*Biochimica et Biophysica Acta*, 339 (1974) 246–257

BBA 76574

# EFFECT OF ATP ON THE INTERMEDIARY STEPS OF THE REACTION OF THE ($Na^+ + K^+$)-DEPENDENT ENZYME SYSTEM

## II. EFFECT OF A VARIATION IN THE ATP/$Mg^{2+}$ RATIO

J. C. SKOU

*Institute of Physiology, University of Aarhus, 8000 Aarhus C (Denmark)*

(Received August 2nd, 1973)

(Revised manuscript received November 26th, 1973)

## SUMMARY

1. It has been investigated how ATP in the presence of varying concentrations of $Mg^{2+}$ (a) influences the apparent affinity for $Na^+$ relative to $K^+$ for the site on the ($Na^+ + K^+$)-dependent enzyme system where $Na^+$ activates – the "$Na^+$-site"; (b) influences the catalytic activity in the presence of optimum concentrations of $Na^+ + K^+$.

2. The "$Na^+$-site" has an apparent affinity for $K^+$ which is at least 2.5 times higher than for $Na^+$ without ATP in the medium. ATP increases the apparent affinity of this site for $Na^+$ relative to $K^+$ and with saturating concentrations of ATP the apparent affinity for $Na^+$ is about 3 times higher than for $K^+$, i.e. ATP increases the apparent affinity for $Na^+$ relative to $K^+$ at least 7–8 times.

3. It is the concentration of total ATP in the solution which determines the effect on the $Na^+$:$K^+$ affinity ratio. At a given concentration of added ATP a wide variation in the concentration of $Mg^{2+}$ has no effect on the apparent $Na^+$:$K^+$ affinity ratio. It means (a) that the effect is independent of the catalytic activity, (b) that free ATP as well as MgATP must have the effect, and (c) that the system must have about the same affinity for free ATP and for MgATP at the site where they exert their effect.

4. With $K^+ + Na^+$, the activity as a function of MgATP gives a bell-shaped curve when the $Mg^{2+}$ concentration is varied at a given ATP concentration. The activity at the maximum increases with the ATP concentration up to 3 mM and the maximum moves towards higher MgATP concentrations. The slope of the ascending part of the curve decreases with an increase in the concentration of added ATP, i.e. the activity at a given MgATP concentration decreases with an increase in the free ATP: free $Mg^{2+}$ ($ATP_f$ : $Mg^{2+}_f$) ratio. For the descending part of the curve the activity decreases with a decrease in the $ATP_f$ : $Mg^{2+}_f$ ratio, showing that maximum activity at a given MgATP concentration is obtained with an intermediary $ATP_f$:$Mg^{2+}_f$ ratio. The relationship between the effects of MgATP, free ATP and free $Mg^{2+}$ on the system is discussed.

## INTRODUCTION

From results of experiments on the effect of ATP, $Na^+$, $K^+$, and $Mg^{2+}$ on the inhibition of the $(Na^+ + K^+)$-activated enzyme system by *N*-ethylmaleimide, it was suggested that ATP increases the apparent affinity for $Na^+$ relative to $K^+$ for the site on the system where $Na^+$ activates, the i-site (i for inside) [1].

The effect of ATP was seen under conditions where no $Mg^{2+}$ was added and where the medium contained 5 mM EDTA to complex the traces of $Mg^{2+}$ in the enzyme preparation, i.e. it seems to be due to an effect of ATP as such and not to a hydrolysis of ATP. The effect seemed to be independent of the reaction with *N*-ethylmaleimide. In the present paper it has been investigated how $Mg^{2+}$ influences the effect of ATP on the apparent affinity for $Na^+$ relative to $K^+$ of the i-site on the system, and under conditions where the hydrolytic activity has been used as a tool to test the effect instead of the inhibition by *N*-ethylmaleimide.

## METHODS

The enzyme was prepared as described in the previous paper [1]; the specific $(Na^+ + K^+)$-activity was 200–290 $\mu$M $P_i$/mg protein per h (3 mM $Mg^{2+}$, 3 mM ATP, 120 mM $Na^+$, 30 mM $K^+$, pH 7.4, 37 °C). The $Mg^{2+}$ activity (equal to the g-strophanthin-insensitive activity) was less than 0.5% of the total activity.

The activity was tested by measuring the amount of $^{32}P$ released from ATP labelled with $^{32}P$ in the $\gamma$ position. The test volume was 1 ml which besides 30 mM Tris–HCl, pH 7.4, 37 °C, contained $[^{32}P]$ATP, $Na^+$, $K^+$, and $Mg^{2+}$ in the concentrations given on the figures. The reaction was started by the addition of the enzyme. The reaction time was in all the experiments adjusted in such a way that the maximum hydrolysis of the added ATP was less than 20%. Under these conditions, the hydrolysis was a linear function of time. The reaction was stopped by the addition of 0.1 ml 50% trichloroacetic acid, and the hydrolysis of $[^{32}P]$ATP was determined according to the method of Lindberg and Ernster [2]. The $^{32}P$ was counted in a liquid scintillation counter [3]. $[^{32}P]$ATP was from The Radiochemical Centre, Amersham, England. ATP, ITP, GTP, and CTP were from Boehringer. $[^{32}P]$ATP, ATP, GTP, ITP, and CTP were purified and converted to the Tris salt by chromatography on a DEAE-sephadex A-25 (Pharmacia) column [4].

