Biochimica et Biophysica Acta 904 (1987) 353–364
Elsevier

BBA 73768

The sided action of Na^+ and of K^+ on reconstituted shark ($Na^+ + K^+$)-ATPase engaged in Na^+–Na^+ exchange accompanied by ATP hydrolysis. I. The ATP activation curve

Flemming Cornelius and Jens Christian Skou

Institute of Biophysics, University of Aarhus, Aarhus (Denmark)

(Received 15 May 1987)

Key words ATPase, ($Na^+ + K^+$)-, Sodium–sodium exchange, Reconstitution, ATP activation, Kinetics, Proteoliposome

The ATP hydrolysis dependent Na^+–Na^+ exchange of reconstituted shark ($Na^+ + K^+$)-ATPase is electrogenic with a transport stoichiometry as for the Na^+–K^+ exchange, suggesting that translocation of extracellular Na^+ is taking place via the same route as extracellular K^+. The preparation thus offers an opportunity to compare the sided action of Na^+ and of K^+ on the affinity for ATP in a reaction in which the intermediary steps in the overall reaction seems to be the same without and with K^+. With Na^+ but no K^+ on the two sides of the enzyme, the ATP-activation curve is hyperbolic and the affinity for ATP is high. Extracellular K^+ in concentrations of 50 μM (the lowest tested) and up gives biphasic ATP activation curves, with both a high- and a low-affinity component for ATP. Cytoplasmic K^+ also gives biphasic ATP-activation curves, however, only when the K^+ concentration is 50 mM or higher ($Na^+ + K^+ = 130$ mM). The different ATP-activation curves are explained from the Albers-Post scheme, in which there is an ATP-dependent and an ATP-independent deocclusion of $E_2(Na_2^+)$ and $E_2(K_2^+)$, respectively, and in which the dephosphorylation of E_2-P is rate limiting in the presence of Na^+ (but no K^+) extracellular, whereas in the presence of extracellular K^+ it is the deocclusion of $E_2(K_2^+)$ which is rate limiting.

Introduction

With unsided preparations of the ($Na^+ + K^+$)-ATPase a major difference between the Na^+-dependent ATP hydrolysis and the ($Na^+ + K^+$)-dependent hydrolysis, apart from the difference in V_{max}, is that with Na^+ the ATP activation curve is hyperbolic with a K_m which is a fraction of μM, while in the presence of K^+ the activation curve is biphasic with components of both high- and low-affinity for ATP [1–8]. The low concentrations of K^+ needed to induce biphasic activation curves suggest that it is an extracellular effect. This is confirmed from experiments with a sided preparation, resealed ghosts, which show that with extracellular K^+ the ATP-activation curve is complex suggesting a high-affinity ($K_m \approx 1$ μM) and a low-affinity effect of ATP ($K_m \approx 100$ μM). With no K^+ and no Na^+ extracellular, i.e. measuring the uncoupled Na^+ efflux, the ATP hydrolysis saturates at about 1 μM ATP suggesting only the high-affinity effect of ATP [9].

Dephosphorylation of E_2-P by K^+ leads to an occlusion of K^+, $E_2(K_2)$ which according to the Albers-Post scheme [10,11] means transfer of K^+ from the extracellular medium to the membrane phase. The low-affinity effect of ATP with $Na^+ + K^+$ is explained from the requirement for ATP to

Correspondence F Cornelius, Institute of Biophysics, University of Aarhus, DK-8000 Aarhus C, Denmark.

increase the rate of release of K^+ from the K^+ occluded conformation $E_2(K_2)$, which probably represents the release of K^+ from the membrane phase to the cytoplasmic medium.

In a previous paper it was shown that reconstituted rectal gland ($Na^+ + K^+$)-ATPase has an ATP hydrolysis dependent Na^+–Na^+ exchange in which extracellular Na^+ acted as a poor K^+ substitute. The stoichiometry for the exchange was as for the Na^+–K^+ exchange and the exchange was electrogenic [12]. This suggests that the intermediary steps in the reaction with Na^+ in this reaction are the same as in the reaction with $Na^+ + K^+$. The rate of the ATP hydrolysis dependent Na^+–Na^+ exchange is about 6% of the rate of the Na^+–K^+ exchange. With extracellular Na^+ acting as a K^+ substitute in the ATP hydrolysis dependent Na^+–Na^+ exchange, Na^+ must go through the same occlusion/deocclusion steps as K^+ in the $Na^+ + K^+$ reaction during the translocation step from the extracellular to the cytoplasmic side. With a sided preparation it therefore seems possible to compare the effect of Na^+ and of $Na^+ + K^+$ on the affinity of ATP under conditions where the reaction with and without K^+ follows the same pathway.

Is there in this Na^+–Na^+ exchange only a high-affinity effect of ATP, and if so what is the explanation of the different effect of ATP with extracellular Na^+ and with extracellular K^+? How is the sided effect of Na^+ and of K^+? K^+ can become occluded not only from the extracellular side by a dephosphorylation of E_2-P but also from the cytoplasmic side of the system [13]. Provided that the low-affinity effect of ATP in the presence of K^+ is due to the requirement for ATP for deocclusion of K^+ from $E_2(K_2)$ there should also be a low-affinity effect of ATP with K^+ on the cytoplasmic side but no K^+ on the extracellular side. In this paper we attempt to answer these questions.

Methods

Materials. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were obtained from Avanti Polar Lipids, AL, U.S.A. Octaethyleneglycol dodecyl monoether ($C_{12}E_8$) was from Nikko Chemicals, Tokyo, Japan. $^{22}NaCl$ and [^{32}P]ATP was obtained from Amersham International. Phosphocreatine, creatine phosphokinase and cholesterol were from Sigma.

Preparation of membrane bound and solubilized ($Na^+ + K^+$)-ATPase from the rectal glands of *Squalus acanthias* was as previously described [14,15]. The specific activity of the enzyme after solubilization was 600–900 μmol P_i/mg per h at 22° C (pH 7).

Preparation of proteoliposomes. Proteoliposomes were prepared as previously described [16] by cosolubilization of lipids and protein using $C_{12}E_8$ followed by adsorption of the detergent to Bio-Beads (Bio-Rad). A 1 : 20 protein to lipid weight ratio was used and the proteoliposomes contained phospholipids and cholesterol in the following proportions (mole fractions) PC/ PE/ PI/ cholesterol (48 : 12 : 1.5 : 38.5).

