ENERGY ASSUMPTION (SEE TEXT)

The equations are based on the model in Fig. 1.

$r \equiv k_3/(k_2 + k_3)$	$k_2 = V_1/r$
$V_{MA} = rk_2E_0 \equiv V_1 \cdot E_0$	$k_1 = V_1/(K_{MA} - rK_{iA})$
$K_{MA} = r \times (k_{-1} + k_2)/k_1$	$k_{-1} = k_1 \times K_{iA}$
$K_M = r \times (k_2 + k'_{-2})/k'_2$	$k_3 = V_1/(1 - r)$
$K_A = rk_2/k'_1$	$k'_1 = V_1/K_A$
$K_{iA} = k'_{-1}/k'_1$	$k'_{-1} = K_{iA} \times k'_1$
$K_{iM} = k'_{-1}(k'_{-2} + k_2)/k_2k'_2$	$k'_2 = V_1/(K_M - rK_T)$
$K_T \equiv M \cdot A/MA$	$k'_{-2} = k'_2 \cdot K_T$
$K'_{MM} \equiv K_{MM}/r$	$K_{MM} = K'_{MM} \cdot r$

The rate constant ratio r. The physical meaning of r is most easily seen by deriving the expressions for the steady-state concentrations of the various intermediates in Fig. 1. At large substrate (MgATP) concentrations we obtain for [Ex]:

$$\frac{[Ex]}{E_0} = \frac{k_2/(k_2 + k_3)}{K_{MA}/MA + 1} \qquad (M \to 0)$$

$$\to k_2/(k_2 + k_3) = 1 - r \qquad (4)$$

if $MA >> K_{MA}$. Hence $1 - r$ is the relative steady-state concentration of Ex at saturating substrate concentrations (and zero Mg^{2+} concentration which, of course, can only be approximated).

Using Table I, we can obtain an upper bound for r. Clearly, $r \leqslant 1$ by definition, but it turns out (see below) that a narrower range for possible values of r is obtained by asserting that since all rate constants must be positive, we have, from the expressions for k_1 and k'_2:

$$r < K_{MA}/K_{iA} \text{ or } K_M/K_T \qquad (5)$$

whichever is smaller.

This relation (5) may be used to discuss a question raised in paper I: While it is known that for the Na^+-enzyme the intermediate Ex exists, and is to be identified with the two phosphorylated intermediates E_1P and E_2P, this is not known for the ($Na^+ + K^+$)-enzyme. In fact, Ex is unnecessary for a qualitative interpretation of the steady-state kinetic data. If Ex is omitted for the ($Na^+ + K^+$)-enzyme, it implies that r is equal to 1. Our data show, however, (see below) that r is less than 0.33 for this enzyme, and hence the intermediate Ex is present at steady state at an appreciable concentration and must be explicitly included in the model.

The complete model. We shall be concerned with

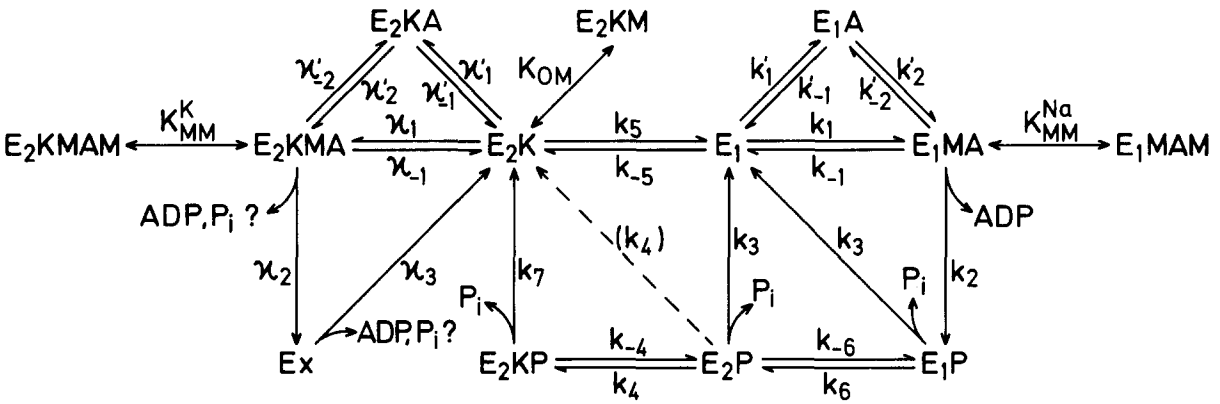

Fig. 2. The complete (minimal) model for $(Na^+ + K^+)$-ATPase with rate constant assignments. Double-headed arrows indicate equilibrium. The model proposed previously (paper III, Ref. 3) uses the path $E_2P \rightarrow E_2K$ (indicated by the broken arrow) instead of $E_2P \rightarrow E_2KP \rightarrow E_2K$. To obtain the steady-state rate for this model (or parts thereof, see text) the rate constants, where appropriate, must be multiplied by ligand concentrations.

the determination of the rate constants for the complete (minimal) model proposed in paper III. This model is shown in Fig. 2, where we have explicitly included the intermediate E_2KP. Note that the rate constants in the right hand part of the model (E_1-E_1A-E_1MA-E_1P-E_2P, the 'Na^+-cycle') are denoted k_i, while the corresponding constants for the left hand part (E_2K-E_2KA-E_2KMA-E_x, the 'K^+-cycle') are denoted κ_i. Similarly, the rate constant ratio r will be denoted ρ when referring to the $(Na^+ + K^+)$-enzyme. Replacing the path E_2P-E_2KP-E_2K by the dotted arrow $E_2P \rightarrow E_2K$ we obtain the scheme discussed in paper III.

There are two problems in connection with this scheme (Fig. 2): (A) How should the rate constants k_i, $|i| \geq 4$, be evaluated? (B) It is apparently possible (see paper I) to represent the steady-state kinetics of the $(Na^+ + K^+)$-enzyme(i.e. at large substrate concentrations) by the simple scheme in Fig. 1, which must then be considered a 'compressed' version of the complete model in Fig. 2, such that E in Fig. 1 comprises not only E_2K but also all the remaining 'Na^+-cycle' states*). What is the relation between the intrinsic rate constants, κ_i, in Fig. 2, and the apparent rate constants obtained on the basis of the compressed scheme, Fig. 1?

ad A: We have shown (paper III) that when including the intermediate E_2K in the 'Na^+-cycle', the kinetic experiments on the K^+ inhibition may be accounted for. We shall therefore examine in more detail to what extent information about the rate constants not determined in Table I for the Na^+-enzyme may be extracted from these experiments.

