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EFFECTS OF ATP AND PROTONS ON THE Na : K SELECTIVITY OF THE ($Na^+ + K^+$)-ATPase STUDIED BY LIGAND EFFECTS ON INTRINSIC AND EXTRINSIC FLUORESCENCE

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Summary

The effect of pH and of ATP on the Na : K selectivity of the ($Na^+ + K^+$)-ATPase has been tested under equilibrium conditions. The $Na^+ : K^+$-induced change in intrinsic tryptophan fluorescence and in fluorescence of eosin maleimide bound to the system has been used as a tool. 1 mol of eosin maleimide per mol of enzyme gives no loss in either ATPase or phosphatase activity and the fluorescence in the presence of Na^+ is about 30% higher than in the presence of K^+.

Choline, protonated Tris, protonated histidine and Mg^{2+} have an 'Na^+' effect on the extrinsic fluorescence, while Rb^+, Cs^+ and NH_4^+ have a 'K^+' effect. Choline and protonated Tris have an Na^+ effect on intrinsic fluorescence.

A close correlation between the effect of Na^+ compared to K^+ on the fluorescence change and on Na^+ activation of hydrolysis indicates that the observed changes in fluorescence are due to an effect of Na^+ and of K^+ on the internal sites of the system.

The equilibrium between the two conformations, which are reflected by the difference in fluorescence with Na^+ and K^+, respectively, is highly influenced by the concentration of protons. At a given $Na^+ : K^+$ ratio, an increase in the proton concentration shifts the equilibrium towards the 'K^+' fluorescence form while a decrease shifts the equilibrium towards the 'Na^+' fluorescence form, i.e., protons increase the apparent affinity for K^+ and vice versa, K^+ increases pK values of importance for the $Na^+ : K^+$ selectivity. Conversely, a decrease in protons increases the apparent affinity for Na^+ and vice versa, Na^+ decreases the pK.

ATP decreases the apparent pK for the protonation-deprotonation, i.e., ATP facilitates the deprotonation which accompanies Na^+ binding.

The results suggest two effects of ATP for the hydrolysis in the presence

of Na^+ and K^+: (i) at low ATP concentrations ($K_{0.5} < 10$ μM) on the K^+-Na^+ exchange on the internal sites and (ii) at higher, substrate, concentrations on the activation by K^+ on the external sites.

Introduction

The apparent $Na^+ : K^+$ selectivity of the internal sites on the $(Na^+ + K^+)$-ATPase depends on pH in the medium, a Bohr effect, and on the ATP concentration. This was concluded from measurements of the effect of pH and ATP on the $Na^+ : K^+$ ratio for half-maximum sodium activation of hydrolysis, i.e., under non-equilibrium conditions [1].

It has been proposed that it is the ATP-induced increase in the rate of release of K^+ from the Na^+-loading site which limits the rate of the overall hydrolysis [2—4]. If this is correct the $Na^+ : K^+$ ratio necessary for half-maximum Na^+ activation of the hydrolysis may not represent equilibrium values, at least not at low ATP concentrations, and conclusions about changes in selectivity based on these values may not be correct.

The observation that intrinsic tryptophan fluorescence of the $(Na^+ + K^+)$-ATPase reacts differently to Na^+ and to K^+ [4] opens a possibility of investigating the effect of pH and ATP on the ligand affinities under equilibrium conditions. Such experiments are reported in the present paper, and the results are compared with the results obtained in the kinetic experiments [1].

The advantage of using the intrinsic fluorescence is clearly that the system is unperturbed. However, as the maximum fluorescence change is very small, 1—3% of total fluorescence [4], it is difficult to titrate the effects of cations at physiological concentrations. The dilution becomes so large that the specific cation effect is unmeasurable.

This problem has been overcome by using an extrinsic fluorescence probe, fluorescein isothiocyanate [5], where the difference in response to Na^+ and K^+ is about 15%. However, the ATPase activity is lost upon labelling with fluorescein isothiocyanate [5].

In search of probes fulfilling the conditions of both having a response to conformational changes and not inactivating the enzyme, we have used eosin maleimide. This probe is very suitable, as the labelled enzyme is fully active and the difference in response to Na^+ and K^+ is about 30% of total fluorescence.

Methods

Enzyme was prepared from the rectal glands from *Squalus acanthias* as previously described [6]. The specific ATPase activity was 1455 ± 27 μmoles P_i/mg protein per h and the specific *p*-nitrophenylphosphatase activity 201 ± 5 μmol *p*-nitrophenol/mg protein per h.

Labelling with eosin maleimide

0.6 mg enzyme protein/ml in 20 mM histidine-HCl buffer, pH 7.4 (20°C) with 6% (w/v) glycerol was incubated in the dark for 30 min at 37°C with

40 μM eosin maleimide. The reaction was stopped by the addition of 200 μM mercaptoethanol. The enzyme was washed three times in 20 mM histidine-HCl, pH 7.0, 25% glycerol. After the final centrifugation the enzyme was resuspended in the histidine/glycerol buffer to a protein concentration of 2—2.5 mg/ml and stored in the dark at −20° C. The specific activity of control enzyme decreased about 20% during the 30 min incubation at 37° C while there was no decrease in activity in the presence of eosin maleimide. The washing of the eosin maleimide-labelled enzyme gave a slight decrease in activity from 1455 ± 60 to 1320 ± 28 μmoles P_i/mg protein per h and with a *p*-nitrophenylphosphatase activity of 191 ± 5 μmoles *p*-nitrophenol/mg protein per h, a ratio of 6.9. Between 0.9 and 1.2 mol of eosin maleimide (ϵ for eosin maleimide $8.3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [7]) were incorporated per mol enzyme (^{32}P-labelling site). Eosin maleimide was obtained from Molecular Probes Inc., Plano, TX.

Fluorescence measurements

The fluorescence was measured on a Perkin-Elmer spectro fluorometer MPF 44A in 3 ml test solution, which at pH 7.2 and lower, consisted of 30 mM histidine-HCl, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid, while at a higher pH the buffer was 30 mM Tris-HCl, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid. The solution was stirred and kept at 22° C during the measurement.

The intrinsic fluorescence was measured by excitation at 295 nm and emission at 325 nm [4], slitwidth 10 nm both for excitation and emission, response time 0.3 s. The protein concentration in the test solution was 25—30 μg/ml. In titration experiments the ions were added by a Hamilton syringe. Correction was made for the dilution.

With eosin maleimide-labelled enzyme, the excitation wavelength was 530 nm and emission was measured at 560 nm, the protein concentration was 75—100 μg/ml. The eosin maleimide excitation maximum increased from 522 to 530 nm when bound to the enzyme; the emission maximum was 542 nm.