The proteoliposomes were prepared to contain intravesicular 0.1 mM $MgCl_2$, 30 mM histidine (pH 7.0) and varying concentrations of NaCl (11 to 130 mM) by isoosmotic replacement of NaCl with sucrose. The extravesicular medium contained either the same concentrations of NaCl + sucrose as on the inside or sucrose was replaced with an isoosmolar concentration of either NaCl or KCl. The orientation of Na^+/K^+ pump molecules after reconstitution was determined as described previously [16]. About 15% of the pump molecules were oriented inside-out (i : o), 65% were right-side out (r : o) and the rest (20%) were incorporated with both extracellular and cytoplasmic side exposed (n-o).

ATPase assay The hydrolytic activity of solubilized ($Na^+ + K^+$)-ATPase was determined at 22° C in a medium containing (mM): 130 NaCl, 20 KCl, 4 $MgCl_2$, 3 ATP, 0.2 EGTA, 20 histidine (pH 7.0) and further 0.66 mg/ml bovine serum albumin and 0.1 mg/ml $C_{12}E_8$. Inorganic phosphate was determined by the method of Baginski et al. [17] with the addition of 5% sodium dodecyl sulfate to the arsinite-citrate reagent. In order to determine the ATPase activity for reconstituted ($Na^+ + K^+$)-ATPase at varying ATP-concentration (the substrate curves) [^{32}P]ATP was employed and the relased ^{32}P determined in a scintillation counter as previously described [18]. The reaction time was adjusted in such a way that less

than 20% of the added ATP was being hydrolysed during the assay.

[^{32}P]ATP, ATP and ADP was purified by chromatography on a DEAE-Sephadex A-25 column [19].

Protein. Protein was determined as described by Peterson [20]. Bovine serum albumin was used as a standard.

Flux-measurement. The proteoliposomes contained Na^+ and no K^+. ATP was added to the medium outside the vesicles together with ATP regenerating system phosphocreatine (1.5 mM) and creatine phosphokinase (5 I.U./ml). At the outside either Na^+ or $Na^+ + K^+$ were present. With this condition a Na^+–Na^+ exchange takes place on i : o-oriented Na^+/K^+ pumps, only. The intensity of the exchange could be measured as an influx of radioactive ^{22}Na as previously described [12].

In essence, the influx was measured by incubating proteoliposomes for varying periods of time in the presence of ATP and ^{22}Na, withdrawing a 50 μl sample and passing it through a cationic resin cartridge containing Bio-Rex 70 in the Tris-form by flushing with 1 ml 260 mM sucrose at 0 °C. Essentially all extraliposomal ^{22}Na was removed by this procedure leaving, in the eluate, only ^{22}Na trapped within the vesicles. Before use the resin was equilibrated with 3 mg/ml bovine serum albumin. A parallel experiment with 1 mM digitoxigenin added to the incubation medium served as a measure of the passive influx of ^{22}Na. The steady-state value of intravesicular ^{22}Na-activity was reached after about 3 h.

The liposome associated ^{22}Na-activity followed a simple monoexponential curve

$$A_t = A_\infty(1 - \exp(-kt))$$

where A_t and A_∞ are the activities at zero time and 3 h respectively. k, the observed rate constant, was calculated from a weighted non-linear regression analysis of A_t vs. t [21,22]. The initial influx could then be calculated from $k \cdot A_\infty$, the specific activity, the protein content of 50 μl sample and the fraction of i : o-oriented pumps.

As described only inside-out pumps become engaged in transport across the vesicle membrane when ATP is added externally. Thus, referring to the cellular situation when cations are bound to the normal extracellular side they are referred to as extracellular and when they are bound to the opposite side as cytoplasmic. Inside and outside the vesicles are equivalent to extracellularly and cytoplasmic, respectively. Vesicular influx and efflux of cations are then cellular effluxes and influxes, respectively.

Statistics. The substrate curves i.e. either the maximum flux or the hydrolytic activity (v) as a function of [ATP] were analysed by non-linear regression analysis [21,22] by fitting of the data to either the first-degree (Eqn. 1) or the second-degree (Eqn. 2) rate equations

$$v = a\ \mathrm{ATP}/(b + \mathrm{ATP}) \tag{1}$$

$$v = (a \cdot \mathrm{ATP}^2 + b\ \mathrm{ATP})/(\mathrm{ATP}^2 + c\ \mathrm{ATP} + d) \tag{2}$$

Distinction between the two equations was aided both by inspection of the variance of the computer fit and by a graphical procedure: for each experiment the data were plotted as v versus $v/(\mathrm{ATP})$ (Eadie-plot). First-degree equations are strictly linear, whereas higher-degree rate functions are non-linear.

For second degree systems it was further tested if the rate equation (Eqn. 2) was equivalent to the sum of two Michaelis-Menten type equations (Eqn. 3)

$$v = V_m(1) \cdot \mathrm{ATP}/(K_m(1) + \mathrm{ATP}) + V_m(2)\ \mathrm{ATP}/(K_m(2) + \mathrm{ATP}) \tag{3}$$

As demonstrated by Rossi and Garrahan [8] this will be the case if both inequalities Eqn. 4 and Eqn. 5 are satisfied

$$c^2 > 4d \tag{4}$$

$$a/2 > c/(c^2 - 4d) \cdot (a/2 - c/d) \tag{5}$$

For all experiments which were found to be second degree systems the inequalities Eqn. 4 and Eqn. 5 were satisfied and therefore their substrate curves could adequately be described by Eqn. 3 as well as Eqn. 2.

Results

The ATP substrate curve of the $(Na^+ + K^+)$-ATPase engaged in Na^+–Na^+ exchange

The ATP hydrolysis, as well as the maximum Na^+ flux, has been used to measure the apparent affinity for ATP for the Na^+–Na^+ exchange with Na^+ but no K^+ extracellular, and with Na^+ or K^+ ($K^+ + Na^+ = 116$ mM) on the cytoplasmic side. The effect of extracellular K^+ is deduced from a comparison of an effect of K^+ on the ATP affinity of an unsided $(Na^+ + K^+)$-ATPase preparation with the effect of cytoplasmic K^+ on the sided preparation.

Na^+–Na^+ exchange parameters

The initial influx (cellular efflux) was estimated from ^{22}Na-influx experiments with and without addition of the lipid soluble digitoxigenin to the flux medium as previously described [12] by best computer fit using the expression, $A_t = A_\infty (1 - e^{-kt})$ for the radioactivity trapped in the proteoliposomes as a function of time (see Methods). The difference between the two calculated initial influxes (±digitoxigenin) represents the $(Na^+ + K^+)$-ATPase-dependent Na^+ influx (cellular efflux) which as previously demonstrated is the result of the Na^+–Na^+ exchange taking place under these conditions with no extracellular K^+, and is driven by a net ATP-hydrolysis [12]. The influx in the presence of digitoxigenin represents a passive leak. The passive leak was in most instances below 10% of total influx.