We consider the scheme E_1-E_1A-E_1MA-E_1P-E_2P-E_2KP-E_2K-E_2KM. As before (see paper III) we make use of the fact that for $M > 3$ mM the pathway E_1-E_1A-E_1MA makes a negligible contribution to the total reaction flux (see paper I for details) and hence we may omit the intermediate E_1A provided the Mg^{2+} concentration is large enough. Under this condition the complete expression for the steady-state rate (in double reciprocal form) may be written:

$$v^{-1} = S(K, M) \cdot MA^{-1} + I(K, M) \tag{6}$$

where

$$S(K, M) = \frac{K_{MA}}{V_{MA}} \left(1 + \frac{K}{K_K^e} F_{KM}\right) \tag{7}$$

$$K_K^e \equiv k_5/k_{-5} \; ; \qquad F_{KM} \equiv 1 + M/K_{OM} \tag{8}$$

* Note that this 'compression' does not necessarily imply that the concentrations at steady state of the Na^+-cycle states are zero, but merely that the Na^+-cycle makes a negligible contribution to the total activity under these conditions.

$$I(K,M)=\left\{(1+\alpha)F'_{MM}+\frac{K}{D_K}\left[1+\alpha r(1+\beta_0+\beta F_{KM})\right.\right.$$
$$\left.\left.+(1+\alpha)\frac{M}{K'_{MM}}\right]\right\}\left\{V_{MA}(1+\alpha)\left(1+\frac{K}{D_K}\right)\right\}^{-1} \quad (9)$$

$$\alpha=k_6/k_3 \quad (10)$$

$$\beta_0=k_2/k_7 \quad (11)$$

$$\beta=k_2/k_5 \quad (12)$$

$$F'_{MM}=1+M/K'_{MM} \quad (13)$$

$$D_K=k_3(k_{-4}+k_7)/k_4k_7 \quad (14)$$

In deriving these equations it has been assumed that $k_{-6} \ll k_6$ (as is the case at 1°C (see paper II)), and has been set to zero, since it will always appear in the combinations (k_6+k_{-6}) or (k_3+k_{-6}).

If the intermediate E_2KP is not explicitly included (i.e. its steady-state concentration is very small), the same formal expressions are obtained, except that the ratio β_0 is not present in Eqn. 9, and D_K assumes the simple form

$$D_K^0=k_3/k_4 \quad (15)$$

(superscript '0' indicates $[E_2KP] \simeq 0$) where k_4 is now the (second order) rate constant governing the transition $E_2P \rightarrow E_2K$ (k_{-4} and k_7 are not explicitly present in the model in this case). We shall see below that it is possible to distinguish between these two possibilities on the basis of experimental observations.

On this basis the data analysis may proceed as follows: From Eqn. 7 it is seen that $S(K,M)$, at fixed M, is a linear function of K, and, at fixed K, a linear function of M. Since K_{MA} and V_{MA} are known (paper I; they are the Michaelis constant for MgATP and the maximal velocity for the Na^+-enzyme, respectively), the constants K_K^e and K_{OM} may be determined from the slope and intercept of such a linear plot of $S(K,M)$ vs. K at fixed M. This was done in paper III, where the values obtained were

$$K_K^e=66 \text{ mM} \quad (16)$$

$$K_{OM}=7.2 \text{ mM} \quad (17)$$

The function $I(K,M)$, Eqn. 9, is more complicated. The unknown quantities are α, β, β_0, and D_K. If these can be estimated, then, since k_2 and k_3 can be calculated using Table I, all the remaining rate constants can be found from Eqns. 10–16.

We define the reduced intercept

$$I_R(K,M) \equiv V_{MA} \cdot I(K,M) \quad (18)$$

and consider first the simplest case, i.e. a model with a direct transition $E_2P \rightarrow E_2K$ (rate constant k_4), β_0 absent from Eqn. 9, and D_K defined by Eqn. 15.

From Eqn. 9 (with $\beta_0=0$) it follows that for large K (M fixed), $K \gg D_K$, a limiting value of $I_R(K,M)$ is approached:

$$I_R(K,M) \rightarrow I_R^0(M)$$
$$=\frac{1+\alpha r(1+\beta F_{KM})+(1+\alpha)M/K'_{MM}}{1+\alpha} \quad (19)$$

From the experiments to be presented below this property obtains for $K>20$ mM. $I_R^0(M)$ is a linear function of M:

$$I_R^0(M)=\frac{1+\alpha r(1+\beta)}{1+\alpha}$$
$$+\frac{(1+\alpha)/K'_{MM}+\alpha\beta r/K_{OM}}{1+\alpha}\cdot M$$
$$\equiv a+b\cdot M \quad (20)$$

which defines a and b, and Eqn. 8 has been used. Since r is considered known (see below), the slope b and intercept a of a plot of $I_R^0(M)$ vs. M can be used to determine α and β. Solving for these, using Eqn. 20, we obtain:

$$\alpha=\frac{1-a+(b-(1/K'_{MM}))K_{OM}}{a-r-K_{OM}(b-1/K'_{MM})} \quad (21)$$

$$\beta=\frac{(b-(1/K'_{MM}))K_{OM}(1+\alpha)}{r\alpha} \quad (22)$$

Having thus determined α and β, we can now use Eqn. 9 at constant M and low values of K to determine D_K:

$$I_R = \frac{(C \cdot K/D_K) + B}{1 + K/D_K} \tag{23}$$

where

$$C \equiv \frac{1 + \alpha r(1 + \beta F_{KM}) + (1 + \alpha) M/K'_{MM}}{1 + \alpha} \tag{24}$$

$$B \equiv F'_{MM} \tag{25}$$

Eqn. 23 may be transformed into

$$\frac{I_R - B}{C - I_R} = K/D_K \tag{26}$$

Hence the quantity $Z \equiv (I_R - B)/(C - I_R)$ is independent of M and, when plotted vs. K, yields a straight line through the origin whose slope determines D_K.

If the intermediate E_2KP is included the situation is less satisfactory due to the presence of the two additional rate constants k_{-4} and k_7. The analysis described above now leads to

$$\alpha = \frac{1 - a + (b - (1/K'_{MM}))K_{OM}}{a - r(1 + \beta_0) - (b - (1/K'_{MM}))K_{OM}} \tag{27}$$

while the expressions for β and Z, Eqns. 22 and 26, are unchanged, but D_K now has the more complicated form of Eqn. 14. Also, Eqn. 24 now has the form

$$C = \frac{1 + \alpha r(1 + \beta_0 + \beta F_{KM}) + (1 + \alpha) M/K'_{MM}}{1 + \alpha} \tag{24'}$$

For a complete determination of the rate constants two additional pieces of experimental evidence are necessary: (a) a determination of the relative concentrations of E_2P and E_1P at steady state, when the phosphorylation is performed at zero K^+-concentrations, such as the experiments reported in paper II at 1°C; and (b) determination of the K^+-dependent dephosphorylation, i.e. of k_4, at 37°C, such as has been described (at 21°C) by Mårdh [4]. That these will suffice is seen as follows:

At steady state, with no potassium, we have

$$\frac{d[E_2P]_s}{dt} = k_6[E_1P]_s - (k_3 + k_{-6})[E_2P]_s = 0 \tag{28}$$

Hence

$$\frac{[E_2P]_s}{[E_1P]_s} = \frac{k_6}{k_3 + k_{-6}} \simeq \frac{k_6}{k_3} = \alpha \tag{29}$$

From a determination in this way of α, we calculate β_0 (and hence k_7, since k_2 is known, see below) using Eqns. 27 and 11. We then obtain, using D_K (Eqn. 14) a relation between k_4 and k_{-4} (k_3 is considered known, see below). If k_4 is determined experimentally, then k_{-4} may be calculated.

ad B: Consider the general sequence of states

$$\rightleftharpoons Y_1 \underset{C_{-1}}{\overset{C_1}{\rightleftharpoons}} Y_2 \underset{C_{-2}}{\overset{C_2}{\rightleftharpoons}} Y_3 \rightleftharpoons \tag{S1}$$

where the interconversions are governed by the first-order rate constants C_i. If we 'compress' this scheme into

$$\rightleftharpoons Y_1 \underset{C^*_{-1}}{\overset{C^*_1}{\rightleftharpoons}} Y^* \rightleftharpoons \tag{S2}$$

then the resulting rate constant C^*_{-1}, to be used when calculating the one-way rate $C^*_{-1} \cdot [Y^*]$, must be less than C_{-1} because the concentration $[Y^*] > [Y_2]$ *.