Results

Intrinsic fluorescence

Enzyme in a histidine buffer at pH 7.2 showed practically no change in fluorescence upon addition of K^+ whereas addition of Na^+ led to a decrease in fluorescence and on a further addition of K^+ the fluorescence again increased (Fig. 1). This is in contrast to the experiments reported by Karlish and Yates [4], who found that K^+ without Na^+ gives an increase in fluorescence while Na^+ without K^+ has no effect. The explanation is that the cited experiments were performed in a Tris buffer. Fig. 1 shows that when the medium in addition to 30 mM histidine contains Tris or choline, the addition of K^+ leads to an increase in fluorescence. The response to K^+ increases with increasing Tris or choline concentrations up to about 150 mM, and is half-saturated at 20—25 mM Tris or choline. The response to Na^+ conversely decreases, the total change in fluorescence being constant at about 2% of total fluorescence.

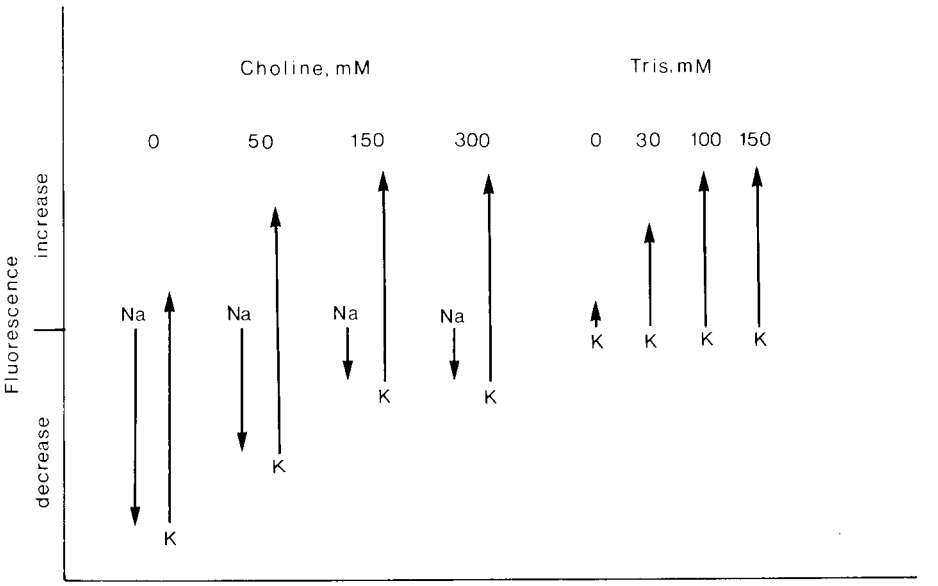

Fig. 1. The effect of choline and of Tris-HCl on the intrinsic fluorescence response to Na^+ and to K^+ in 30 mM histidine, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid, pH 7.2, 22°C. In the experiments with choline, 10 mM Na^+ was added followed by 10 mM K^+, with Tris no Na^+ was added prior to K^+. The maximum difference in response with Na^+ and with K^+ is about 2% of the total fluorescence.

In 30 mM histidine, pH 7.2, the main part of the system thus has the conformation seen with K^+ in the medium, while with 150 mM Tris or choline the conformation is the one seen with Na^+ in the medium, i.e., Tris as well as choline has an 'Na^+' effect (see also below). Choline decreases the apparent affinity for K^+ [8] which may suggest that the choline and Tris effects are due to removal of bound K^+.

In the following experiments on intrinsic fluorescence, the test solution contained 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid and 150 mM choline besides 30 mM histidine or Tris in order to obtain the same ionic strength and the same buffers as in previous hydrolysis experiments [1]. Choline turns the system into the 'low' fluorescence form seen with Na^+ in the medium, and when K^+ is added the fluorescence increases. With Na^+ and choline in the medium, Na^+ + choline = 150 mM.

With 150 mM choline, the concentration of K^+ which gives half-maximum increase in fluorescence ($K_{0.5}$ for K^+) has a minimum between pH 6.5 and 7.2 and is 0.12 ± 0.01 mM (n = 4) at pH 7.2 (Fig. 2A); this value is higher than that of 0.05 mM (pH 7.0, Tris 100 mM) obtained by Karlish and Yates [4] by the same method. The half-maximum value for fluorescence change can be determined with reasonable accuracy, but it is not possible to obtain titration curves which can be used for an analysis of the K^+ binding.

As was expected from the effect of K^+ on the affinity for ATP [9,10] and in agreement with previous experiments with intrinsic fluorescence [4], the $K_{0.5}$ value for K^+ increases with 10 μM ATP (Fig. 2A). 10 mM Na^+ and 10 mM Na^+ plus 10 μM ATP give a further increase in $K_{0.5}$ for K^+ (Fig. 2B). An unexpected finding, however, is that the effect of 140 mM choline which is pronounced

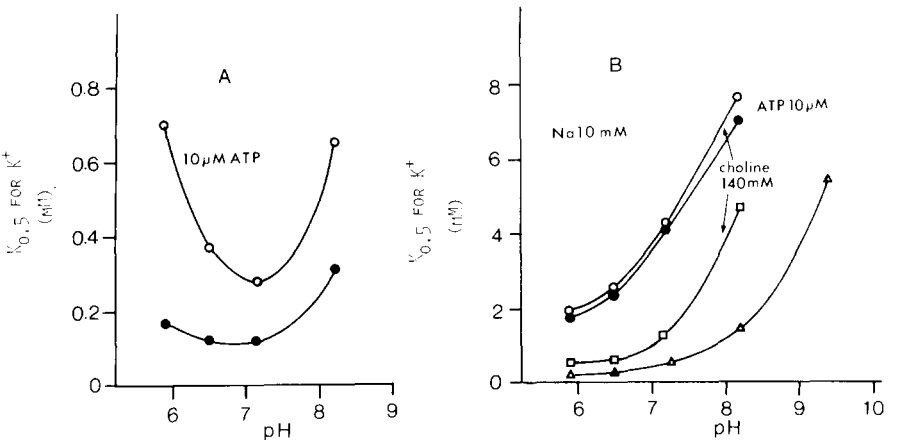

Fig. 2. The effect of pH on $K_{0.5}$ for K^+ for change in intrinsic fluorescence: (A) with 150 mM choline without (•) and with 10 μM ATP (○); (B) with 10 mM Na^+ without (△) and with 140 mM choline (□) with 10 mM Na^+ + 10 μM ATP without (•) and with 140 mM choline (○), 22°C. At pH 7.2 and lower, 30 mM histidine, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid and at higher pH, 30 mM Tris-HCl, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid. The curves are averages of three to five experiments. S.E. varies between ±3 to ±10% of the values (see text).

with 10 mM Na^+ is practically eliminated when 10 μM ATP is added.

With Na^+ and/or choline there is no longer a minimum for $K_{0.5}$ for K^+ inside the tested pH interval both with and without ATP. If there is a minimum it is shifted towards a pH lower than 6.0.