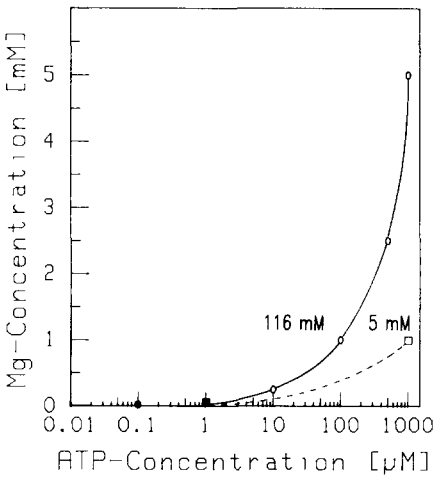


Fig 1 The total Mg concentration needed to obtain maximum hydrolytic activity of 1 o-oriented reconstituted $(Na^+ + K^+)$-ATPase engaged in Na^+–Na^+ exchange at different total ATP concentrations at two cytoplasmic Na^+ concentrations (116 mM and 5 mM) The proteoliposomes contained 130 mM Na^+ internally (extracellular) and isoosmolarity was achieved by addition of sucrose The temperature was 22° C and pH = 7 0

Since the present Na^+–Na^+ exchange is associated with ATP hydrolysis the substrate curves were also determined by measuring the ATP hydrolysis associated with Na^+–Na^+ exchange as a function of the ATP concentration However, in contrast to measurement of flux, non-oriented reconstituted enzyme (see Methods) will contribute to the hydrolysis unless proper precautions are taken. As shown previously [16,23] the inhibition by ouabain is not instantaneous. Therefore in order to measure ATP hydrolysis catalyzed by 1. o-oriented enzyme molecules only, the proteoliposomes were preincubated with ouabain in the presence of Mg (1 mM) and P_i (0.2 mM), before testing in the presence of ouabain (1 mM)

Interaction with Mg

MgATP seems to be the substrate for the enzyme. Free ATP inhibits the hydrolytic activity of the enzyme and so does free Mg^{2+} but the inhibitory effect of free Mg^{2+} is not very pronounced, i.e. a slight variation in the concentration of free Mg^{2+} has little effect on the activity [24]. In the present experiments at each ATP concentration the Mg^{2+} concentration chosen is the concentration which gives optimum effect at a fixed ATP concentration and a fixed concentration of cations. This means that the concentration of free Mg^{2+} is not the same with different ATP concentrations. The optimum Mg^{2+} concentration does not only vary with the ATP concentration but at a given ATP concentration it is also dependent on the concentration of the monovalent cations, Fig. 1.

The substrate curve for Na^+–Na^+ exchange using sided preparations

(A) Interaction with cytoplasmic Na^+

Using proteoliposomes with 130 mM Na^+ internally (extracellular) the maximum hydrolytic activity was determined as a function of ATP at

different external (cytoplasmic) Na^+ concentrations. The osmolarity was kept constant by isoosmotic replacement of Na^+ with sucrose.

Fig. 2A shows the substrate curves at 116 mM cytoplasmic Na^+ (plus sucrose to obtain an osmolarity of 260 mosM) with 130 mM extracellular Na^+. Such substrate curves were analysed in order to determine if they were biphasic or simple hyperbolic. This could be decided from both the goodness of a direct non-linear computer fit using either a first-degree (Eqn. 1) or a second-degree (Eqn. 2) rate equation and by replotting the data in the form of an Eadie plot as shown in Fig. 2B (see Methods). The curves presented in Figs. 2A and 2B are the best computer fit: the relation was found to be simple monophasic. Similar experiments were carried out at four additional cytoplasmic Na^+ concentrations (5, 25, 46 and 80 mM) with 130 mM extracellular Na^+ (data not shown). At all cytoplasmic Na^+ concentrations tested the substrate curve was found to be monophasic, i.e. the kinetics were of a simple Michaelis-Menten type. In Fig. 3 and Table I are shown the K_m and V_m fitting parameters calculated from the non-linear regression analysis of the substrate curves at varying cytoplasmic Na^+ between 5 mM and 116 mM. The extracellular Na^+-concentration was 130 mM in all cases. As seen, both the K_m and V_m value increase with increasing cytoplasmic Na^+ concentration.

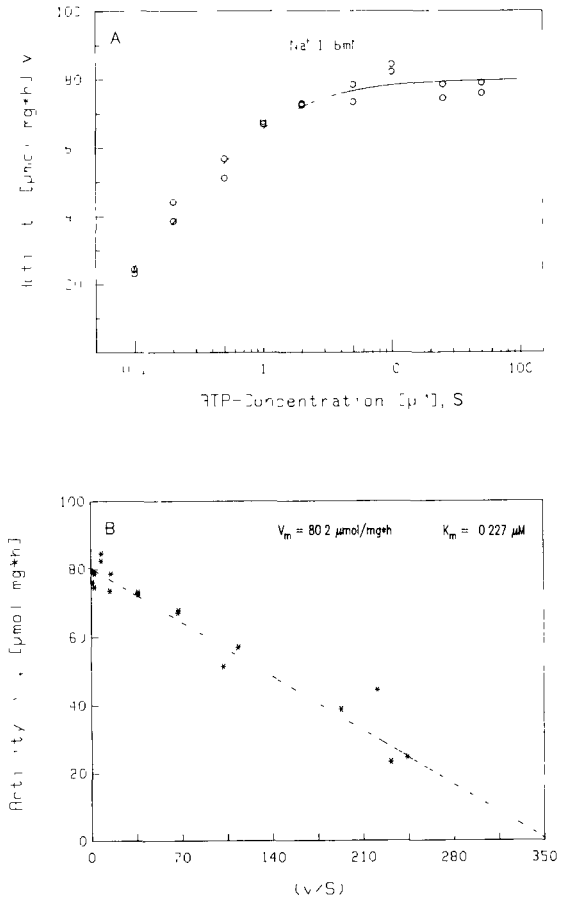


Fig. 2 (A) The ATP activation curve (substrate curve) for 1:0-oriented reconstituted ($Na^+ + K^+$)-ATPase engaged in Na^+–Na^+ exchange The hydrolytic activity (v) is plotted against the ATP concentration (S) on a logarithmic scale The extracellular concentration of Na^+ was 130 mM and the cytoplasmic Na^+ concentration was 116 mM 28 mM sucrose was added to the medium to obtain isoosmolarity The line represents the best fit to the equation, $v = V_m$ ATP/(K_m + ATP) using a weighted non-linear regression analysis (see Methods) (B) The same data replotted in the linearized Eadie plot (i.e. v versus v/S) The straight line represents the best fit as shown in panel A The fitting parameters were $V_m = 80.2 \pm 1.2$ µmol/mg (1.0) protein per h and $K_m = 0.227 \pm 0.011$ µM