In our case, when studying the kinetics of the $(Na^+ + K^+)$-enzyme we 'compress' the complete model, Fig. 2, into the simple scheme shown in Fig. 1, i.e. the entire set of 'Na^+-cycle intermediates' are represented as the single species E (= E_2K). This means that when using the simple scheme (and Table I) to calculate κ_1 (and hence κ_{-1}), the values obtained will be smaller (by the same factor) than the actual values κ_1 and κ_{-1} that should be used in the complete model, Fig. 2 **.

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* In particular, if Y_2 is a transient, i.e. its steady-state concentration is very small, we have $C_{-2} << C_{-1} + C_2$, and the rate constant C^*_1 in this case is [5]:

$$C^*_{-1} = \frac{C_{-1} C_{-2}}{C_{-1} + C_2} < C_{-1} .$$

** This argument applies to the rate constants κ'_1 and κ'_{-1} as well. We have not, in the results, made corrections in these constants, since all computations are performed at $[Mg^{2+}]$ = 15 mM, and in that case the flux in the step $E_2K \rightarrow E_2KA$ is negligible compared to the total reaction flux (see paper I). Hence the computations described are insensitive to changes in these rate constants.

We shall use a simple computational procedure to remedy this situation:

(i) Based on an additional assumption ('the activation energy assumption') and using the procedures described above we first obtain a complete set of rate constants in Fig. 2. Of these, the values, κ_1^0 and κ_{-1}^0 for κ_1 and κ_{-1}, are to be considered first approximations.

(ii) The complete set of nine independent differential equations for the model in Fig. 2 are solved (together with the mass conservation equation) for the case of steady state, and the steady-state concentrations of enzyme intermediates are used to obtain the rate of hydrolysis.

(iii) The rate, v, is computed for values of M, K, and a series of values of MA appropriate for the $(Na^+ + K^+)$-enzyme. (e.g. $M = 15$ mM, $K = 20$ mM, 1 mM $\leqslant MA \leqslant$ 10 mM).

(iv) The results are plotted ($1/v$ vs. $1/MA$). For the reasons discussed above, K_{MA}, and hence the slope (proportional to K_{MA}/V_{MA}) of the double reciprocal plot (see Table I), will be larger than the experimental value.

(v) The computations (ii) and (iii) are repeated with new values $\kappa_1 = f \cdot \kappa_1^0$ and $k_{-1} = f \cdot \kappa_{-1}^0$ ($f > 1$), and the slope of the double reciprocal plot is obtained as a function of f.

(vi) That value of f is chosen (and hence the values of κ_1 and κ_{-1} for the complete model (Fig. 2)) which corresponds to the slope value obtained experimentally.

Numerical evaluation

Enzyme concentration

To obtain values of the rate constants, a knowledge of the total concentration of enzyme is necessary. Measurements of the specific enzyme activity, and assuming one ATP-site per enzyme unit, yields for preparations with 1 mg protein/ml

$$E_0 = 3.3 \cdot 10^{-7}\ \mathrm{M} \tag{30}$$

For use in the present work (where the enzyme preparation contained 2 mg/ml) we then have

$$V_1^{Na} = 282\ \mathrm{min}^{-1} \qquad (Na^+\text{-enzyme}) \tag{31}$$

$$V_1^{K} = 7880\ \mathrm{min}^{-1} \qquad ((Na^+ + K^+)\text{-enzyme}) \tag{32}$$

TABLE II

VALUES OF KINETIC CONSTANTS DETERMINED IN PAPER I [1]

$K_T = 85\ \mu$M.

	$(Na^+ + K^+)$-enzyme	Na^+-enzyme
V_{MA} (μM · min^{-1})	5 208	186
K_{MA} (μM)	224	0.69
K_M (μM)	28	11
K_{iM} (μM)	39	53
K_{iA} (μM)	202	0.76
K_A (μM)	145	0.16
K'_{MM} (μM)	66 000	38 000
K_{OM} (μM)	7400 [a]	

[a] The value given for this contant is the average of that determined in paper I [1] and, from different experiments, in paper III [3].

where the values of V_{MA} determined earlier and presented in Table II have been used.

From the expressions for k'_1 and k'_{-1} (Table I) we note that these are completely determined by the steady state kinetic parameters. We thus have

$$k'_1 = 1.8 \cdot 10^9\ \mathrm{M}^{-1} \cdot \mathrm{min}^{-1} \tag{33}$$

$$k'_{-1} = 1340\ \mathrm{min}^{-1} \tag{34}$$

for the Na^+-enzyme, and

$$\kappa'_1 = 5.4 \cdot 10^7\ \mathrm{M}^{-1} \cdot \mathrm{min}^{-1} \tag{35}$$

$$\kappa'_{-1} = 1.1 \cdot 10^4\ \mathrm{min}^{-1} \tag{36}$$

for the $(Na^+ + K^+)$-enzyme.

We now turn to a consideration of the ratio r (denoted by ρ when referring to the $(Na^+ + K^+)$-enzyme). From Table I, since we must have k_1, $k'_2 > 0$, r must satisfy the conditions

$$r < K_{MA}/K_{iA} = 0.908 \tag{37}$$

and

$$r < K_M/K_T = 0.129 \tag{38}$$

TABLE III

UPPER AND LOWER LIMITS FOR RATE CONSTANTS IN Fig. 1

Rate constant (units)	$(Na^+ + K^+)$-enzyme [a]	Na^+-enzyme	Na^+-enzyme (symmetry assumption)
r	0–0.326	0–0.129	0.118–0.129
$k_2(min^{-1})$	$(\infty-2.4)\cdot 10^4$	$(\infty-2.2)\cdot 10^3$	$(2.4-2.2)\cdot 10^3$
$k_1(M^{-1}\cdot min^{-1})$	$(3.5-5.6)\cdot 10^7$	$(4.1-4.8)\cdot 10^8$	$(4.7-4.8)\cdot 10^8$
$k_{-1}(min^{-1})$	$(7.1-10)\cdot 10^3$	$(3.1-3.6)\cdot 10^2$	$3.6\cdot 10^2$
$k_3(min^{-1})$	$(7.9-12)\cdot 10^3$	$(2.8-3.3)\cdot 10^2$	$(3.2-3.3)\cdot 10^2$
$k'_1(M^{-1}\cdot min^{-1})$	$5.4\cdot 10^7$	$1.8\cdot 10^9$	$1.8\cdot 10^9$
$k'_{-1}(min^{-1})$	$1.1\cdot 10^4$	$1.3\cdot 10^3$	$1.3\cdot 10^3$
$k'_2(M^{-1}\cdot min^{-1})$	$(2.8-8\,300)\cdot 10^8$	$(2.6-810)\cdot 10^7$	$(2.8-81)\cdot 10^8$
$k'_{-2}(min^{-1})$	$(2.4-7\,100)\cdot 10^4$	$(2.2-680)\cdot 10^3$	$(2.4-68)\cdot 10^4$

[a] These values are obtained using the 'compressed' model, Fig. 1, also for the $(Na^+ + K^+)$-enzyme, see text.

whence the allowable range for r is

$$0 < r \leqslant 0.129 \tag{39}$$

The corresponding conditions for the $(Na^+ + K^+)$-enzyme are

$$\rho < 1.109 \text{ (fulfilled by definition)} \tag{40}$$

and

$$\rho < 0.329 \tag{41}$$

and hence

$$0 < \rho < 0.329 \tag{42}$$

is the allowable variation.