With ITP, CTP, and GTP as substrate, the activity was tested by measuring the $P_i$ released by the method of Fiske and SubbaRow [5].

For the calculation of the concentration of free $Mg^{2+}$ ($Mg^{2+}{}_f$), free ATP ($ATP_f$), ATP complexed with $Mg^{2+}$ and with $Na^+$ and $K^+$, the following formation constants were used: $MgATP^{2-}$, $10^4$ $M^{-1}$ [6], sodium and potassium ATP, 15 $M^{-1}$ [7].

The figures show typical results, which have all been reproduced 3–5 or more times; for variability in the experimental results, see legend to Fig. 1 and see also Table I.

## RESULTS

In Fig. 1 is shown the effect of a variation in the $Na^+$ and $K^+$ concentration on the catalytic activity with different concentrations of ATP (and $Mg^{2+}$). The sum

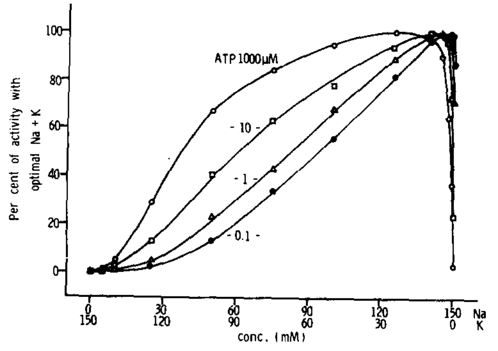


Fig. 1. The effect of $Na^+ + K^+$ on the catalytic activity of the $(Na^+ + K^+)$-dependent enzyme system with ATP 0.1 µM, $Mg^{2+}$ 10 µM; ATP 1 µM, $Mg^{2+}$ 100 µM; ATP 10 µM, $Mg^{2+}$ 250 µM; ATP 1000 µM, $Mg^{2+}$ 1000 µM, respectively. The ordinate shows per cent of the activity with optimum concentrations of $Na^+ + K^+$. The test has been made in 30 mM Tris–HCl, pH 7.4, at 37 °C. From a number of such experiments the mean concentration of $Na^+$ ± S.E. for half maximum $Na^+$ activation (the left part of the curve) was for 1 mM ATP 37 ± 1.2 mM ($n = 3$), for 0.01 mM ATP 55 ± 2.4 mM ($n = 3$), for 0.001 mM ATP 79 ± 1.8 mM ($n = 3$) and for 0.0001 mM ATP 93 ± 1.1 mM ($n = 4$) ($Na^+ + K^+ = 150$ mM).

of $Na^+$ and $K^+$ was kept constant at 150 mM. For comparison the activity has been expressed in percent of the maximum obtained with optimum concentrations of $Na^+$ and $K^+$.

The figure shows that less $Na^+$ (a lower $Na^+$:$K^+$ ratio) is necessary to give half maximum activity (the left part of the curve) when the ATP concentration is increased. Furthermore, that the stimulating effect, which can be obtained with $K^+$ in the presence of $Na^+$, decreases with the ATP concentration. With 0.1 µM ATP, the activity with 150 mM $Na^+$ is 87% of the maximum activity with $Na^+ + K^+$, while with 1000 µM ATP it is only 3%. It means that $K^+$ with the low concentrations of ATP only activates when the $K^+$:$Na^+$ ratio is low, at higher ratios the activity with $K^+ + Na^+$ is lower than with $Na^+$ alone, see the figure. Fig. 1 finally shows that more $K^+$ is necessary to give half maximum activity (the right part of the curve) when the ATP concentration is increased (this last point will not be further discussed in the present paper).

In the experiments shown in Fig. 1, the ATP concentration given on the figure is the concentration of added ATP. ATP exists, however, as a mixture of free ATP and of ATP complexed with $Mg^{2+}$ and also with $Na^+$ and $K^+$ in the medium. In order to see if the effect of ATP found in Fig. 1 is due to free or to complexed ATP, the experiments were repeated, but with a fixed concentration of added ATP and a varied concentration of $Mg^{2+}$. The sum of $Na^+$ and $K^+$ was kept constant at 150 mM. The result of these experiments is shown in Fig. 2 and in Table I. In Fig. 2 the concentration of added ATP was 10 µM and the $Mg^{2+}$ concentration 2 µM, 35 µM, and 1000 µM, respectively. In Table I the $Na^+$:$K^+$ values for half maximum activation by $Na^+$ are given for the following concentrations of added ATP, 1 µM, 10 µM, and 500 µM, respectively, and for each ATP concentration for varying concentrations of $Mg^{2+}$. The values are read from experiments as shown in Fig. 2.