When flux is being measured there is more scatter in the experimental points which makes it more difficult to decide from the Eadie plots if the substrate curves were purely monophasic or biphasic with a small component of low affinity. However, the general tendencies are the same as found in experiments measuring ATP hydrolysis

(B) Interaction with cytoplasmic K^+

Fig. 4 shows Eadie plots from a serie of substrate curves determined at increasing cytoplasmic K^+ when the sum of $Na^+_{cyt} + K^+_{cyt} = 116$ mM and extracellular Na^+ is 130 mM. The lines in the figure present the best computer fit to either first- or second-order rate equation and the fitting parameters are shown in Figs. 5A, 5B and Table I. The general tendencies are: (i) as cytoplasmic K^+ increases and becomes greater than 50 mM the substrate curve becomes biphasic, i.e. curved in the Eadie plot; (ii) the maximum activity decreases with increasing cytoplasmic K^+ (and concomitant decrease in Na^+); and (iii) the apparent affinity for ATP is rather constant for cytoplasmic K^+ concentrations up to 50 mM and then it decreases with increasing cytoplasmic K^+ (see Fig. 5A).

When the initial influx is measured as a func-

tion of the ATP concentration in media with increasing K^+ (K^+ substituted for Na^+) the same substrate curves resulted as when measuring ATP hydrolysis directly: The Eadie plots are clearly curved at the higher cytoplasmic K^+ concentrations, but linear with low cytoplasmic K^+. The fitting parameters (K_m, V_m) for the flux experiments are shown in Figs. 5A and 5B together with the data from hydrolysis. As indicated the apparent K_m (Fig. 5A) increases drastically with increasing cytoplasmic K^+ going from 1.5 μM at 2 mM cytoplasmic K^+ to 56.5 μM at 105 mM cytoplasmic K^+. The maximum influx of Na^+ decreased in parallel with the hydrolytic activity with increased cytoplasmic K^+ (Fig. 5B), indicating a fixed stoichiometry.

ATP substrate curves using unsided preparations

(A) Interaction with cytoplasmic Na^+

It should be noted that rather high cytoplasmic K^+ concentrations are needed (approx 50 mM) in order to induce biphasic substrate curves (Fig. 4). In previous experiments with unsided preparations the substrate curve was biphasic in the presence of much lower (10 mM) K^+ concentrations [6–8] indicating that it was an extracellular K^+ effect. This was also the case in experiments with resealed ghosts where the substrate curve was clearly biphasic at 1 mM extracellular K^+ [9].

Using i·o-oriented reconstituted ($Na^+ + K^+$)-ATPase it is difficult to investigate the effect of extracellular K^+; however, by comparing the effects of K^+ on unsided and on sided preparations it is possible to evaluate the effect of extracellular K^+. We therefore studied the hydrolytic activity of several unsided preparations as a function of the Na^+ concentration alone and as a function of a combination of $Na^+ + K^+$. In order to make the comparison as complete as possible, we used both unreconstituted ($Na^+ + K^+$)-ATPase (solubilized or membrane bound) and reconstituted ($Na^+ + K^+$)-ATPase in the absence of ouabain In the latter, both (i:o)-oriented and (n-o) enzyme are active, however, the fraction of (n-o) enzyme is higher than of (i.o)-oriented enzyme, and in the presence of K^+ only (n-o)enzyme is activated (see next paragraph).

Fig. 6 compares the Eadie plots of substrate curves for the three unsided preparations mentioned above with reconstituted (i.o)-oriented enzyme in the absence of K^+ and with 130 mM Na^+. As shown the computed regression lines are all apparently rectilinear in the linearized Eadie plot

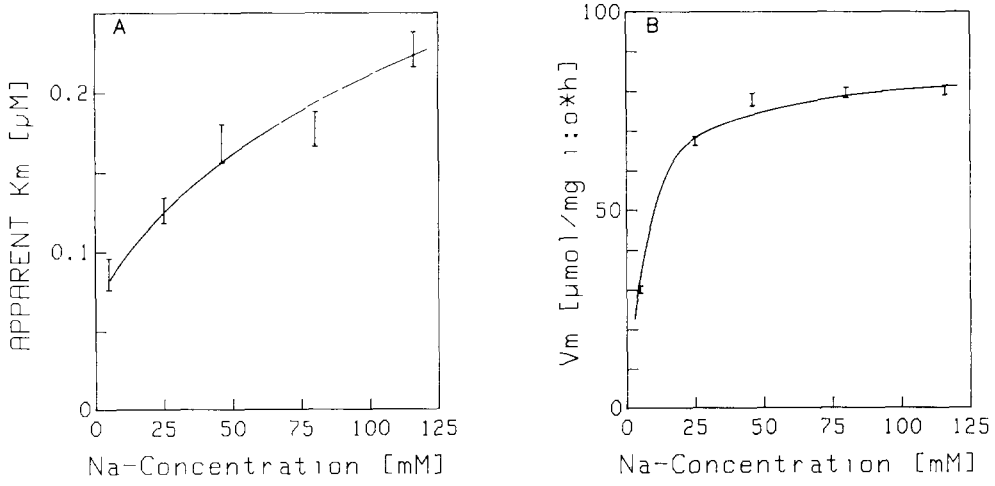


Fig 3. The fitting parameters (K_m and V_m) computed from the hydrolytic activity (v) of reconstituted i o-oriented ($Na^+ + K^+$)-ATPase at different ATP concentrations (S), at five different cytoplasmic Na^+-concentrations The extracellular (inside the proteoliposomes) Na^+ concentration was 130 mM in all cases Enzyme reconstituted as n-o (non-oriented) with both sides exposed was inhibited by ouabain in the test solution after preincubating the proteoliposomes with ouabain in the presence of Mg (1 mM) and P_i (0 2 mM) Bars indicate ± 1 S E from regression analysis

indicating simple Michaelis-Menten kinetics. However, the apparent ATP affinity was slightly different for the different preparations: 0.22 μM for membrane bound enzyme (not treated with the detergent $C_{12}E_8$), 0.74 μM for $C_{12}E_8$ solubilized enzyme and 0.32 μM for reconstituted (sided as well as unsided) enzyme.