Recalling that $1-\rho$ is the relative concentration of the intermediate Ex under optimal conditions, i.e. saturating substrate concentration and $M \sim 0$, we have

$$\frac{[Ex]_{opt}}{E_0} = 1-\rho \geqslant 0.671 \tag{43}$$

Though, as mentioned in paper I, the intermediate Ex is not necessary for the qualitative interpretation of the steady state kinetic experiments, the values obtained for the kinetic parameters, resulting in the conditions presented in Eqns. 41 and 42, necessitates its inclusion in the model.

It is interesting that with the conditions for r, Eqn. 39 and ρ, Eqn. 42, rather narrow ranges for several of the remaining rate constants may be obtained. Thus, as seen in Table III *, first and second column, respectively, κ_1, κ_{-1}, and κ_3 are determined within a factor less than 1.6, and k_1, k_{-1}, and k_3 within a factor less than 1.2. As expected (see Table I), for k_2 and κ_2 only lower bounds are determined, and the possible ranges for k'_2 and k'_{-2}, as well as for κ'_2 and κ'_{-2}, are rather large.

On the other hand, the fact that the ranges for k'_2 and κ'_2 are only partially overlapping suggests a possibility for a closer determination of r: if we assume that the enzyme-ATP complexes E_2KA and E_1A in Fig. 2 for the $(Na^+ + K^+)$-enzyme and the Na^+-enzyme, respectively, behave 'symmetrically' towards Mg^{2+}, i.e. that the rate constants for Mg^{2+}-addition κ'_2 and k'_2 should be equal, it follows that the lower bound for k'_2 is $2.8\cdot 10^8\ M^{-1}\cdot min^{-1}$ and the upper bound for κ'_2 is $8.1\cdot 10^9\ M^{-1}\cdot min^{-1}$. Using the expressions (Table I) for these rate constants we then obtain

$$0.118 \leqslant r \leqslant 0.129 \tag{44}$$

* It should be noted that the values in Table III for κ_1 and κ_{-1} are the 'apparent' values for these rate constants, i.e. they are the values obtained when the complete mechanism, Fig. 2, is 'compressed' into the simpler scheme in Fig. 1 to represent the kinetic data for the $(Na^+ + K^+)$-enzyme.

$$0 < \rho \leqslant 0.318 \qquad (45)$$

While the range of possible values for ρ is practically unchanged, that for r is considerably narrowed, resulting in the rate constants for the Na^+-enzyme presented in the third column of Table III.

The range for the r variation, Eqn. 44, is actually sufficiently narrow to estimate r to within less than 10%. As seen from Eqns. 20 and 24, the quantities a, b, and C, to be used in the data evaluation below, are not very sensitive to the precise value of r. Certainly a variation of less than 10% is of no consequence, since the experimental error in the determination of a and b will be of about this magnitude. Hence we can, using a value of r within the range given in Eqn. 44, obtain good estimates of the rate constants k_4, k_{-4}, k_5, k_{-5}, and k_6 (see below).

Clearly a precise value of r implies a precise value of k_2', and, when the 'symmetry assumption' discussed above is imposed. a specific value of κ_2' and hence of ρ is obtained. This in turn leads to precise values of all the rate constants for the $(Na^+ + K^+)$-enzyme.

An intuitively attractive choice of k_2' (and κ_2') is obtained as a special case of the 'symmetry assumption', namely if we assume that the rate constant governing the addition to ATP of Mg^{2+} is independent of whether or not ATP is bound to an enzyme unit. This seems a reasonable assumption to make since it is known [6], for several cases in which MgATP or MgADP are substrates, that the enzyme-substrate complex is a 'substrate-bridge' complex, i.e. the nucleotide forms a bridge between the enzyme and the metal. Hence, if the addition of the enzyme to the ATP ion results in only small changes in that part of the nucleotide to which Mg^{2+} adds, the above assumption is obtained. In terms of the transition state theory, this assumption is concerned with equality of activation energies. Consequently, we shall refer to this assumption as the 'activation energy assumption'.

The rate constant k for the process

$$Mg^{2+} + ATP^{-4} \xrightarrow{k} MgATP^{-2} \qquad (S3)$$

has been measured directly at 26°C by Diebler et al. [7]. The value obtained was $k = 1.2 \cdot 10^7\ M^{-1} \cdot s^{-1} = 7.2 \cdot 10^8\ M^{-1} \cdot min^{-1}$. The activation energy for the process is not available, nor is a value at 37°C. We shall assume that

$$k(37°) = 4 \times k(26°) \simeq 2.8 \cdot 10^9\ M^{-1} \cdot min^{-1} \qquad (46)$$

The value $Q_{10} = 4$ is of course somewhat arbitrary, but calculation shows that, apart from the rate constants directly involved (k_2' and κ_2'), the constants obtained with $Q_{10} = 2$ are changed only a few percent. With Eqn. 46 the values obtained for r and ρ are

$$r = 0.128 \qquad (47)$$

$$\rho = 0.296 \qquad (48)$$

These values have been used in the analysis to follow.

In the previous section (Theoretical background) we indicated a possible route for the determination, on the basis of kinetic experiments on K^+ inhibition of the Na^+-enzyme, of the remaining rate constants in the Na^+-cycle. Knowledge of these is necessary prior to a determination of the actual (rather than appar-

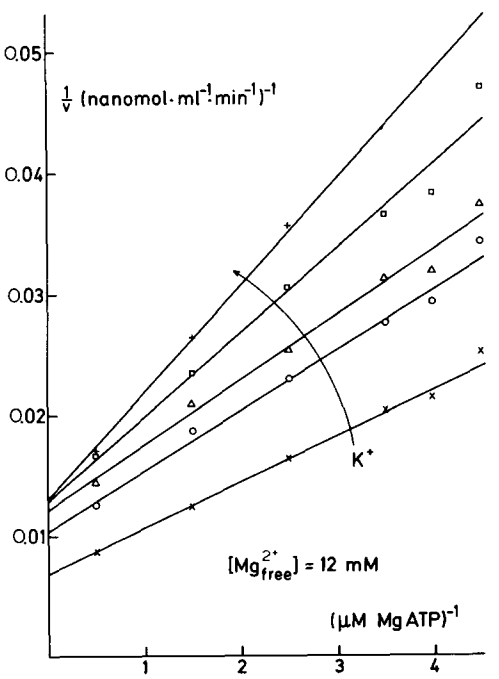

Fig. 3. K^+ inhibition of the Na^+-enzyme. Double-reciprocal plot. $[Na^+]$ = 150 mM; $[Mg^{2+}_{free}]$ = 12 mM. 30 mM histidine buffer, pH = 7.4, 37°C. $[K^+]$: 0 mM (x); 5 mM (o); 10 mM (△); 20 mM (□); 30 mM (+).