In Fig. 3A is shown how a change in pH influences the Na^+ : K^+ ratio which

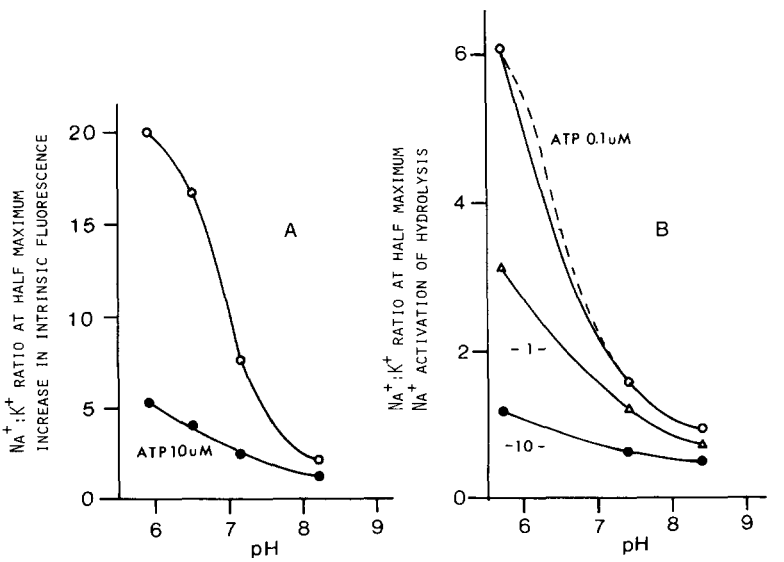

Fig. 3. The effect of pH on the Na^+ : K^+ ratio (A) which corresponds to $K_{0.5}$ for changes in intrinsic fluorescence with 10 mM Na^+, 140 mM choline, without (○) and with 10 μM ATP (•). (B) For half-maximum Na^+ activation of hydrolysis with Na^+ + K^+ = 150 mM and with 0.1 μM ATP (○), 1 μM ATP (△) and 10 μM ATP (•), respectively, buffers as in Fig. 2. The values in B are replotted from Fig. 6 in Ref. 1.

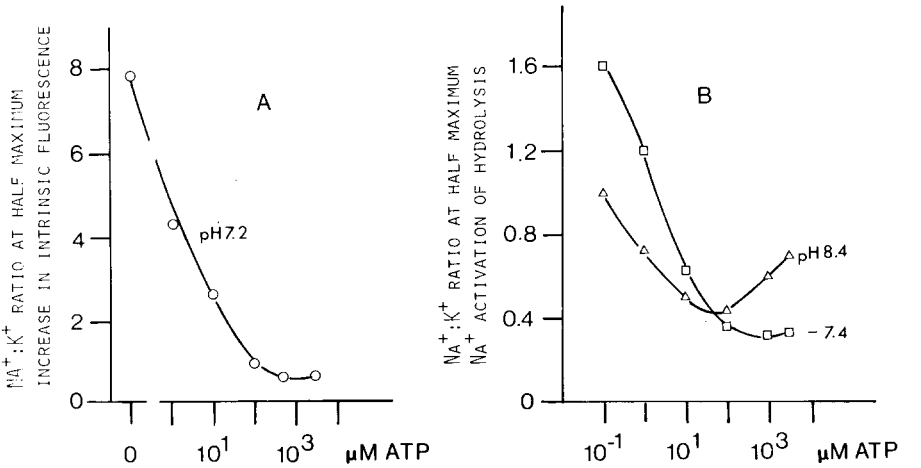

Fig. 4. The effect of ATP on the Na^+ : K^+ ratio (A) which corresponds to $K_{0.5}$ for K^+ with 10 mM Na^+, 140 mM choline, pH 7.2, in 30 mM histidine, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid, (B) for half-maximum Na^+ activation of hydrolysis with $Na^+ + K^+ = 150$ mM at pH 7.4, 30 mM histidine (□) and at pH 8.4 with 30 mM Tris-HCl (△). The values are taken from Fig. 6 in Ref. 1.

corresponds to $K_{0.5}$ for K^+ at 10 mM Na^+, 140 mM choline, with and without 10 μM ATP. For comparison is shown the Na^+ : K^+ ratio for half-maximum activation by Na^+ of the ATP hydrolysis at 0.1, 1.0 and 10 μM ATP (Fig. 3B). In Fig. 4 is shown how the Na^+ : K^+ ratio for half-maximum effect depends on ATP in the fluorescence (A) and in the hydrolysis experiments (B). Fig. 3B is replotted and Fig. 4B is reproduced from Fig. 6 in Ref. 1.

The fluorescence experiments are at pH 7.2, 22°C, and the hydrolysis experiments at pH 7.4 (and 8.4, see below), 37°C.

The pH as well as the ATP effect on the Na^+ : K^+ ratio follow the same pattern in the fluorescence and in the hydrolysis experiments. The slope of the pH curve suggests that in the fluorescence experiments without ATP and in the hydrolysis experiments with 0.1 μM ATP, the Na^+ : K^+ ratio is influenced by the dissociation of a group with an apparent pK between 6.5 and 7.0. 10 μM ATP seems to shift the pH curve towards lower pH values.

The ATP effect levels off with 100—500 μM ATP at pH 7.2 (Fig. 4A) and pH 7.4 (Fig. 4B) (for pH 8.4, see below). Half-maximum effect on the Na^+ : K^+ ratio for the fluorescence change is obtained with 1—2 μM ATP which is of the same order as for the ATP effect on the Na^+ : K^+ ratio for half-maximum Na^+ activation of the hydrolysis at pH 7.4, less than 10 μM ATP (no value for zero ATP) [1].

However, the Na^+ : K^+ ratio giving half-maximum change is greater by a factor of 5 in the fluorescence experiments (Figs. 3A and 4A) than in the hydrolysis experiments (Figs. 3B and 4B).

The ionic strength and the buffers used were the same in the two kinds of experiment. The lower temperature in the fluorescence experiments cannot be the cause as the Na^+ : K^+ ratio for the fluorescence effect is only slightly lower at 37 than at 22°C (not shown).

The discrepancy between the hydrolysis and fluorescence experiments may

arise from a difference in the saturation of the enzyme with Na^+ and K^+. In the kinetic experiments, Na^+ was varied against K^+ with $Na^+ + K^+$ constant at 150 mM in order to keep the ionic strength constant at a physiological level without introducing a third cation.

To perform such experiments it is necessary to mix a test solution for each combination of the cations and compare their fluorescence. This cannot be done with intrinsic fluorescence as a probe because of the small change in the signal. For this reason, the effects of Na^+ and K^+ have been investigated using the eosin maleimide-labelled enzyme, where the change in fluorescence amounts to about 30% of the total.

Eosin maleimide fluorescence

Labelling of the enzyme with eosin maleimide under mild conditions with about 1 mol of the probe bound per mol of enzyme has no effect on activity (see Methods).