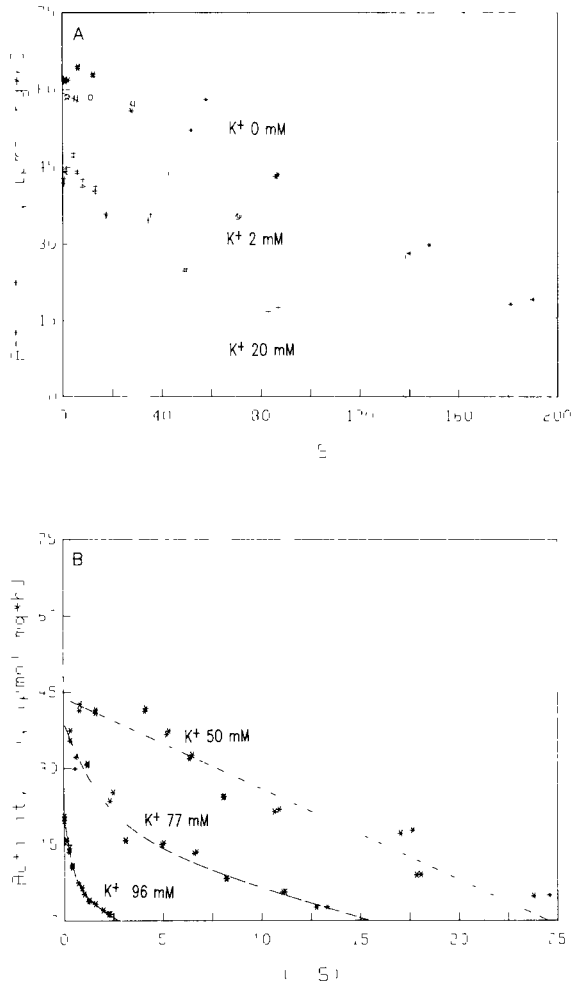


Fig 4 Eadie plots of hydrolytic activity of i o-oriented ($Na^+ + K^+$)-ATPase at increasing cytoplasmic K^+ concentrations. Six different cytoplasmic K^+ concentrations are analyzed. The cytoplasmic concentration of $Na^+ + K^+$ was 116 mM and 28 mM sucrose was added to obtain isoosmotic conditions The extracellular concentration of Na^+ was 130 mM The fitting parameters are shown in Fig. 8, lower curve At cytoplasmic K^+ concentrations greater than 50 mM the Eadie plots are clearly curved, i e the substrate curves are not hyperbolic.

(B) Interaction with K^+

Fig. 7 depicts Eadie plots for different K^+ concentrations using a reconstituted preparation without ouabain and similar results were obtained using solubilized enzyme and membrane bound enzyme (data not shown). For both preparations the curves are only rectilinear when K^+ is completely omitted in the medium. Substitution of as little as 50 μM Na^+ with K^+ causes the Eadie plots to become curves with a component of high apparent affinity and a component of lower affinity (data not shown).

In Fig. 8 the effect of increasing the K^+ concentration simultaneously on the two sides of the enzyme on the affinity for ATP (upper curve) is shown for comparison with the sided preparation (lower curve). For the sided preparation the K^+ concentrations refer to cytoplasmic concentrations alone, whereas only Na^+ is present extracellularly. The two curves clearly demonstrate the vast difference in sensitivity towards cytoplasmic and extracellular K^+, with respect to the ATP affinities.

Effect of extracellular Na^+

With 130 mM Na^+ extracellular the ATP-activation curve is hyperbolic at all the tested cytoplasmic concentrations of Na^+. However, when both the cytoplasmic and the extracellular Na^+ are lowered to below 25 mM, with sided as well as with unsided preparations, biphasic substrate curves result. That this is due to a decrease in the extracellular concentration of Na^+ is seen from experiments with sided preparations, in which the cytoplasmic concentration of Na^+ is 130 mM and the extracellular concentration of Na^+ is 25 mM. Under these conditions the ATP activation curve is also biphasic. There is always a contamination of K^+ in the solutions and it amounts to 3–6 μM in the present experiments. As discussed above 50 μM K^+ in the presence of 150 mM Na^+ gives biphasic curves with unsided preparations, and when added to the external medium with sided preparations. Considering this it seems likely, that a contamination of 3–6 μM of K^+ is enough to induce biphasic substrate curves when the Na^+ concentration is lowered to 25 mM Na^+ on the extracellular side of the enzyme. In agreement with this view it is found, that with unsided

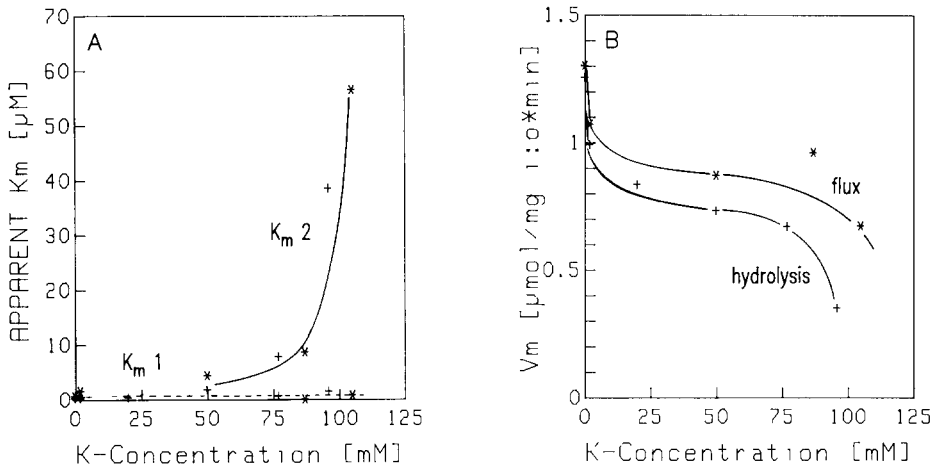


Fig 5 Comparison of the fitting parameters (V_m and K_m) from the substrate curves in the presence of cytoplasmic K^+ using either Na^+ flux (*) or ATP hydrolysis (+) as a measure of v Above 50 mM cytoplasmic K^+ concentration the curves were clearly biphasic, however, the fractions representing the high-affinity component are very small (see Table I) Panel A shows the apparent affinities (K_m) for ATP at increasing cytoplasmic K^+ ($Na^+ + K^+ = 116$ mM) Panel B shows the maximum active influx of Na^+ and the maximum hydrolytic activity at increasing cytoplasmic K^+