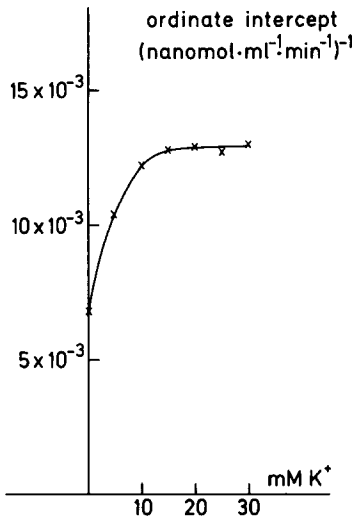

Fig. 4. Ordinate intercepts, $I(K,M)$, from lines as in Fig. 3, vs. $[K^+]$ at constant $[Mg^{2+}] = 12$ mM. The curve is drawn through the experimental points as an 'aid to the eye'.

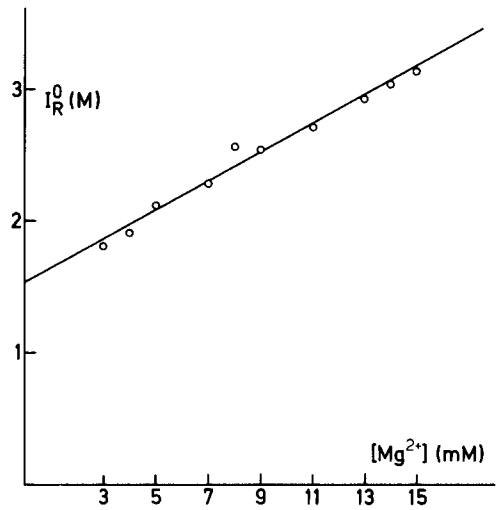

Fig. 5. The (reduced) intercept $I_R^0(M)$ at large (25 mM) $[K^+]$ vs. $[Mg^{2+}_{free}]$. The line is the least squares regression line: $I_R^0 = 1.54 + 0.109\ [Mg^{2+}]$.

ent) values of κ_1 and κ_{-1}, in the K-cycle of Fig. 2, as also described in that section.

Experiments at constant $[Mg^{2+}] = 12$ mM and various K^+ concentrations are presented in double reciprocal plots in Fig. 3. In Fig. 4, a secondary plot of the ordinate intercepts $I(K,M)$ vs. $[K^+]$ is shown. As mentioned above, the slope vs. $[K^+]$ variation was used in paper III to determine K_K^e and K_{OM}. Note that, in accordance with Eqn. 9, $I(K,M)$ rises and approaches a limiting value as K is increased to about 25 mM. Similar experiments, not shown, were performed, each at seven different values of K, at $M = 6$ mM (two runs), $M = 9$ mM (two runs), $M = 15$ mM (three runs).

As indicated in the previous section, the analysis of these experiments proceeds in two stages: (i) a consideration of $I^0(M)$ (Fig. 4b) (i.e. the limiting value of $I(K,M)$ at $K = 25$ mM), vs. M to obtain α, β, and if necessary, β_0; and (ii) a determination of D_K (see Eqns. 23–25) from $I(K,M)$ at low (<20 mM) values of K and at fixed M.

The quantity $I_R^0(M)$ determined from the experiments at $K = 25$ mM is plotted vs. M in Fig. 5. A weighted linear regression analysis yields

$$I_R^0(M) = a + b \cdot M \qquad \text{with}$$

$$a = 1.54 \pm 0.19 \tag{49}$$

$$b = 0.109 \pm 0.014 \tag{50}$$

where the standard deviations are indicated. We consider first the simplest case, obtained when E_2KP is suppressed. The quantities α $(= k_6/k_3)$ and β $(= k_2/k_5)$ may then be calculated, using Eqns. 21–22. We obtain

$$\alpha = 0.09 \Rightarrow k_6 = \alpha k_3 = 0.09 \cdot 324 = 29.2\ \text{min}^{-1} \tag{51}$$

$$\beta = 57 \Rightarrow k_5 = k_2/\beta = 2.2 \cdot 10^3/57 = 38.6\ \text{min}^{-1} \tag{52}$$

and hence (see Eqns. 8 and 16)

$$k_{-5} = k_5/K_K^e = 7 \cdot 10^3\ \text{M}^{-1} \cdot \text{min}^{-1} \tag{53}$$

For the next stage the data treatment proceeds as follows: From the data at a fixed value of M, the reduced intercept $I_R(K,M)$ is calculated for each value of K. Using the values obtained for α and β above, the quantities C and B are computed using Eqns. 24 and 25, and the quantity Z (Eqn. 26) is calculated. This calculation is performed for each value of M. For each value of K the average $\bar{Z}$ is obtained by simple

TABLE IV

EXPERIMENTAL VALUES USED FOR DETERMINATION OF D_K (SEE TEXT)

I_R = Reduced ordinate intercept. Averages are indicated by bars. $Z \equiv (I_R - B)/(C - I_R)$.

$[K^+]$ (mM)	$[Mg^{2+}]$ = 6 mM $C = 2.19, B = 1.16$			$[Mg^{2+}]$ = 9 mM $C = 2.52, B = 1.24$			$[Mg^{2+}]$ = 12 mM $C = 2.85, B = 1.32$			$[Mg^{2+}]$ = 15 mM $C = 3.18, B = 1.40$			$\bar{Z}$
	I_R	$\bar{I}_R$	Z	I_R	$\bar{I}_R$	Z	I_R	$\bar{I}_R$	Z	I_R	$\bar{I}_R$	Z	
0	1.14			1.29			1.32			1.37			
		1.16	0		1.23	−0.008		1.37	0.03	1.55	1.51	0.07	0.02
	1.18			1.17			1.42			1.60			
5	1.86			1.94			2.32			2.02			
		1.80	1.64		1.93	1.17		2.30	1.78	2.57	2.36	1.17	1.44
	1.73			1.91			2.28			2.48			
10	2.06			2.25			2.68			2.27			
		1.92	2.81		2.10	2.05		2.68	8.0	2.94	2.61	2.12	3.75
	1.77			1.93			2.67			2.61			
15	1.98			2.27			2.64			2.25			
		1.90	2.55		2.23	3.41		2.72	10.8	2.89	2.70	2.70	4.87
	1.82			2.19			2.80			2.96			
20	2.21			2.56			2.54			2.43			
		2.10	10.44		2.33	5.75		2.68	8.0	3.03	2.76	2.83	6.75
	1.99			2.09			2.82			2.81			

algebraic averaging of the individual Z values. These calculations are shown in Table IV. In Fig. 6 is shown a plot of $\bar{Z}$ vs. K. Linear regression yields

$$\bar{Z} = 0.02 + 0.34\,K \tag{54}$$

whence

$$D_K^0 = 2.94 \cdot 10^{-3}\ \mathrm{M} \tag{55}$$

Using Eqn. 15 we thus obtain

$$k_4 = k_3/D_K^0 = 1.1 \cdot 10^5\ \mathrm{M^{-1}} \cdot \mathrm{min^{-1}} \tag{56}$$

This completes the calculation of rate constants, assuming the essential absence of E_2KP at steady state.