K^+ decreases while Na^+ increases the extrinsic fluorescence of eosin maleimide-labelled enzyme; this is identical to the effect on the fluorescein isothiocyanate-labelled enzyme [5] but opposite to the effect of the cations on intrinsic fluorescence.

The magnitude but not the direction of the eosin fluorescence response to Na^+ and to K^+ is, at a given pH, different in a histidine and in a Tris buffer and it is influenced by the pH of the buffer, see Fig. 5.

In a histidine as well as in a Tris buffer with no Na^+ or K^+ present, a decrease

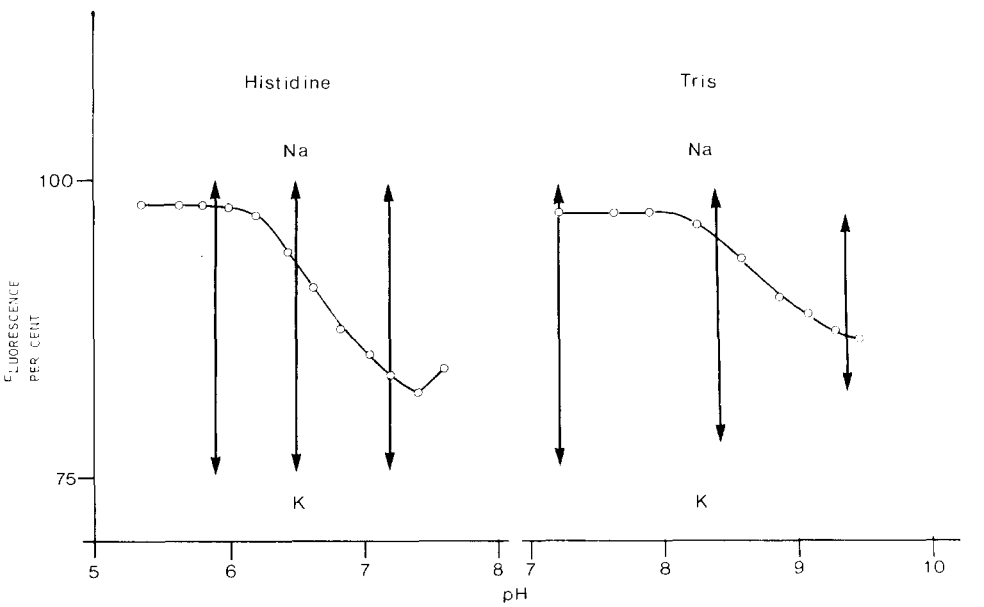

Fig. 5. The effect of pH on the eosin maleimide fluorescence response to Na^+ and to K^+ in 30 mM histidine, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid and in 30 mM Tris-HCl, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid, respectively. The curves show the fluorescence in the two buffers and the arrows the effect of addition of Na^+, the increase, and K^+, the decrease, in fluorescence. 100% is total fluorescence.

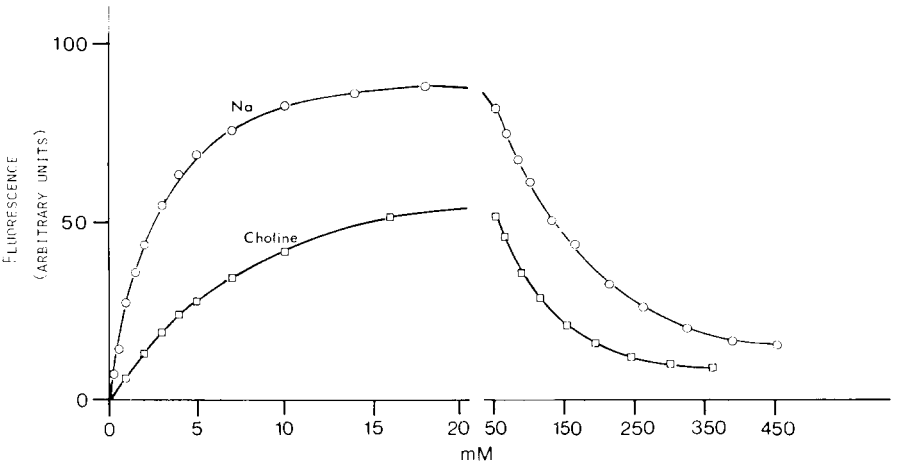

Fig. 6. The effect of Na^+ and of choline on the eosin maleimide fluorescence at pH 7.2 in 30 mM histidine, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid (compare Fig. 5) at 22° C.

in pH transfers the system into the high fluorescence form and, in parallel, the response to Na^+ decreases and to K^+ increases (Fig. 5). However, by further decreasing the pH, the fluorescence again decreases and the decrease sets in at a higher pH and is more pronounced the lower the concentration of the buffer (not shown). This indicates that the 'sodium' effect of the protonated buffer can be overcome by a protonation of the system, i.e., the effect of a decrease in pH in the presence of histidine or Tris is due to a sum of two opposing effects, an Na^+ effect of the protonated buffer and a K^+ effect of a protonation of the system (see below).

Rb^+, Cs^+ and NH_4^+ have a K^+ effect on the fluorescence while the effect of Li^+ at low concentrations is like K^+ but at higher concentrations like Na^+. Mg^{2+} has an Na^+ effect ($K_{0.5}$ 0.24 mM in 30 mM histidine-HCl, pH 7.2).

In 30 mM histidine, pH 7.2, the fluorescence response to Na^+ increases with the Na^+ concentration up to a certain level and thereafter decreases (Fig. 6). It seems likely that the decrease is due to an ionic strength effect.

Choline has, like the protonated form of histidine and Tris, an Na^+ effect and gives an increase in fluorescence followed by a decrease, but the slope of the increase and the maximum value obtained are lower (Fig. 6). Due to the lower affinity for choline, the ionic strength effect sets in before the full Na^+ effect is obtained.

The ascending part of the Na^+ curve in Fig. 6 is a rectangular hyperbola, giving a straight line in a double-reciprocal plot and with a K_m value of 2.0 ± 0.2 mM ($n = 5$). This agrees with a K_{Na^+} of 1.5 mM at pH 7.5 obtained by Karlish [5] using fluorescein isothiocyanate-labelled enzyme.

K^+ increases the $K_{0.5}$ for Na^+, and the Na^+ titration curve becomes S-shaped and more so the higher the K^+ concentration (not shown). The $K_{0.5}$ for Na^+ with 1 mM K^+ is 11 mM, with 2 mM K^+ 16 mM and with 5 mM K^+ present 25 mM.

When the system is turned into the high fluorescence form by 30 mM Tris-HCl at pH 7.2 (see Fig. 5), by choline or by Na^+, titration with K^+ gives S-

shaped curves (not shown). This is in contrast to the experiments with fluorescein-labelled enzyme where the K^+ titration curve in the presence of Tris and Na^+ is hyperbolic [5].