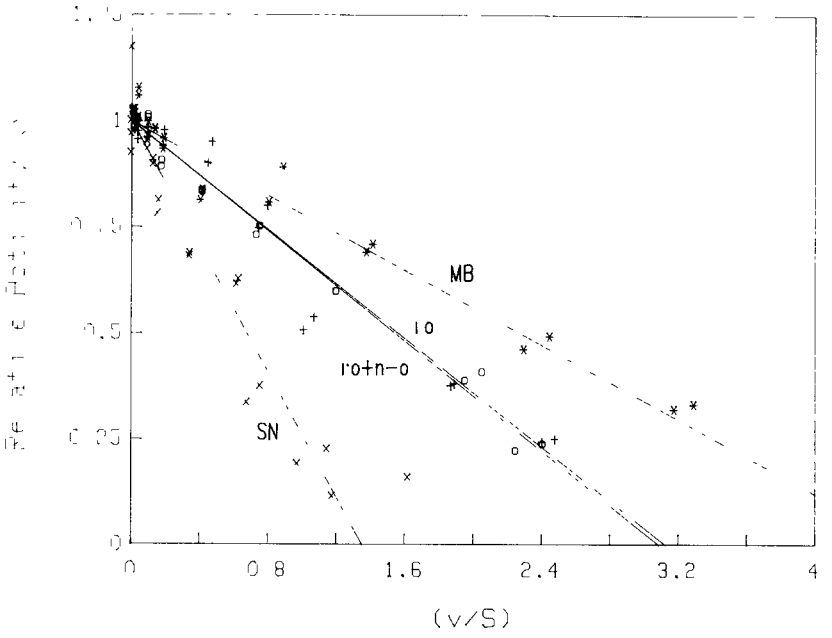


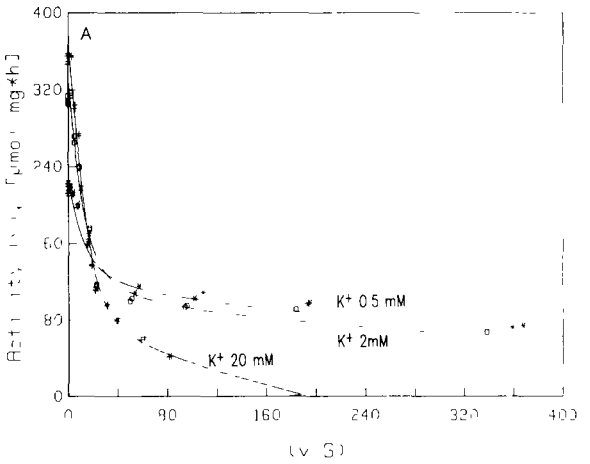
Fig 6 Eadie plots of hydrolytic activity using different unsided preparations compared to a sided preparation The activity (v) is given relative in order to compare the different preparations The labels on the curves identify the following preparations MB, membrane-bound enzyme, i o+n-o, inside-out plus non-oriented reconstituted enzyme, i o, inside-out oriented reconstituted enzyme, and SN, solubilized enzyme The K_m values given by the fitting procedure were, MB 0 22 μM, i o and i o+n-o 0 32 μM; SN 0 74 μM All experiments were carried out with 116 mM cytoplasmic and 130 mM extracellular Na^+ 28 mM sucrose was added to the medium to obtain isoosmotic conditions

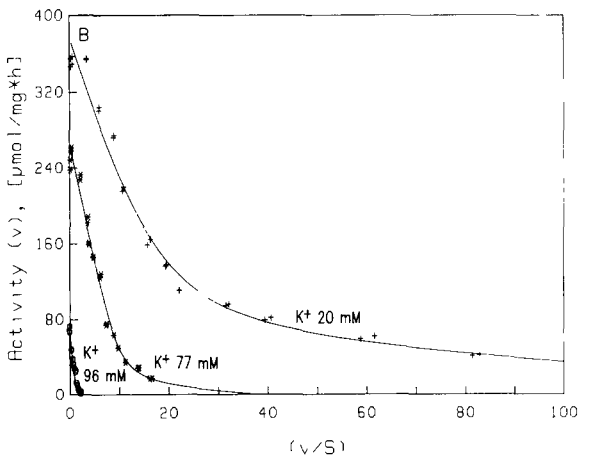
TABLE I

FITTING PARAMETERS FOR ATP ACTIVATION CURVES USING RECONSTITUTED SHARK $(Na^+ + K^+)$-ATPase

The hydrolytic activity is measured at varying cytoplasmic cation concentrations (either Na^+ alone or a combination of $Na^+ + K^+$) and a constant extracellular Na^+ concentration All figures are presented as means ± S E V_m is expressed in μmol/mg per h, K_m is expressed in μM

Na^+ (mM)	Na^+ media		Na^+/K^+ (mM/mM)	$Na^+ + K^+$ media			
	V_m	K_m		V_m (1)	V_m (2)	K_m (1)	K_m (2)
116	80 2 ± 1 2	0 227 ± 0 011	116/0	63 8 ± 0 62	–	0 242 ± 0 007	–
80	79 6 ± 1 3	0 177 ± 0 011	114/2	59 4 ± 1 3	–	0 254 ± 0 016	–
46	77 9 ± 1 6	0 168 ± 0 012	96/20	51 4 ± 0.5	–	0 344 ± 0 015	–
25	71 8 ± 0 7	0 138 ± 0 004	66/50	43 9 ± 1 3	–	1 79 ± 0 11	–
5	34 0 ± 1 0	0.081 ± 0 008	38/77	3 26 ± 1 0	37 9 ± 2 1	0 220 ± 0.014	7 8 ± 0 6
			20/96	3 95 ± 0.9	17 0 ± 1 12	1 49 ± 0 6	35.3 ± 3 3





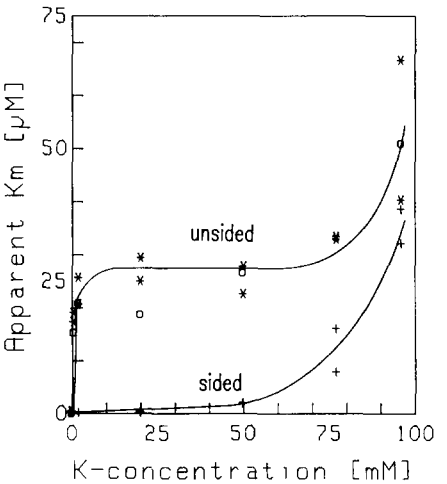


Fig 8 The K_m values computed using either a sided preparation (1 o-oriented reconstituted enzyme) or an unsided preparation (non-oriented reconstituted enzyme) (○), membrane-bound enzyme (★) or solubilized enzyme (★) at different external K^+ concentrations with $Na^+ + K^+ = 116$ mM