However, we note the very low value of α. At 1°C (recalling that α may be determined as the ratio of the steady state concentrations of E_2P and E_1P with no K^+ added (see Eqn. 29) $\alpha \sim 1.5$ (see paper II), and estimates on the basis of Mårdh's data [4] yields $\alpha \sim 2.5$ at 21°C. In addition, estimates by Mårdh [4] based on K^+-dependent dephosphorylation of the phosphorylated enzyme indicate that

$$k_4 \cdot [K^+] > 14\,000\ \mathrm{min^{-1}}$$

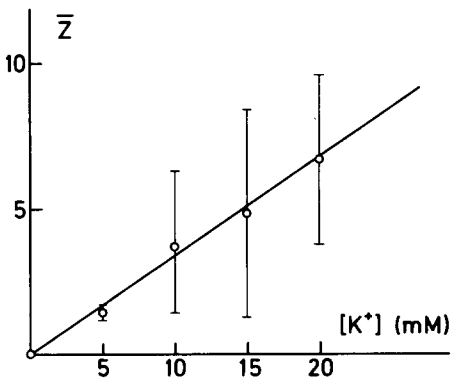

Fig. 6. The average, $\bar{Z}$, of the quantity $Z = (I_R - B)/(C - I_R)$ from Table IV vs. $[K^+]$. The vertical lines indicate the standard deviations, which are (expectedly) rather large because Z is a ratio of differences between quantities of the same order of magnitude. The large standard deviations of Z are not representative of the experimental error in determining I_R, which, as seen from Table IV, is rather small.

and, since $[K^+]$ was 0.01 M,

$$k_4(21^\circ) > 1.4 \cdot 10^6\ M^{-1} \cdot min^{-1} \quad (57)$$

Thus the value obtained above is too small at least by a factor of 15.

These considerations lead us to propose the existence at steady state of the intermediate E_2KP at non-zero concentration. To account for the rapid dephosphorylation in the presence of K^+ observed by Mårdh [4], we must then assume that E_2KP is acid labile and is therefore not detected in phosphorylation experiments such as those, in which the amount of phosphorylated enzyme is measured by acid precipitation.

In this case we have three unknowns, α, β, β_0, but only two equations, Eqns. 27 and 22. With Eqn. 27 we can establish an upper bound for β_0 and hence a lower bound for k_7. Since $\alpha > 0$ we have, with the values for a, b, derived from the data above, that the numerator is positive and hence

$$r(1 + \beta_0) < a - (b - (1/K'_{MM}))K_{OM} = 0.92 \quad (58)$$

which leads to

$$\beta_0 < 6 \Rightarrow k_7 > 367\ min^{-1} \quad (59)$$

We can now make use of the fact that Eqn. 27 contains only α and β_0. Lacking evidence regarding the value of α at 37°C we can, as an example, assume that it is close to the estimate at 21°C, $\alpha \sim 2.5$ [4]. Solving for β and β_0 we then obtain

$$\beta_0 = 5.92 \Rightarrow k_7 = 372\ min^{-1} \quad (60)$$

$$\beta = 6.59 \Rightarrow k_5 = 334\ min^{-1} \quad (61)$$

which in turn leads to

$$k_{-5} = 5060\ M^{-1} \cdot min^{-1} \quad (62)$$

With these values of α, β, and β_0 we must now repeat the evaluation of D_K from the data, since C (Eqn. 24') contains these quantities. It turns out that C is practically unchanged by this procedure, so the same value of D_K is obtained.

We now consider Eqn. 14, using the value of D_K just determined. Since k_7 and k_3 are known, this results in a relation between k_4 and k_{-4}

$$k_{-4} = \frac{k_7}{k_3} \cdot D_K \cdot k_4 - k_7 \quad (63)$$

$$= 3.4 \cdot 10^{-3} \cdot k_4 - 372$$

The condition $k_{-4} > 0$ then yields

$$k_4 > 1.1 \cdot 10^5\ M^{-1} \cdot min^{-1} \quad (64)$$

i.e. the value obtained with a model lacking significant steady state amounts of E_2KP, Eqn. 56, is just the lowest possible value in the present model, which is thus capable of explaining a rapid K^+-dependent dephosphorylation.

In view of the lack of experimental determination of k_4 at 37°C we are forced to assume a value and then calculate k_{-4} from Eqn. 63. It turns out that the steady-state properties of the model are independent of the choice of k_4, provided it is large enough and satisfies Eqn. 63.

The only remaining rate constant in the Na^+-cycle of Fig. 2 is k_{-6}, which, as discussed above, has been assumed small. Here we use the estimate at 1°C (paper II)

$$k_{-6} \simeq k_6 \cdot 0.05 \quad (65)$$

The results of the above calculations are presented in Table V.

With a knowledge of all the Na^+-cycle rate constants we can now obtain precise values of κ_1 and κ_{-1} using the iterative procedure described in the previous section. The value of the experimental slope in a double reciprocal plot may be calculated using Table II since (see paper I) for the $(Na^+ + K^+)$-enzyme (at M = 15 mM used in the computations)

$$\text{slope} = V_{MA}^{-1} K_{MA}(1 + M/K_{OM}) \quad (66)$$

The results are shown in Fig. 7, where the slope, computed as described (see Theoretical background), is plotted against the factor f

$$\kappa_1 = f\kappa_1^0$$

$$\kappa_{-1} = f\kappa_{-1}^0$$

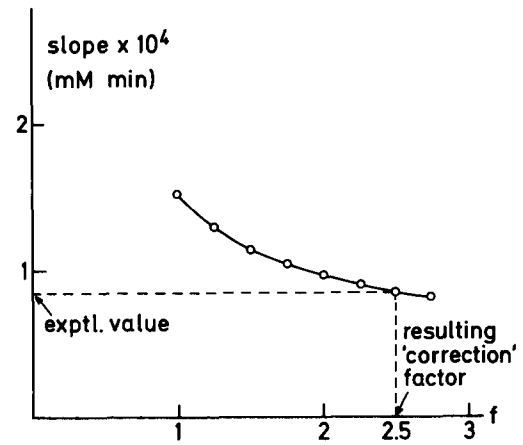

Fig. 7. The slope of double-reciprocal plots of computed $(Na^+ + K^+)$-enzyme activities vs. substrate (MgATP) concentration, as a function of the 'correction' factor f (see text).

TABLE V

RATE CONSTANTS OBTAINED BY THE PROCEDURES DESCRIBED IN THIS ARTICLE

Temperature 37°C. $[Na^+]$ = 150 mM.

	$r = 0.128; \rho = 0.296; \alpha = 2.5$ [a]		
V_1^{Na}	$2.8 \cdot 10^2$ min^{-1}	V_1^K	$7.9 \cdot 10^3$ min^{-1}
k_2	$2.2 \cdot 10^3$ min^{-1} [b]	κ_2	$2.7 \cdot 10^4$ min^{-1}
k_1	$4.8 \cdot 10^8$ M^{-1} · min^{-1}	κ_1	$1.2 \cdot 10^8$ M^{-1} · min^{-1}
k_{-1}	$3.6 \cdot 10^2$ min^{-1}	κ_{-1}	$2.4 \cdot 10^4$ min^{-1}
k_3	$3.2 \cdot 10^2$ min^{-1}	κ_3	$1.1 \cdot 10^4$ min^{-1}
k'_1	$1.8 \cdot 10^9$ M^{-1} · min^{-1}	κ'_1	$5.4 \cdot 10^7$ M^{-1} · min^{-1}
k'_{-1}	$1.3 \cdot 10^3$ min^{-1}	κ'_{-1}	$1.1 \cdot 10^4$ min^{-1}
k'_2	$2.8 \cdot 10^9$ M^{-1} · min^{-1} [c]	κ'_2	$2.8 \cdot 10^9$ M^{-1} · min^{-1} [c]
k'_{-2}	$2.4 \cdot 10^5$ min^{-1}	κ'_{-2}	$2.4 \cdot 10^5$ min^{-1}
k_4	$1 \cdot 10^7$ M^{-1} · min^{-1} [d]		
k_{-4}	$3.4 \cdot 10^4$ min^{-1}	K_{MM}^{Na}	$4.9 \cdot 10^{-3}$ M
k_5	$3.3 \cdot 10^2$ min^{-1}	K_{MM}^{K}	$2.0 \cdot 10^{-2}$ M
k_{-5}	$5.1 \cdot 10^3$ M^{-1} · min^{-1}		
k_6	$8.1 \cdot 10^2$ min^{-1}		
k_{-6}	$4.1 \cdot 10^1$ min^{-1}		
k_7	$3.7 \cdot 10^2$ min^{-1}		