TABLE I

THE EFFECT OF A VARIATION IN THE $Mg^{2+}$ CONCENTRATION AT DIFFERENT CONCENTRATIONS OF ATP ON THE CONCENTRATION OF $Na^+$ NECESSARY FOR HALF MAXIMUM ACTIVATION

The values are read from curves as those shown in Fig. 2.

| ATP (μM) | $Mg^{2+}$ (μM) | $Na^+$ for half maximum activation ($Na^+ + K^+$ = 150 mM) mM |
|---|---|---|
| 1 | 1 | 83 |
| 1 | 10 | 84 |
| 1 | 10 | 88 |
| 1 | 100 | 82 |
| 1 | 100 | 76 |
| 1 | 100 | 80 |
| 1 | 500 | 90 |
| 1 | 500 | 84 |
| 1 | 2 000 | 86 |
| 1 | 3 000 | 84 |
| 1 | 3 000 | 96 |
| 1 | 5 000 | 88 |
| | | mean 85 |
| 10 | 2 | 62 |
| 10 | 2 | 58 |
| 10 | 2 | 64 |
| 10 | 5 | 61 |
| 10 | 10 | 66 |
| 10 | 35 | 57 |
| 10 | 35 | 56 |
| 10 | 35 | 58 |
| 10 | 50 | 62 |
| 10 | 50 | 57 |
| 10 | 50 | 59 |
| 10 | 100 | 54 |
| 10 | 250 | 54 |
| 10 | 500 | 52 |
| 10 | 500 | 54 |
| 10 | 500 | 60 |
| 10 | 1 000 | 57 |
| 10 | 2 500 | 60 |
| 10 | 3 000 | 60 |
| 10 | 10 000 | 63 |
| 10 | 10 000 | 65 |
| 10 | 10 000 | 64 |
| | | mean 59 |
| 500 | 1.5 | 33 |
| 500 | 2.5 | 37 |
| 500 | 12 | 35 |
| 500 | 30 | 38 |
| 500 | 100 | 37 |
| 500 | 250 | 35 |
| 500 | 500 | 37 |
| 500 | 1 000 | 37 |
| 500 | 10 000 | 40 |
| | | mean 37 |

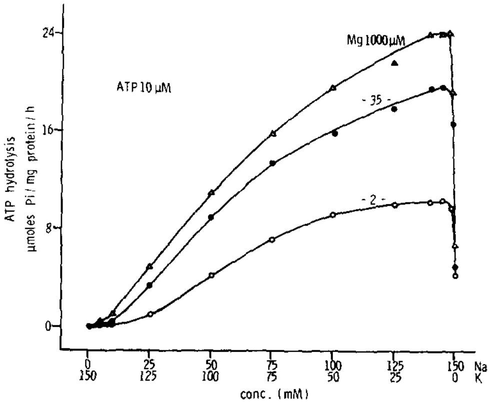


Fig. 2. The effect of $Na^+ + K^+$ on the catalytic activity of the $(Na^+ + K^+)$-dependent enzyme system with 10 µM ATP and with 2 µM, 35 µM, and 1000 µM $Mg^{2+}$, respectively. The ordinate in Figs 2–6 gives the activity in µM $P_i$ hydrolyzed from ATP per mg protein per h.