Fig 7 Eadie plots of hydrolytic activity using reconstituted enzyme without the presence of ouabain, i e the hydrolytic activity corresponds to 1 o-oriented and non-oriented enzyme However, since K^+ is present the activity is primarily arising from n-o enzyme, since 1 o-oriented enzyme is devoid of extracellular K^+ Six Eadie plots are shown at increasing cytoplasmic K^+ $(Na^+ + K^+ = 116$ mM) and 130 mM extracellular Na^+ The fitting parameters (K_m) are shown in Fig 8, upper curve All curves including the lowest cytoplasmic K^+ concentration (0 5 mM) used indicate biphasic substrate curves

preparations the sensitivity for the effect of K^+ on the biphasic shape of the ATP-activation curve is increased, when the Na^+ concentration is decreased from 150 mM to 25 mM.

Discussion

In the present paper we have investigated the effect of cytoplasmic and extracellular Na^+ and K^+ on the shape of the substrate curve of reconstituted shark $(Na^+ + K^+)$-ATPase engaged in Na^+–Na^+ exchange accompanied by ATP hydrolysis. The same results were obtained when either the rate of exchange (measured as cellular Na^+ efflux) or the hydrolysis of ATP was used as a measure of enzyme activity (v) as a function of ATP concentration at the various cation conditions.

The substrate curves at the different ionic conditions were analyzed by using the Eadie plot which is considered superior to other plots (especially the Lineweaver-Burk plot) in order to discriminate between mono- and biphasic substrate curves [25].

The ATP activation in the presence of extracellular K^+

Assuming that the reaction with the cytoplasmic and the extracellular cations is consequetive as it is described in the recent modified Albers-Post scheme [26,12] (Fig. 9) it is a dephosphorylation of E_2-P which leads to the transition of K^+ from the extracellular to the cytoplasmic side This step involves an occlusion of K^+ due to the dephosphorylation followed by a deocclusion, the rate of which is increased by ATP. With extracellular K^+, the rate of dephosphorylation is high and it is the rate of deocclusion of K^+ from $E_2(K_2)$ which is rate limiting. The biphasic ATP activation curve with extracellular K^+ [1–9] can be explained form a branched deocclusion reaction [7,27,28]: One deocclusion which is independent of ATP and in which $E_2(K_2)$ is converted to a form with high ATP affinity and which will dominate with a low ATP concentration. Another in which ATP with a low affinity increases the rate of deocclusion (see Fig. 9). In the scheme suggested by Moczydlowski and Fortes [7] the high-affinity form is shown as E_1 with no cations

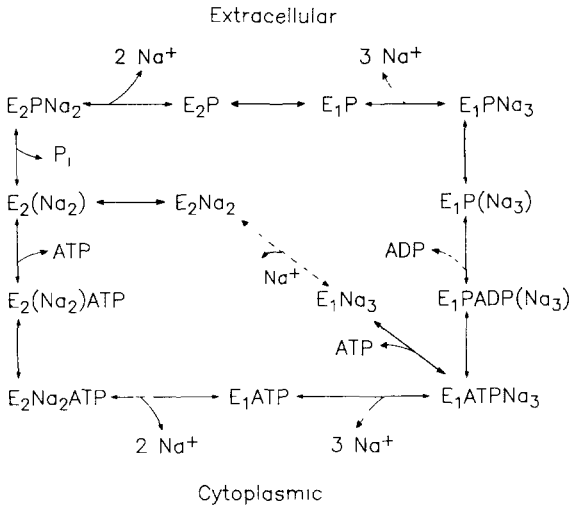


Fig 9 The extended Albers-Post scheme for the $(Na^+ + K^+)$-ATPase reaction in the absence of K^+ (Na^+–Na^+ exchange accompanied by ATP hydrolysis). The scheme is based on the Albers-Post model [10,11] and the modifications by Karlish et al [26] and Nørby et al [33]. The symbols E_1 and E_2 refer to the two different conformations of the enzyme Parenthesis indicate that the cations are occluded within the enzyme The stippled line from E_2Na_2 to E_1Na_3 indicates that this reaction probably involves several steps

bound. However, the enzyme with no cations bound is on an E_2-conformation [29,30] and only with Na^+ bound the enzyme has the E_1-conformation. This suggests that it is the ATP-independent spontaneous deocclusion of K^+ from $E_2(K_2)$ to E_2K_2 followed by the exchange of K^+ for Na^+ which leads to E_1Na_3 and it is this conformation which has the high affinity for ATP (Fig. 9). With a low ATP concentration the deocclusion follows this route while with a higher ATP concentration it is the ATP-dependent deocclusion which becomes dominant

ATP-activation in the absence of extracellular K^+

With Na^+ extracellular instead of K^+ the rate of dephosphorylation of E_2-P is very low, a few percent of the rate of dephosphorylation by K^+. The electrogenic effect and the stoichiometry of the ATP-dependent Na^+–Na^+ exchange suggest that extracellular Na^+ is translocated via the same steps as extracellular K^+ and that the dephosphorylation of E_2-P by extracellular Na^+ leads to an occlusion of extracellular Na^+ followed by its deocclusion to the cytoplasmic side. With Na^+,

but no K^+, nearly all of the enzyme is in the phosphoform in the steady state indicating that it is the dephosphorylation of E_2-P which is rate limiting in the reaction and not the deocclusion of Na^+ from $E_2(Na_2)$.

There seem to be at least two possible explanations of the lack of low affinity effect of ATP with Na^+. One is that the Na^+ occluded form $E_2(Na_2)$ has a high affinity for ATP. The other is that $E_2(Na_2)$ has a low affinity like $E_2(K_2)$, but the rate of spontaneous deocclusion of $E(Na_2^+)$ is so high that it can cope with the low rate of dephosphorylation. Therefore the low affinity effect of ATP is not seen when the rate of hydrolysis, or the rate of flux of Na^+ is measured.

The effect of cytoplasmic K^+

The shape of the ATP-activation curve is changed from hyperbolic to nonhyperbolic (biphasic) at a very low (50 μM) concentration of extracellular K^+ while with Na^+ extracellular but no K^+ the concentration of K^+ needed in the cytoplasmic medium to induce biphasic activation curve is higher than 50 mM (with $Na^+ + K^+ = 116$ mM) (see Fig. 4).