[a] Value estimated on the basis of experiments at 21°C [4].
[b] The reported experimental value at 21°C, and at $[Na^+]$ = 120 mM, is 9 000–13 000 min^{-1} [9].
[c] Based on the 'activation energy assumption' (see text) and the reported rate constant at 26°C for M + ATP → MATP [7].
[d] Assumed value, consistent with experiments [4].

where κ_1^0 and κ_{-1}^0 are the values obtained from the 'compressed' scheme in Fig. 1. The experimental slope value is obtained for $f = 2.5$, and the actual values of κ_1 and κ_{-1} for Fig. 2 may now be calculated. The results are shown in Table V.

Finally, the influence on f of the particular choices of α and k_4 must be examined. For α varying from 1.5 (the value found at 1°C, see paper II) to 3.5 the value of C, Eqn. 24', is unchanged. Hence D_K is the same in all cases. For each value of α (and the corresponding values of β_0 and β, Eqns. 27 and 22), the above calculation may be performed (at fixed k_4). The results show that f is independent of the choice of α. Likewise, for a particular value of α, k_4 and hence k_{-4} has been varied over a 32-fold range, with no change in the value of f. The observed steady-state properties are thus independent of the particular values of the rate constants k_6, k_7, k_4, and k_{-4}, provided, of course, that the values are consistent with the equations derived for the model. With these reservations concerning the particular values of the rate constants k_6, k_7, k_4, k_{-4}, the results presented in Table V represent the quantitative aspects at 37°C of the model in Fig. 2.

Discussion

The crucial first step in the evaluation of the rate constants in terms of the complete model is a determination of the ratios r and ρ. Although upper bounds for both quantities are suggested from the values of the kinetic parameters (Table II), it was necessary, in order to obtain precise values, to make the 'symmetry assumption' (which essentially determines r) and, as a special case, the activation energy assumption. This appears to us very reasonable, but it lacks experimental support.

The value obtained for $r \simeq 0.13$ means that under optimal conditions, i.e. saturating substrate concentration, $[Mg^{2+}] \sim 0$ and no K^+, the relative concentration (at steady state) of phosphorylated enzyme is

$$\frac{[EP]_{opt}}{E_0} = 0.87 \qquad (67)$$

It is interesting to note that when estimating the enzyme activity using phosphorylation sites (i.e. measuring the amount of $(E_1P + E_2P)$), the value ob-

tained is 80–85% of the activity obtained by measuring ATP binding (i.e. measuring the maximum obtainable E_1A concentration) (Klodos, I., personal communication).

Allowing for differences in the media and the temperature, our calculated value, Eqn. 67, seems in good agreement with this result. It therefore tends to support the assumption on which our calculation is based.

The values of r and ρ also allow a calculation of the intrinsic Mg^{2+}-inhibition constants K_{MM}. The values are included in Table V. We note that they differ for the two enzyme forms by a factor of 4. The intrinsic constant $K_{MM}^{Na} \sim 5$ mM indicates that when phosphorylation is studied without K^+ in the medium, Mg^{2+} has an appreciable effect while in hydrolysis experiments only the 'operational' constant $K'_{MM} \sim 38$ mM is 'felt'.

The value of $\rho \sim 0.30$ was shown above to indicate that significant amounts of the intermediate Ex would be present at steady state for the $(Na^+ + K^+)$-enzyme. However, the nature of this intermediate is not specified. It is tempting to suppose that it is a phosphorylated enzyme intermediate, and is identical to E_2KP. That this cannot be the case is seen as follows: if $Ex = E_2KP$, then the rate constant $k_7 = \kappa_3$, where the latter is determined from the kinetic parameters as described above. Instead of Eqn. 63 we now have

$$k_{-4} = \frac{\kappa_3}{k_3} \cdot D_K \cdot k_4 - \kappa_3$$

$$= 0.098\, k_4 - 1.1 \cdot 10^4 \qquad (63')$$

This again leads to $k_4 > 1.1 \cdot 10^5$, which is qualitatively satisfactory. However, if k_4 is large and we know that at least the inequality given in Eqn. 57 must hold, say $k_4 \sim 10^7\ M^{-1} \cdot min^{-1}$, we obtain from Eqn. 63'

$$k_{-4} \sim 0.1 \cdot k_4 = 10^6\ min^{-1}$$

With $K^+ = 10$ mM the pseudo-first order rate constant governing the transition $E_2P \rightarrow E_2KP$ is $k_4 \cdot 0.01 = 10^5\ min^{-1}$ while that for the reverse process is 10-times larger. As a result, even at large (i.e. millimolar) ATP concentrations, the concentration of E_2P at steady state would not be decreased relative to the situation at $[K^+] = 0$, and hence the Na^+-cycle flux would predominate. In other words, we would not, even at $[K^+] = 10$ mM and large substrate concentrations, observe an activity of a sufficient magnitude to be a $(Na^+ + K^+)$-enzyme activity. This qualitative observation has been corroborated by computations of the steady state rate under these conditions (results not shown). We conclude that E_2KP is different from Ex, whose nature cannot be determined on this basis. We expect that a detailed kinetic analysis of the inhibition of the $(Na^+ + K^+)$-enzyme by P_i and ADP may throw some light on this question.

We mentioned above that in order for our model to explain the apparently rapid dephosphorylation of the phosphorylated enzyme observed by Mårdh [4] it is necessary to postulate that E_2KP is unstable in the presence of strong acid and therefore is not precipitated. This means that the observed process is not necessarily a dephosphorylation but may just represent a depletion of the acid stable phosphorylated species when K^+ is added to the medium.

It is worth while reconsidering the property of two distinct hydrolysis cycles in our model in the light of the results reported here. Is it for instance conceivable, as has been implied in the model of Karlish et al. [8], that the enzyme-substrate complex E_2KMA must be converted to E_1MA prior to hydrolysis? This would imply that the step $E_1MA \rightarrow E_1P$ would be common to the two activities. Clearly the largest possible flux in this step would be obtained if, at steady state, essentially all the enzyme was present in the form E_1MA. Then

$$\text{Max. flux} = k_2 \cdot E_0 = 1500\ \mu M \cdot min^{-1} \qquad (68)$$

However, the maximum measured flux for the $(Na^+ + K^+)$-enzyme is

$$V_{MA}^K = 5200\ \mu M \cdot min^{-1} \text{ (see Table II)} \qquad (69)$$

which would be obtained at saturating (i.e. millimolar) substrate concentrations. Thus the maximum obtainable flux is too low by a factor 3.5. Furthermore, we saw above that under saturating substrate conditions, since $\rho \sim 0.3$, about 70% of the enzyme is in the form Ex (which in the case under consideration would correspond to $(E_1P + E_2P)$), making the flux in the step $E_1MA \rightarrow E_1P$ even lower. This argu-

ment lends further support to the supposition that the 'Na$^+$-cycle' and the 'K$^+$-cycle' are completely distinct, with very different kinetic properties, and that the two cycles are connected by processes involving the addition of K$^+$ to the enzyme.