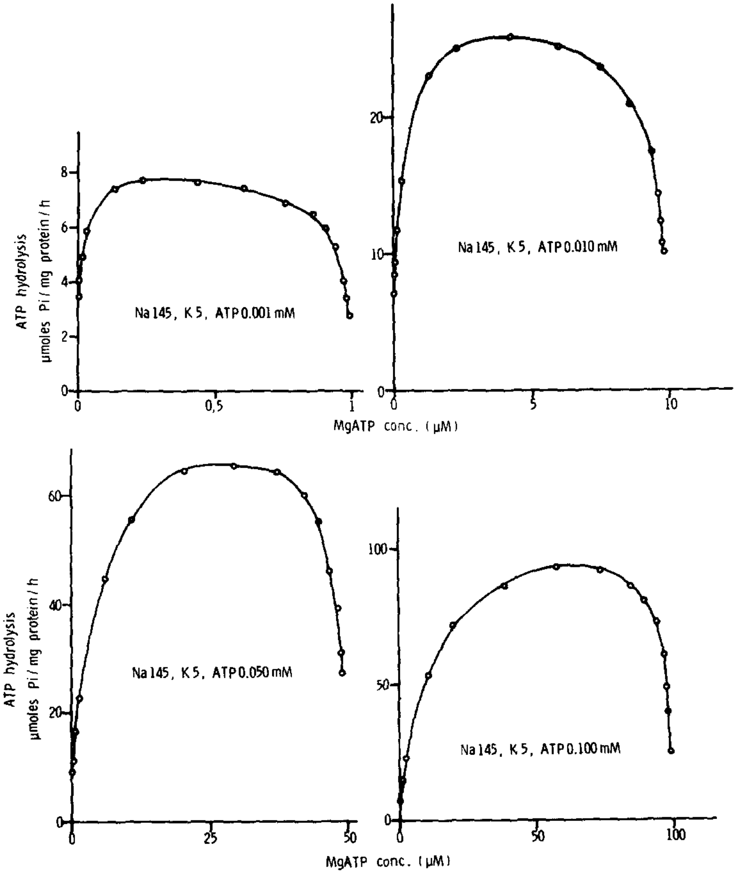


Fig. 3. The catalytic activity of the $(Na^+ + K^+)$-dependent enzyme system as a function of the MgATP concentration. The concentration of added ATP was kept constant at 1 µM, 10 µM, 50 µM, and 100 µM, while the concentration of added $Mg^{2+}$ was varied. For calculation of the MgATP concentration, see Methods. The $Na^+$ and $K^+$ concentrations given on the curves were the concentrations which gave maximum activity.

The figure and the table show that there is no systematic variation in the $Na^+$ : $K^+$ concentration ratio for half maximum activation with a variation in the $Mg^{2+}$ concentration when the total ATP concentration is kept constant. At a given ATP concentration the ratios seem to scatter around a common value independent of the $Mg^{2+}$ concentration. This value is for 1 μM ATP 85:65, for 10 μM ATP 59:91, and for 500 μM ATP 37:113. It seems thus as if the $Na^+$:$K^+$ concentration ratio for half maximum $Na^+$ activation is determined by the total concentration of ATP in the medium independent of a wide variation in the $Mg^{2+}$ concentration. This means that it is independent of the ratio between uncomplexed and complexed ATP and independent of the catalytic activity.

A variation in the $Mg^{2+}$ concentration at a given ATP concentration has, however, a pronounced effect on the rate of hydrolysis in the presence of $Na^+ + K^+$. This is shown in Figs 3 and 4 for concentrations of total ATP from 1 μM up to 6.000 μM. The activity is measured with the concentrations of $Na^+ + K^+$ which give optimum activity for each of the ATP concentrations, and the activity is plotted as a function of the MgATP concentration. There is a certain low activity without added $Mg^{2+}$. It can be decreased to zero by adding EDTA to the test medium, but not by extensive washing of the enzyme preparation with or without EDTA in the washing medium. EDTA has not been added to the test medium to avoid a complicating factor and the MgATP concentrations have been calculated from the added $Mg^{2+}$.

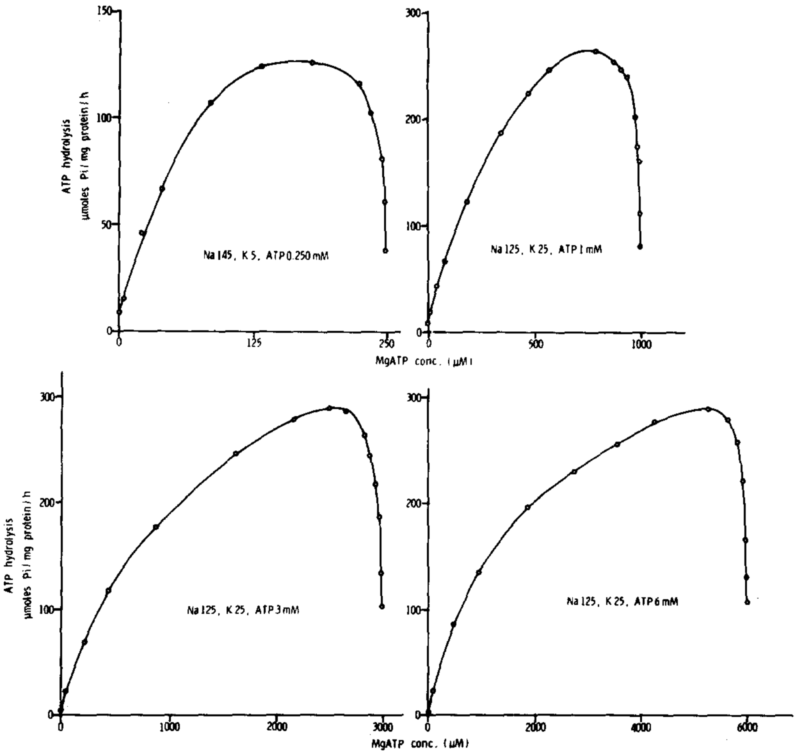


Fig. 4. As Fig. 3, but with a concentration of added ATP of 250 μM, 1000 μM, 3000 μM, and 6000 μM.