Extracellular K^+ becomes occluded by dephosphorylation of E_2-PK_2^+ [31], but K^+ can also become occluded from the cytoplasmic side by a backwards reaction (see Ref. 13). Extracellular K^+ competes for extracellular Na^+ for the dephosphorylation and the apparent affinity for K^+ is at least 100-times higher than the apparent affinity for extracellular Na^+. On the cytoplasmic side the $K^+ : Na^+$ ratio for equal distribution between the $E_2K_2^+$ and the $E_1Na_3^+$ form is 10 : 140. This much lower apparent affinity for cytoplasmic K^+ may explain that more cytoplasmic K^+ is needed to occlude enough molecules on the K^+ form that the spontaneous rate of deocclusion can no longer cope with the rate of dephosphorylation. Another factor which may be of importance is that with Na^+ but no K^+ extracellular the rate of dephosphorylation is lower than with K^+, and therefore a higher fraction of the molecules must be on the K^+ occluded form for the spontaneous deocclusion to become rate limiting, i.e. a higher cytoplasmic than extracellular K^+ is needed in order to observe the effect on the affinity for ATP.

Ionic strength effect

With Na^+ but no K^+ Rossie and Garrahan [8] in unsided preparations found V_m as well as K_m to increased with increase in Na^+. K_m increased from about 0.05 μM with 20 mM Na^+ to about 0.27 μM with 130 mM Na^+. In the present paper a similar effect is observed (Table I). K_m increases from about 0.08 μM with 5 mM Na^+ to 0.23 μM with 116 mM Na^+. From the present experiments it is seen that this is due to an effect of cytoplasmic Na^+. K_d for ATP increases with an increase in ionic strength [32], this may explain that K_m for ATP increases when the concentration of Na^+ is increased in a replacement for glucose.

Simultaneous and consecutive models

The results reported in the present paper have been discussed with reference to the widely accepted consecutive model of Albers and Post [10,11] including the modifications proposed by Karlish et al. [26] and the formation of at least three different phosphoenzymes proposed by Nørby et al. [33]. The model does not include the possibility of separate dephosphorylation of each phosphoenzyme intermediate [33] or cation fluxes associated with each phosphorylation-dephosphorylation cycle as proposed by Lee and Fortes [34] and by Yoda and Yoda [35]. This does not, however, with respect to cations exclude that other models simultaneous or consecutive with transmembrane allosteric effects [36] could explain the results as well. This will be discussed in more detail in a proceeding paper.

Acknowledgements

We wish to thank Birte Westergaard and Hanne Zakarias for excellent technical help. The investigation was supported by the Danish Medical Research Council, 'Ingeborg and Leo Dannin's Foundation for Scientific Research' and P. Carl Petersen's Foundation.

References

1 Neufeld, A H. and Levy, H M (1969) J Biol Chem 244, 6493–6497
2 Kanazawa, T , Saito, M. and Tonomura, Y (1970) J Biochem (Tokyo) 67, 693–711
3 Robinson, J D (1976) Biochim Biophys Acta 429, 1006–1019

4 Froehlich, J P., Albers, R W , Koval, G J , Goebel, R. and Berman, M (1976) J. Biol Chem 251, 2186–2188
5 Cantley, L C , Cantley, L G and Josephson, L (1978) J Biol Chem. 253, 7361–7368
6 Garrahan, P J , Horenstein, A H and Rega, A F (1979) in Na,K-ATPase Structure and Kinetics (Skou, J C and Nørby, J G , eds), pp 261–274, Academic Press, London
7 Moczylowski, E G and Fortes, P A. (1981) J Biol Chem 256, 2357–2366
8 Rossi, R C and Garrahan, P J (1985) in The Sodium Pump (Glynn, I M and Ellory, J C , eds), pp 443–455, The Company of Biologists, Cambridge
9 Glynn, I M and Karlish, S.J D (1976) J Physiol 256, 465–496
10 Albers, R W (1967) Annu Rev Biochem 36, 727–756
11 Post, R L , Kume, T , Tobin, T , Orcutt, B and Sen, A K. (1969) J Gen Physiol 54, 306–326
12 Cornelius, F and Skou, J C (1985) Biochim Biophys Acta 818, 211–221
13 Glynn, I M (1985) in The Enzymes of Biological Membranes (Martonosi, A N , ed), pp 35–114, Plenum Press, New York
14 Skou, J C and Esmann, M (1979) Biochim Biophys Acta 567, 436–444
15 Esmann, M , Skou, J C and Christiansen, C (1979) Biochim Biophys Acta 567, 410–420
16 Cornelius, F and Skou, J C (1984) Biochim Biophys Acta 772, 357–373
17 Baginski, E S , Foa, P P and Zak, B (1966) Clin Chem 13, 326–332
18 Skou, J C (1979) Biochim Biophys Acta 567, 421–435
19 Nørby, J G and Jensen, J (1971) Biochim Biophys Acta 233, 104–116
20 Peterson, G L. (1977) Anal Biochem 83, 346–356
21 Johnston, a (1985) J. Pharmacol Methods 14, 323–329
22 Duggleby, R G (1981) Anal Biochem. 110, 9–18
23 Goldin, S M (1977) J Biol Chem 252, 5630–5642
24 Hexum, T , Samson, F E and Himes, R H (1970) Biochim Biophys Acta 212, 322–331
25 Hofstee, B H J (1959) Nature 184, 1296–1297
26 Karlish, S J D , Yates, D W and Glynn, I.M (1978) Biochim Biophys Acta 525, 252–264
27 Neet, K E and Green, N M (1977) Arch Biochem Biophys 1978 588–597
28 Reynolds, J A , Johnson, E A and Tanford, C (1985) Proc Natl Acad Sci USA 82, 3658–3661
29 Skou, J C and Esmann, M (1983) Biochim Biophys. Acta 746, 101–113
30 Esmann, M and Skou, J C (1983) Biochim Biophys Acta 748, 413–417
31 Poste, R L , Heyvary, C and Kume, S (1972) J Biol Chem 247, 6530–6540
32 Nørby, J G and Jensen, J (1974) Ann N Y Acad Sci 242, 158–167
33 Nørby, J G , Klodos, I and Christiansen, N O (1983) J Gen Physiol 82, 725–759
34 Lee, J A and Fortes, P A G (1985) in The Sodium Pump (Glynn, I M and Ellory, J C , eds), pp 277–282, The Company of Biologists, Cambridge
35 Yoda, A and Yoda, S (1987) J Biol Chem 262, 110–115
36 Karlish, S J D and Stein, W D (1985) J Physiol 359, 119–149