In the present experiments at pH 7.2 with 30 mM histidine, $K_{0.5}$ for K^+ was 0.15 ± 0.01 mM (n = 4) in the presence of 150 mM choline, which is about the same as the value of 0.12 mM found in the experiments on the intrinsic fluorescence but lower than the value of 0.4 mM (pH 7.5 and with 100 mM Tris) found by Karlish [5] using fluorescein isothiocyanate-labelled enzyme. As was observed in experiments on binding of K^+ [8], the $K_{0.5}$ value for K^+ decreases with a decreasing choline concentration and is 0.08 ± 0.006 mM (n = 3) with 50 mM choline and 0.024 ± 0.005 mM (n = 3) without choline. In the presence of Na^+, the $K_{0.5}$ value for K^+ increases along an S-shaped curve with the Na^+ concentration (not shown). The $K_{0.5}$ value for K^+ with, for example, 10 mM Na^+ present is 0.63 ± 0.002 mM (0.5 ± 0.04 mM with intrinsic fluorescence), 8.7 ± 0.3 mM with 50 mM Na^+ and 28.3 ± 0.4 mM with 100 mM Na^+ (n = 3). This comes close to the value of 6 mM K^+ at 80 mM Na^+, pH 7.0, 20°C, obtained by Karlish and Yates [4] using intrinsic fluorescence and 12 mM K^+ at 80 mM Na^+, pH 7.7, 20°C, obtained by Karlish et al. [3] using formycin nucleotides.

However, as there is a buffer effect, probably an ionic strength effect and no inert ion to keep the ionic strength constant, the interpretation of the curves in terms of affinities requires a more detailed analysis. We shall return this in another paper.

In the following experiments, the ionic strength has been kept constant by titrating Na^+ against K^+ with $Na^+ + K^+$ constant in order to avoid the effect of a third cation. It is not possible to avoid the buffer effect but it is relatively decreased by increasing $Na^+ + K^+$ to the physiological concentration range, 150 mM.

$Na^+ + K^+$ constant

In Fig. 7 is shown the effect on the fluorescence when Na^+ is titrated against K^+ with $Na^+ + K^+$ constant at 10, 50 and 150 mM, respectively, in a 30 mM histidine, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid buffer, pH 7.2, at 22°C. With $Na^+ + K^+$ = 10 mM, the abscissa values have to be divided by 15 and for $Na^+ + K^+$ = 50 mM by 3.

The $K_{0.5}$ value for the $Na^+ : K^+$ ratio for the fluorescence change decreases from 9.3 ± 0.67 = 14 to 42 : 8.3 = 5 to 107 : 43 = 2.5 with the increase in the sum of the two cations.

Fig. 7 also shows the effect of an Na^+ against K^+ titration on the hydrolytic activity with 0.1 μM ATP at pH 7.4 and with $Na^+ + K^+$ = 150 mM. The fluorescence curve at 150 mM closely follows that part of the hydrolysis curve which shows the activation by Na^+ of the hydrolysis, the left part of the curve. There is a slight difference in the $Na^+ : K^+$ ratio for half-maximum effect, 107 : 43 for fluorescence and 91 : 47 for hydrolysis. However, when pH and temperature in the fluorescence experiment are increased to the values used in the hydrolysis experiment, pH 7.4 and 37°C, the ratio for fluorescence is decreased to 96 : 54.

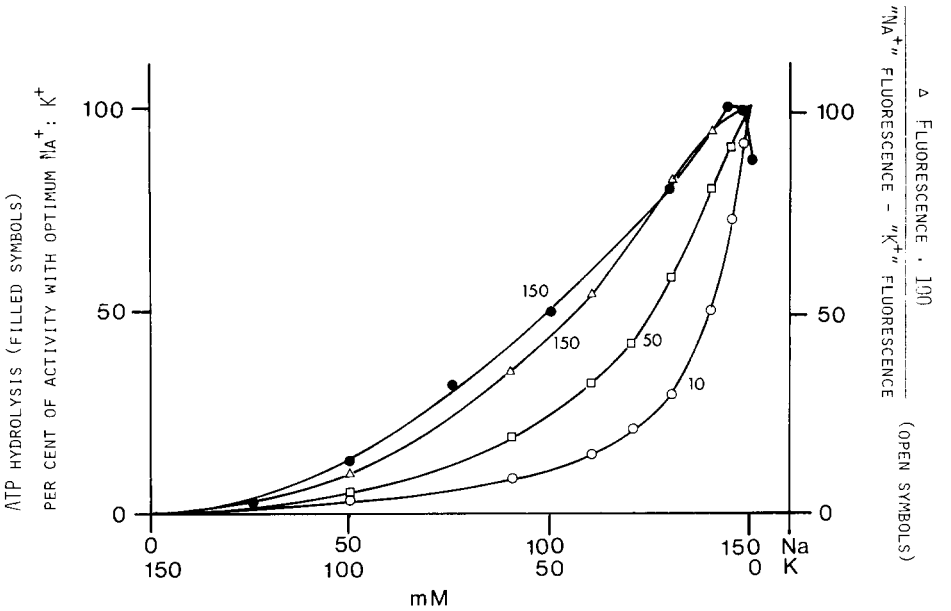

Fig. 7. The effect of a titration of Na^+ against K^+ on the eosin fluorescence with Na^+ plus K^+ = 10 mM (○), 50 mM (□), and 150 mM (△), pH 7.2, 30 mM histidine, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid. For $Na^+ + K^+$ = 10 mM the abscissa value has to be divided by 15 and for $Na^+ + K^+$ = 50 by 3. The ordinate shows the change in fluorescence and it is given as per cent of the difference in fluorescence between 150 mM Na^+ and 150 mM K^+, the maximal change. For comparison is shown the effect of a titration of Na^+ against K^+ on the hydrolytic activity with 0.1 μM ATP, pH 7.4, 30 mM histidine (●), 37°C. The activity is given in per cent of the activity with the optimum Na^+ : K^+ ratio; the values are taken from Fig. 5 in Ref. 1. Each of the curves is an average of at least three experiments, the S.E. varied between ±0.4 and ±2.0 for each of the values given.

ATP effect

ATP shifts the titration curve towards lower values of $K_{0.5}$ for Na^+ for activation of the hydrolysis [1]. Such a shift by ATP is also observed in the fluorescence experiments. However, ATP decreases the maximum of the fluorescence obtained with Na^+, and thereby decreases the difference between the low and the high fluorescence seen with K^+ and with Na^+, respectively. It makes it difficult to carry out Na^+ against K^+ titrations with more than a few μM ATP present.

At pH 7.4 and 37°C, the $K_{0.5}$ value for the fluorescence change ($Na^+ + K^+$ = 150 mM) was decreased from 96 : 54 without ATP to 86 : 64 with 1 μM ATP, compared to a decrease from 91 : 57 with 0.1 μM ATP to 82 : 68 with 1 μM ATP in the hydrolysis experiments.