The second-order rate constants in Table V are all in the same range (10^7–10^9), somewhat smaller than would be expected if the processes were only diffusion controlled. Excepted from this is k_{-5}, governing the transition $E_1 \rightarrow E_2K$. The probable reason for this is that this transition actually consists of two steps

$$E_1 \underset{k_{-a}}{\overset{k_a \cdot K}{\rightleftharpoons}} E_1K \underset{k_{-b}}{\overset{k_b}{\rightleftharpoons}} E_2K \qquad \text{(S4)}$$

If we assume that the first step is at equilibrium (i.e. k_a, $k_{-a} >> k_b$, k_{-b}) we obtain, for the apparent rate constants k_5 and k_{-5} in the simple one-step process used in Fig. 2

$$k_5 = k_{-b}\,; \quad k_{-5} = \frac{k_a k_b}{k_a K + k_{-a}} \qquad (70)$$

This possibility has been discussed also by Karlish et al. [8]. Thus the apparent second-order rate constant k_{-5} may have a small value, even though the actual second-order constant k_a is large, if k_b, the rate constant governing the conformational change, is small. At sufficiently small K$^+$-concentrations ($k_a \cdot K << k_{-a}$) the psuedo-first order rate constant $k_{-5}\,K$ is proportional to K, as found by Karlish et al. [8] for $K < 15$ mM. The alternative two-step scheme

$$E_1 \rightleftharpoons E_2 \rightleftharpoons E_2K \qquad \text{(S5)}$$

is less attractive a priori even though the resulting pseudo-first order rate constant is strictly proportional to [K$^+$], because the assumption of rapid equilibrium in the first step of (S5) implies that the rate constants governing the conformational change are large compared to those of the second step.

From the values obtained for k_1 and κ_1 we note that the probability of adding MgATP is only four times larger for the (empty) Na$^+$-enzyme E_1 than for the (Na$^+$ + K$^+$)-enzyme E_2K, whereas the probability that E_2KMA loses MgATP (to become E_2K) is about 100-fold larger than the corresponding probability for E_1MA ($\kappa_{-1} \sim 100\,k_{-1}$). This is the feature mainly responsible for the rather large difference between the Michaelis constants (K_{MA}, see Table I) for the two forms of enzyme determined in paper I and shown here in Table II.

The two rate constants most sensitive to assumptions in the model are k_2 and κ_2 since they depend on r and ρ in an inverse manner (see Table I). With no assumptions (leading to estimates of the latter quantities) only lower bounds for k_2 and κ_2 can be obtained, cf. Table III. An experimental estimate of k_2 has been reported by Mårdh and Zetterqvist [9]. The average value obtained was about 11 000 min^{-1} at 21°C. This is at variance with our value of 2200 min^{-1} at 37°C. These experiments therefore tend to indicate that the assumption (the 'activation energy assumption') on which our value is based may not be valid, On the other hand, if a value of k_2 at 37°C could be obtained, it could be used to determine r, using the expression in Table I for k_2, making such assumptions unnecessary. We note that a large value of k_2 (resulting in a small value of r), would not affect the argument above (Eqns. 68 and 69), since in that case almost all the enzyme at steady state (and at saturating ATP concentrations) would be present as the phosphorylated species, and the steady state flux in the step $E_1MA \rightarrow E_1P$ would still be too small to account for the activity with K$^+$ present in the medium.

The rate constant k_4 in Table V has somewhat arbitrarily been set to 10^7 M^{-1} · min^{-1}. As discussed above the steady state properties are independent of the particular value chosen. A direct experimental determination is required. It appears that the time resolution of existing methods is insufficient to follow the details of the kinetics of depletion of the phosphorylated (acid stable) enzyme at 37°C. For the same reason the quantity α, yielding the rate constant k_6 in Table V, has been arbitrarily set to 2.5, the value estimated at 21°C from the reported measurements of Mårdh [4].

The rate constants $k_3 = 324$ min^{-1} and $k_6 = 810$ min^{-1}, governing the dephosphorylation (to E_1) of the phosphoenzymes E_1P and E_2P and their interconversion, respectively, can be directly compared to other estimates. At 1°C it was found (paper II) that $k_3 = 0.13$ s^{-1} = 7.8 min^{-1} and k_6 (= k_2 of paper II, Table III) = 0.21 s^{-1} = 12.6 min^{-1}. Our value of k_{-6} is obtained by assuming that the ratio $k_6/k_{-6} \sim 20$, at 37°C is identical to the value at 1°C. At 21°C,

Mårdh [4] obtained $k_3 \sim 180$–240 min^{-1}, so the trend and order of magnitude of our value of k_3 is consistent with more direct experimental determinations.

In kinetic experiments with the $(Na^+ + K^+)$-enzyme at small (< 5 mM) K^+ concentrations we have noted a decrease (with increasing K^+) of the intercept (i.e. the reciprocal apparent maximal velocity) of the primary double reciprocal plot of the data to a limiting value. Similarly, a slight apparently linear increase * of the slope with increasing K^+ is seen. These effects are not accounted for by the model in Fig. 2.

One possible explanation of these features is that E_2K and E_2KMA may add an extra potassium ion, and that the resulting adduct also has hydrolytic activity. This is seen as follows:

Consider the general scheme (S6)

$$\begin{array}{ccc} E & - & ES \xrightarrow{k} \text{Products} \\ {\scriptstyle \kappa_1}\big| & & \big|{\scriptstyle \kappa_2} \\ EK & - & EKS \xrightarrow{k'} \text{Products} \end{array} \qquad \text{(S6)}$$

Assuming for simplicity equilibrium except in the product forming steps, the slope and intercept in a double reciprocal plot for this mechanism is

$$S = \frac{K_m}{kE_0} \frac{1 + K/K_1}{1 + \gamma K/K_2} \qquad (70)$$

$$I = \frac{1}{kE_0} \frac{1 + K/K_2}{1 + \gamma K/K_2} \qquad (71)$$

$$\gamma \equiv k'/k \qquad (72)$$

where K_m and E_0 are Michaelis constant and total enzyme concentration, respectively. From Eqns. 70 and 71 it is seen that the intercept I decreases and the slope S increases with increasing K if

$$1 < \gamma < \frac{K_2}{K_1} \qquad (73)$$

This question requires further investigation.

We emphasize that all the results reported here, including the values of the rate constants, have been obtained at constant Na^+ concentration (150 mM). Nothing can be said, therefore, about the precise influence of Na^+ on the kinetics of ATPase. In particular, since the model in Fig. 2 is used here exclusively to account quantitatively for kinetic experiments in vitro and is not a transport model, we do not, at this stage, associate any 'sidedness' (with regard to ion transport) with the various intermediates in the model. Work aiming at elucidating this, as well as the above mentioned problem, is presently in progress.

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* This fact would seem to interfere with the determination of κ_1 and κ_{-1} as described in the previous section. However, for that computation we have used K = 20 mM because that was the K^+-concentration employed in the previous determination, in paper I, of the kinetic constants K_{MA} and V_{MA}.