The activity at a given total ATP concentration increases with MgATP up to a maximum and thereafter again decreases when the $Mg^{2+}$:ATP ratio becomes high. The activity at the maximum increases with the ATP concentration up to 3 mM, and the MgATP concentration, which gives the maximum, increases with the ATP concentration. There is no fixed $Mg^{2+}$:ATP ratio for the top of the curves; it decreases from about 100:1 with 1 $\mu$M ATP to 10:1 with 50 $\mu$M ATP, 4:1 with 250 $\mu$M ATP, 2:1 with 1000 $\mu$M ATP and 4:3 with 3000 $\mu$M ATP.

The slope of the ascending part of the curves decreases with an increase in the total ATP concentration; this is shown for concentrations of total ATP of 100 $\mu$M and higher in Fig. 5, but is also seen with the lower concentrations of ATP. It means that the activity at a given MgATP concentration decreases with an increase in the concentration of total ATP, which again means with an increase in the concentration of free ATP ($ATP_f$) and a decrease in the concentration of free $Mg^{2+}$ ($Mg^{2+}{}_f$) i.e. with an increase in the $ATP_f$:$Mg^{2+}{}_f$ ratio.

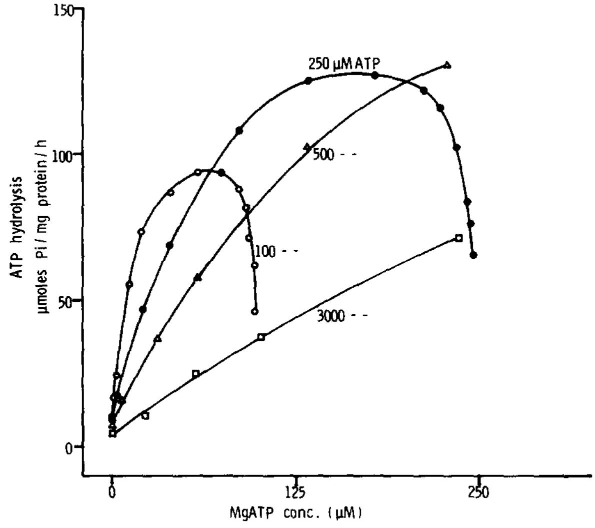


Fig. 5. The catalytic activity as a function of MgATP with ATP 100 $\mu$M, 250 $\mu$M, 500 $\mu$M, and 3000 $\mu$M. The activity was measured with the concentrations of $Na^+$ and $K^+$ which gave optimum activity (see Figs 3 and 4).

When the $Mg^{2+}$:ATP ratio becomes high at a given ATP concentration, the activity decreases with an increase in the concentration of MgATP (Figs 3–5). This is seen both with low and high concentrations of MgATP suggesting that it is not due to an inhibitory effect of MgATP. It suggests that it is due either to the increase in the concentration of $Mg^{2+}{}_f$ or to the decrease in the concentration of $ATP_f$ or both, which follows from the increase in the $Mg^{2+}$ concentration. At the point of half maximum decrease in activity the concentration of $Mg^{2+}{}_f$ and of $ATP_f$ is higher, the higher the MgATP concentration, but the concentration of $Mg^{2+}{}_f$ increases less with MgATP than $ATP_f$ does. For example from 10 to 3000 $\mu$M ATP the concentration of MgATP increases 300 times from 9.8 to 2900 $\mu$M, while $Mg^{2+}{}_f$ increases 3 times from 14990 to 41900 µM, and $ATP_f$ 100 times from 0.07 to 7.2 µM. It means that the higher the MgATP concentration, the less is the decrease necessary in the $ATP_f$:$Mg^{2+}{}_f$ ratio to give an inhibition.

It seems thus as if an increase as well as a decrease in the $ATP_f$:$Mg^{2+}{}_f$ ratio at a given MgATP concentration decreases the activity, meaning that at a given MgATP concentration maximum activity is obtained with an intermediary $ATP_f$:$Mg^{2+}{}_f$ ratio.

In Fig. 6 is shown the effect of a variation in the $Na^+$:$K^+$ ratio on the activity with 500 µM, 10 µM, and 1 µM total ATP, respectively, and with concentrations of $Mg^{2+}$ which give about the same MgATP concentration, namely 0.95, 0.91, and 0.94 µM, respectively. The figure furthermore shows the effect of $Na^+$ and $K^+$ on the activity with 500 µM total ATP and with a concentration of $Mg^{2+}$, 30 µM (18 µM MgATP), which with optimal concentrations of $Na^+$ and $K^+$ gives about the same rate of hydrolysis as with 10 µM total ATP and 35 µM $Mg^{2+}$ (0.95 µM MgATP).