The scale difference seen between A and B in Fig. 3 and between A and B, in Fig. 4, thus disappears when the effect of hydrolysis and fluorescence is compared under identical conditions.

pH effect

The Na^+ versus K^+ titration curves are shifted towards lower Na^+ values for half-maximum effect on fluorescence when pH is increased; this is shown for the pH interval from 5.9 to 9.4 and with $Na^+ + K^+$ = 150 mM (Fig. 8). The

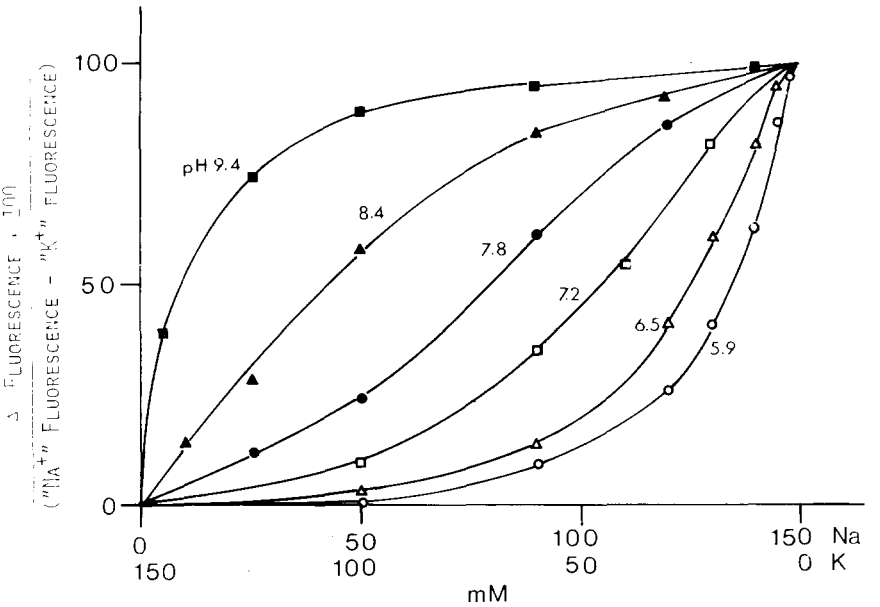

Fig. 8. Na^+ vs. K^+ titration curves of the eosin fluorescence at different values of pH and with $Na^+ + K^+$ = 150 mM. At pH 7.2 and lower, 30 mM histidine, 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid, and at pH 7.8 and higher, 30 mM Tris-HCl and 2 mM *trans*-1,2-cyclohexylenedinitrilotetraacetic acid, 20° C. See legend to Fig. 7 for axes.

effect is pronounced, the Na^+ : K^+ ratio is decreased from 135 : 15 at pH 5.9 to 10 : 140 at pH 9.4.

Fig. 9A shows the effect of pH on the fluorescence with different Na^+ : K^+ ratios. From Figs. 8 and 9A it is seen that an increase in K^+ as well as in H^+

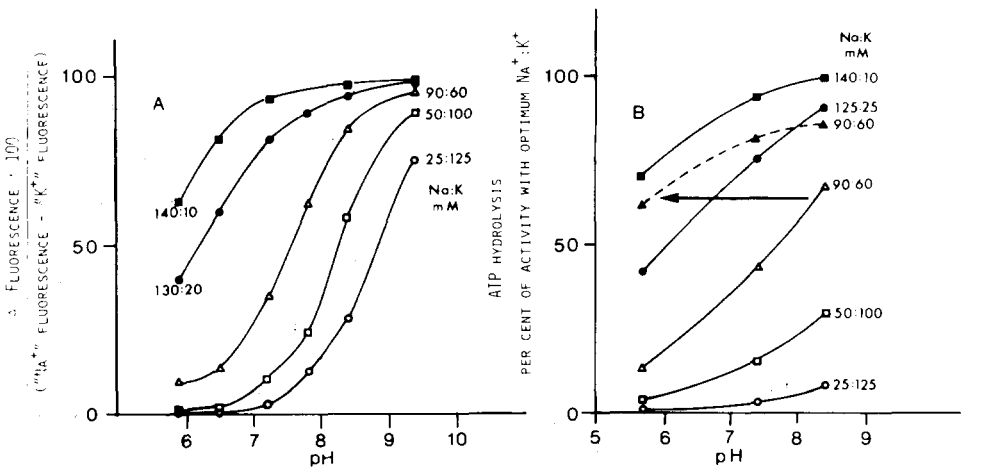

Fig. 9. (A) The effect of pH on the eosin fluorescence at different Na^+ : K^+ ratios and with $Na^+ + K^+$ = 150 mM. Buffers as in Fig. 8, 22° C. See legend to Fig. 7 for axes. (B) The effect of pH on the hydrolytic activity at different Na^+ : K^+ ratios and with $Na^+ + K^+$ = 150 mM. The hydrolytic activity is given as per cent of the activity with the optimum Na^+ : K^+ ratio at each pH value. Buffers as in Fig. 8, 37° C. The ATP concentration was 0.1 μM for all the Na^+ : K^+ ratios and for Na^+ : K^+ of 90 : 60 also 10 μM (dashed line).

shifts the equilibrium towards the low fluorescence form while an increase in Na^+ as well as a decrease in H^+ shifts the equilibrium towards the high fluorescence form. This means that K^+ increases the affinity for H^+ and vice versa, K^+ increases pK values while Na^+ decreases the affinity for H^+ and vice versa, Na^+ decreases pK values.

An extrapolation of the curves in Fig. 9A suggests that at a high enough pH (more than 9.5), the system will be in the high fluorescence deprotonated form even in the presence of K^+, and at a low enough pH (less than 5.5) on the low fluorescence protonated form even in the presence of Na^+. If this is correct, the system basically exists in a protonated and deprotonated form and K^+ shifts the equilibrium towards the protonated and Na^+ towards the deprotonated form.

A comparable way of showing the effect of pH on the Na^+ activation of the hydrolysis is to plot as a function of pH, the activity at a given suboptimal $Na^+ : K^+$ ratio in per cent of the activity with optimum $Na^+ : K^+$ ratio. A change in the fractional activity at a given suboptimal $Na^+ : K^+$ ratio reflects a change in the ability of Na^+ to compete for K^+ for Na^+ activation of hydrolysis. Such a plot is shown in Fig. 9B; the values are taken from the curves used for deter-