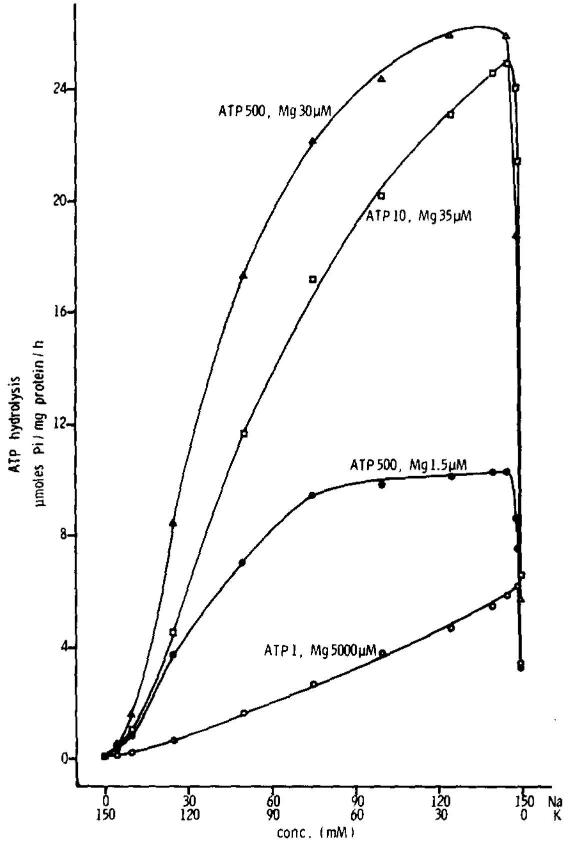


Fig. 6. The effect of $Na^+ + K^+$ on the activity of the $(Na^+ + K^+)$-dependent enzyme system with 1 µM ATP, 10 µM ATP, and 500 µM ATP, and with concentrations of $Mg^{2+}$ which give about the same concentration of MgATP, namely 0.94 µM MgATP with 1 µM ATP and 5000 µM $Mg^{2+}$; 0.91 µM MgATP with 10 µM ATP and 35 µM $Mg^{2+}$; 0.95 MgATP with 500 µM ATP and 1.5 µM $Mg^{2+}$. The figure furthermore shows the effect of $Na^+ + K^+$ on the activity in the presence of 500 µM ATP, and with a concentration of $Mg^{2+}$, 30 µM (MgATP 18 µM), which gives about the same activity as with 10 µM ATP and 35 µM $Mg^{2+}$ (MgATP 0.91 µM).

The results of the experiment show on the figure lend further support to the view that it is not the MgATP concentration neither the rate of hydrolysis which determines the $Na^+:K^+$ ratio for half maximum $Na^+$ activation, but the concentration of total ATP. With the same MgATP concentration the $Na^+:K^+$ ratio for half maximum $Na^+$ activation decreases from 88:62 with 1 μM ATP to 54:96 with 10 μM ATP and to 36:114 with 500 μM ATP. With 500 μM ATP, this last value stays constant when the MgATP concentration is increased, i.e. the rate of hydrolysis is increased. The figure furthermore shows that at a given MgATP concentration the activity with optimum concentrations of $Na^+ + K^+$ varies with the $ATP_f$: $Mg^{2+}{}_f$ ratio. When this ratio is high, the activity is low (500 μM ATP, 1.5 μM $Mg^{2+}$, $ATP_f$:$Mg^{2+}{}_f$ = 1541:0.6). With a lower ratio (10 μM ATP, 30 μM $Mg^{2+}$, $ATP_f$: $Mg^{2+}{}_f$ = 2.8:29) the activity is higher. When the ratio becomes low, (1 μM ATP, 5000 μM $Mg^{2+}$, $ATP_f$:$Mg^{2+}{}_f$ = 0.01:4999) it again becomes lower, cf. the bell shape of the curves in Figs 3–5.

In Fig. 7 is shown the effect of a variation in the $Na^+ + K^+$ concentration on the activity with 3 mM GTP, ITP, CTP, and ATP, respectively. With 3 mM of the triphosphates, the $Na^+:K^+$ ratio for half maximum $Na^+$ activation is 106:44 with