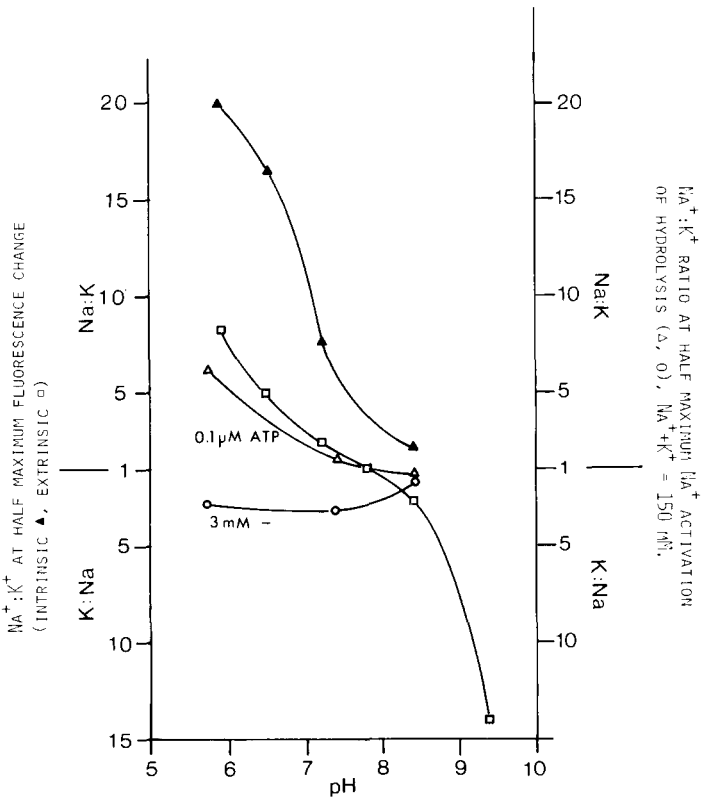

Fig. 10. The effect of pH on the $Na^+ : K^+$ ratio for half-maximum change in intrinsic fluorescence (▲) (from Fig. 2A), in eosin fluorescence with $Na^+ + K^+ = 150$ mM (□), 22°C, and for half-maximum Na^+ activation of hydrolysis with $Na^+ + K^+ = 150$ mM, 0.1 μM ATP (△), and with 3 mM ATP (○), 37°C. Buffers as in Fig. 8. The values for the hydrolysis experiment are taken from Fig. 5 in Ref. 1.

mination of the $K_{0.5}$ values for Na^+ activation described in Ref. 1.

A comparison between Fig. 9A and B shows that the pattern for the effect of a change in pH at different $Na^+ : K^+$ ratios is the same in the fluorescence and in the hydrolysis experiments (note that the effect is titrated to one pH unit higher in the fluorescence experiment). It is also seen from Fig. 9B that at a given $Na^+ : K^+$ ratio, e.g., 90 : 60, an increase in ATP from 0.1 to 10 μM shifts the titration curve towards a lower pK value.

The experiments on extrinsic fluorescence suggest that the $Na^+ : K^+$ selectivity depends on at least two H^+ dissociation constants, one with an acidic and another with an alkaline pK (Fig. 10). However, the increase in the $Na^+ : K^+$ selectivity when the alkaline group is dissociated is not seen in the hydrolysis experiment. With 0.1 μM ATP, the effect of an increase in pH levels off above pH 7.8 (Fig. 10) and with a high ATP concentration the $Na^+ : K^+$ selectivity decreases (shown for 3 mM ATP in Fig. 10, see also Fig. 4B at pH 8.4). This is not due to an effect of ATP as such. In the fluorescence experiments at pH 8.4 and 9.4, 1 μM ATP does not increase the $K_{0.5}$ value for the $Na^+ : K^+$ ratio but gives a slight decrease, i.e., it increases the selectivity (not shown).

Fig. 10 also shows the pronounced effect of an increase in the sum of $Na^+ + K^+$ on the $K_{0.5}$ for the $Na^+ : K^+$ ratio (compare the curve for intrinsic and extrinsic fluorescence).

Discussion

The different effects of Na^+ and of K^+ on the reactivity of the $(Na^+ + K^+)$-ATPase towards sulfhydryl inhibitors [11—15], inactivation by Be^{2+} and F^- [16], trypsin digestion [17], binding of nucleotides [3,9,10] and on the fluorescence response, intrinsic [4] as well as extrinsic [5], suggests that Na^+ has an effect on the conformation of the system different from that of K^+.

Fluorescence and hydrolysis

The effect of pH and of ATP, which was observed in hydrolysis experiments [1], are also observed under equilibrium conditions when the change in fluorescence is used as a tool to test the effect. The correlation between the effect of Na^+ compared to K^+ on the fluorescence change and on the Na^+ activation of hydrolysis indicates that the change in conformation which is reflected by the fluorescence change is due to an effect of the cations on the internal sites on the cytoplasmic side of the membrane.

The extrinsic probe used is a maleimide derivative. When the reactivity of the SH groups towards another maleimide derivative, *N*-ethylmaleimide, is used as a tool to test the effect of Na^+ compared to K^+ on the conformation in the presence of ATP, the same close correlation is obtained between Na^+ activation of hydrolysis and Na^+ protection against *N*-ethylmaleimide inactivation [15] as was observed for the Na^+ effect on fluorescence. K^+ increases the reactivity towards *N*-ethylmaleimide and decreases the fluorescence of the bound eosin maleimide while Na^+ decreases the reactivity towards *N*-ethylmaleimide and increases the fluorescence which may mean that in the presence of Na^+, SH groups are transferred to a more protected, probably hydrophobic environment.

pH effect

H^+ has a pronounced effect on the equilibrium between the two conformations of the system. The results shown in Fig. 10 suggest that there are two H^+ dissociation constants involved, one with an acidic and another with an alkaline pK, which means two deprotonation steps, H_2E—HE—E. However, in the hydrolysis experiments, the effect of the second dissociation is not seen as an apparent increase in Na^+ selectivity as was observed in the fluorescence experiments but as a decrease in activity [1]. It suggests that it is H_2E which is selective towards K^+ while HE and E are selective to Na^+, but HE has catalytic activity while E and H_2E are catalytically inactive.

On the assumption that the number of cations bound are also the number of cations transported, the 3 Na^+ to 2 K^+ ratio for transport (see Ref. 18) then suggests that the K^+-leaving site must be an H_2EK_2 form and only the molecules which are transformed into an $HENa_3$ form have catalytic activity. It means that the hybrid K^+-Na^+ forms are catalytically inactive and according to the observed correlation between fluorescence and hydrolysis, also have K^+ fluorescence. On the other hand, the hyperbolic titration curve with Na^+ in the absence of K^+ suggests that the Na^+ fluorescence is not confined to HE forms with three Na^+. However, in the presence of K^+, the Na^+ titration curve becomes S-shaped, supporting the view that in the presence of K^+ more than one Na^+ is necessary to transform the system into the Na^+ form and according to the correlation between fluorescence and hydrolysis into the catalytically active form.

The transformation of the internal sites from the K^+ form with two sites to the Na^+ form with three sites is thus related to an H^+ release either by an H^+-Na^+ exchange or what seems more likely the H^+ release is related to a change in quarternary structure due to a reorganization of the interaction between the polypeptide chains and this opens up a new site.

ATP effect

ATP at low concentrations decreases the $K_{0.5}$ value for the Na^+ : K^+ ratio, both for the fluorescence change and for the Na^+ activation of hydrolysis. In agreement with the view that a deprotonation shifts the equilibrium towards the form seen with Na^+ in the medium, ATP decreases the pK for the equilibrium between the protonated and the deprotonated form at a given Na^+ : K^+ ratio.