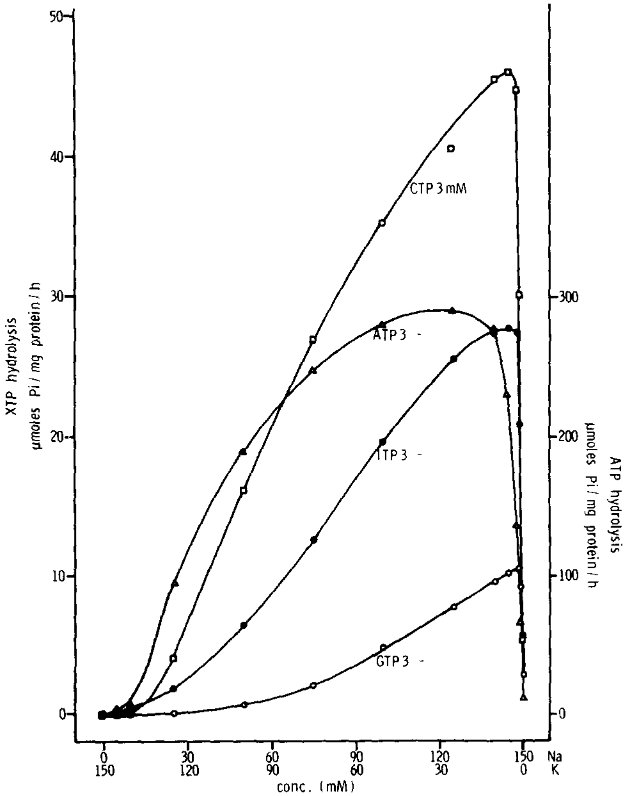


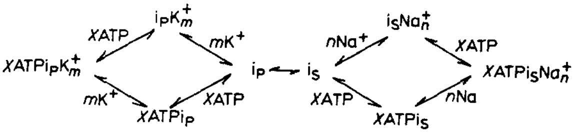
Fig. 7. The effect of $Na^+ + K^+$ on the activity in the presence of 3 mM ATP, 3 mM CTP, 3 mM ITP, and 3 mM GTP, respectively. The left ordinate gives the activity in μM $P_i$ hydrolyzed from CTP, ITP, and GTP per mg protein per h, and the right ordinate the activity in μM $P_i$ hydrolyzed from ATP per mg protein per h.

GTP, 80:70 with ITP, 66:84 with CTP, and 37:113 with ATP, i.e. the apparent $Na^+$:$K^+$ affinity ratio is 0.4:1 with GTP, 0.9:1 with ITP, 1.3:1 with CTP and 3:1 with ATP.

## DISCUSSION

In a previous paper [1] it was found that ATP without $Mg^{2+}$ and under conditions where there was no measurable hydrolysis of ATP (5 mM EDTA) decreases the $Na^+$:$K^+$ ratio for half maximum effect of $Na^+$ on the "$Na^+$-site" of the system from 108:42 without ATP to 36:114 with saturating concentrations of ATP. In the present experiments it is found that ATP also in the presence of $Mg^{2+}$ decreases the $Na^+$:$K^+$ ratio for half maximum $Na^+$ effect on activation of the system and that the effect of a given ATP concentration is widely independent of a variation in the $Mg^{2+}$ concentration. The limiting value at saturating concentrations of ATP is found to be 37:117 as in the previous experiments (36:114). In the present experiments where the catalytic activity is used to measure the effect of ATP on the $Na^+$:$K^+$ affinity ratio, it is not possible to do the experiments without ATP; but with the triphosphate which has the lowest effect, GTP, the ratio was 106:44 to compare with the 108:42 value found in the previous experiments without ATP. It shows that ATP in the presence of $Mg^{2+}$ increases the $Na^+$:$K^+$ ratio for half maximum $Na^+$ activation inside at least the same limits as were found for ATP without $Mg^{2+}$, and that ATP increases the apparent affinity for $Na^{2+}$ relative to $K^+$ at least from about 0.4:1 (106:44) to about 3:1 (37:113), i.e. by a factor of 7–8. As it is an effect on the site where $Na^+$ activates, the "$Na^+$-site", it is on the site which is located on the inside of the intact membrane [8–10], the i-site in the following.

The lack of effect of a variation in the $Mg^{2+}$ concentration at a given ATP concentration shows that the effect of ATP is independent of the hydrolytic activity, and that ATP complexed with $Mg^{2+}$ must have the same effect as free ATP on the affinity ratio. This must mean that there is a site on the system which can react with free ATP ($ATP_f$) as well as with complexed ATP and with about the same affinity, and that the reaction with complexed as well as with uncomplexed ATP has the same effect on the $Na^+$:$K^+$ affinity ratio. It means that ATP in the scheme given in the previous paper to explain the effect of ATP on the affinity ratio [1] must be replaced by *X*ATP where *X*ATP can be either uncomplexed or complexed ATP, and where *X*ATP, as it was found for ATP without $Mg^{2+}$, has a lower affinity for the system on the $K^+$ form, $i_P$, than on the $Na^+$ form, $i_S$, (i for inside, P for potassium, S for sodium).



According to the scheme it is the total concentration of ATP independent of $Mg^{2+}$ which influences the ratio between the i-sites which have reacted with $Na^+$ and with $K^+$ at a given $Na^+$:$K^+$ concentration ratio. The fraction of the system with the i-site on the $Na^+$ form which has reacted with free ATP or with MgATP is given by the ratio between these two components in the solution. It is, however, only that fraction of the system with the i-site on the $Na^+$ form which has reacted with the one of the two components which is the substrate, which can hydrolyse ATP.

The effect of ATP on the apparent $Na^+:K^+$ ratio is seen under conditions where the concentration of $K^+$ relative to $Na^+$ is high enough to saturate the site on the system where $K^+$ activates, the o-site (o for outside). Assuming that the o- and the i-site exist simultaneously, it means that $X$ATP influences the ${}^{o}K_m^+/{}^{i}K_n^+ \leftrightarrow {}^{o}K_m^+/{}^{i}Na_n^+$ equilibrium and shifts it towards the ${}^{o}K_m^+/{}^{i}Na_n^+$ form, which is the form which has the catalytic activity.