ATP facilitates the rate by which K^+ is released from the internal sites and it has been proposed that it is this effect which sets the requirement for ATP as substrate [2—4] with a $K_{0.5}$ which is much higher with $Na^+ + K^+$ (a fraction of 1 mM (see Ref. 19)) than with Na^+ without K^+ (K_D 0.12—0.2 μM, Refs. 9 and 10). The calculated K_D for ATP from the effect on the rate of exchange is 0.45 mM [4].

However, the observed correlation between the equilibrium (fluorescence) and steady-state (hydrolysis) situation suggests that during hydrolysis the K^+ : Na^+ exchange on the internal sites comes to an equilibrium even with the low concentration of ATP. The intrinsic fluorescence experiments and the hydrolysis experiments [1] show that the ATP concentration necesssary ($K_{0.5} < 10$ μM) is lower than the substrate concentration and previous experi-

ments showed that the effect is independent of the Mg^{2+} concentration [20], i.e., the effect is not related to hydrolysis, ATP as well as MgATP has the effect. This suggests that it is not the ATP requirement for the K^+-Na^+ exchange on the internal sites which sets the requirement for ATP as substrate.

It seems more to be that the requirement for ATP is necessary to overcome an inhibition due to K^+ on the external sites [1].

With Na^+ and low concentrations of MgATP, 'external' concentrations of K^+ inhibit [1,21—24] by an effect which is distinctly different from the inhibition by 'internal' concentrations of K^+ [1]. This can most simply be explained from a model with coexisting sites. The fraction of enzyme molecules which are transferred from the $^oNa_n/^iNa_n$ form into the $^oK_m/^iNa_n$ form by external K^+ concentrations has a lower MgATP affinity and with low MgATP concentrations this leads to an activity which is lower with $Na^+ + K^+$ than with Na^+ alone [25]. Increase in K^+ to internal concentrations transfers the molecules on the $^oK_m/^iNa_n$ form into the $^oK_m/^iK_m$ form which has no ATPase activity, i.e., to a further decrease in activity.

The inhibition by external K^+ is pH dependent, K^+ increases the pH optimum relative to the optimum with Na^+, meaning that at certain MgATP concentrations the inhibition by external K^+ can be overcome by an increase in pH (see

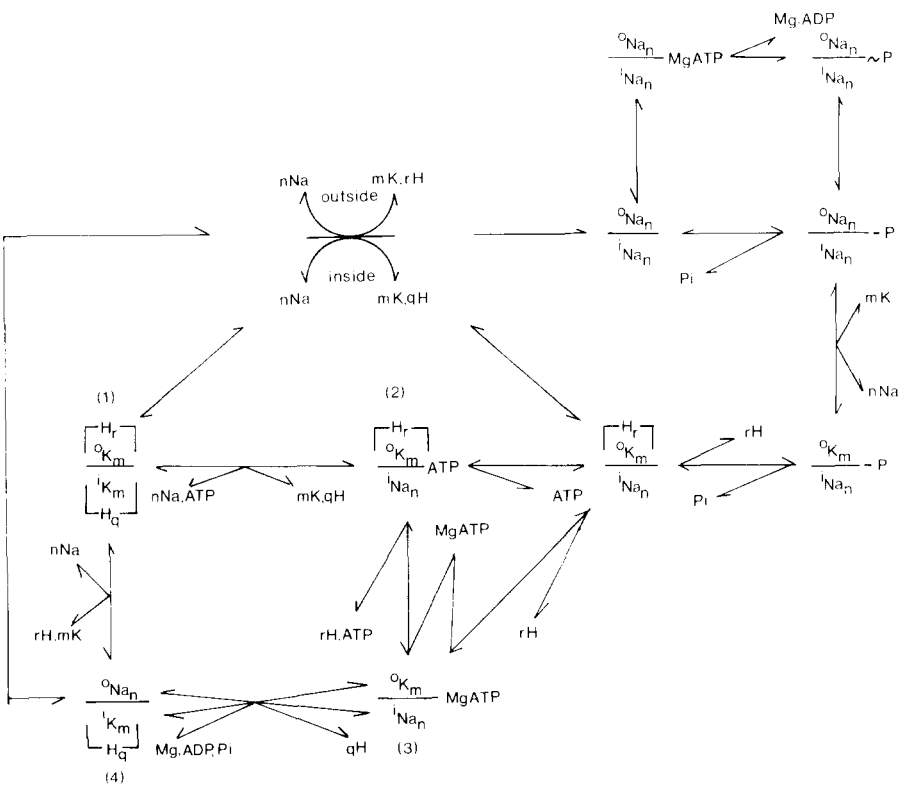

Fig. 11. Model to describe two consecutive effects of ATP. Coexisting internal and external sites (see Ref. 30). Formation of two consecutive phosphoenzymes on the Na^+/Na^+ pathway but no acid-stable phosphoenzyme on the K^+/Na^+ pathway (see Ref. 31); *m*, *n*, *r* and *q* are numbers; i for inside, o for outside. For further details see Ref. 25.

Fig. 3 in Ref. 1); MgATP antagonizes the effect of K^+ on the pH optimum [1]. It indicates that the apparent decrease in affinity for MgATP by external K^+ is due to a protonation of the system which follows from the binding of K^+ to the external sites. It suggests that the effect of external K^+ on the requirement for MgATP as substrate [26] is due to an MgATP-proton antagonism due to K^+ on the external sites. K^+ on the external as well as on the internal sites thus leads to binding of protons, i.e., increases the pK values of the system.

The suggestions about two ATP effects find support from inactivation studies which show a high- and a low-affinity effect of ATP [27—29] and with a K'_D of 2—3 μM [29] and 0.35—0.4 mM [28,29], respectively.

It leads to the conclusion that ATP at low concentrations facilitates the K^+-Na^+ exchange on the internal sites (step 1 to 2, Fig. 11). The 2 form which is protonated due to K^+ on the external sites is catalytically inactive because of lack of interaction between the internal and external sites. MgATP at higher concentrations release these protons and transforms the system into the catalytically active form (step 2 to 3). The following hydrolysis leads to the translocation (step 3 to 4).

According to this there are three affinities for ATP. One very high (K_D 0.12—0.2 μM [9,10]) on the $^oNa_n/^iNa_n$ exchange pathway. Two on the $^oK_m/^iNa_n$ exchange pathway, one with an apparent K_D of a few μM and another with an apparent K_D of a fraction of 1 mM. The high-affinity site on the two pathways may be the same, but the affinity depends on the conformation of the system which is set by the combination of the cations and the protonation.

But, is the low-affinity site a new site, which is opened when the system functions on the $^oK_m/^iNa_n$ pathway or is there only one site but a transition between two forms with a high- and a low-affinity for ATP, respectively?

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