What is then the substrate for the hydrolysis of ATP? Is it $ATP_f$, or is it MgATP? The slope of the ascending part of the activity curve decreases when the concentration of added ATP is increased (Fig. 5); it means that at a given MgATP concentration, the activity decreases when the $ATP_f$ concentration increases, and the $Mg^{2+}{}_f$ concentration decreases. This is in a simple way explained by the above discussed competition between $ATP_f$ and MgATP for a common site and with MgATP as the substrate. The decrease in the concentration of $Mg^{2+}{}_f$ may besides the increase in the competition between $ATP_f$ and MgATP add to the decrease in the slope. It would mean that $Mg^{2+}{}_f$ was necessary for activation besides MgATP as suggested by Fahn et al. [11].

The affinity of the system for $ATP_f$ is high [4, 12]. An equal affinity for $ATP_f$ and MgATP must therefore mean that the affinity for MgATP must also be high. As seen from Figs 4–6 this seems to be the case. The slope of the ascending part of the activity curve as a function of MgATP becomes very steep when the total ATP concentration is decreased (cf. Figs 4–6), which means when the competitive effect of $ATP_f$ is decreased. At the lowest concentration used, 1 $\mu$M ATP, the slope is very steep. It shows that if it is correct that MgATP is the substrate, then the affinity of the system for MgATP is very high.

Having reached an optimum, the activity as a function of MgATP decreases when the $Mg^{2+}$ concentration becomes high at a given ATP concentration. This decrease in activity is seen both with low and with high concentrations of MgATP, which means that it is not due to an inhibitory effect of MgATP. If MgATP is the substrate, it must be due either to an inhibitory effect of $Mg^{2+}{}_f$, which increases with the $Mg^{2+}$ concentration or due to a lack of $ATP_f$ which decreases with an increase in the $Mg^{2+}$ concentration; this last would mean that $ATP_f$ should be necessary for activation of the catalytic activity of the ${}^{o}K_m^+/{}^{i}Na_n^+$ form of the system. The concentration of $ATP_f$ at half maximum inhibition increases more with the MgATP concentration than does the concentration of $Mg^{2+}{}_f$; it may suggest a competition between MgATP and $ATP_f$ and suggest that it is $ATP_f$ which is required for activation and not $Mg^{2+}{}_f$ which inhibits. It would suggest two sites on the system, a MgATP substrate site, where $ATP_f$ competes for MgATP, and where MgATP as well as $ATP_f$ increases the apparent affinity for $Na^+$ relative to $K^+$ on the "$Na^+$-site", and with a high affinity for $ATP_f$ as well as for MgATP. And an $ATP_f$ activator site with a high affinity for $ATP_f$ and where MgATP competes for $ATP_f$.

Such a view is, however, in disagreement with the results of studies on the binding of ATP without $Mg^{2+}$ to the system [4, 12]. It is found that the system at 0 °C per enzyme unit only binds 1 molecule of ATP with a high affinity.

Does it mean that the binding experiments at 0 °C do not show the binding of ATP at higher temperatures?

Or is $\text{ATP}_\text{f}$ not necessary for activation, is it $\text{Mg}^{2+}{}_\text{f}$ which inhibits when the $\text{ATP}_\text{f}:\text{Mg}^{2+}{}_\text{f}$ ratio becomes low at a given MgATP concentration?

Or does it mean that $\text{ATP}_\text{f}$ is the substrate, that MgATP, which competes for $\text{ATP}_\text{f}$, inhibits, and as $\text{Mg}^{2+}$ is required for activity that the system reacts with $\text{Mg}^{2+}{}_\text{f}$ at a site different from the $\text{ATP}_\text{f}$ substrate site. This would give a bell-shaped curve for the activity as a function of MgATP without implying more than one site for $\text{ATP}_\text{f}$, and $\text{ATP}_\text{f}$ would be necessary for activation because it is the substrate.

There is at present no answer to the problem.

The ability of $\text{K}^+$ to activate in the presence of $\text{Na}^+$ relative to the ability of $\text{Na}^+$ alone to activate is lower, the lower the ATP concentration (Fig. 1) (cf. refs 13–16). A simple way to explain this is that the system in the ${}^{0}\text{Na}_m^+/{}^{i}\text{Na}_n^+$ form has a higher affinity for the substrate than in the ${}^{0}\text{K}_m^+/{}^{i}\text{Na}_n^+$ form. With a low concentration of ATP, 0.1 $\mu$M e.g., $\text{K}^+$ not only gives a low increase in activity, but the effect is only seen in a very narrow and low concentration range; in higher concentrations $\text{K}^+$ inhibits, cf. Fig. 1. The inhibitory effect is most likely due to a displacement of $\text{Na}^+$ from the i-site of the system by $\text{K}^+$, i.e. the transformation of the ${}^{0}\text{K}_m^+/{}^{i}\text{Na}_n^+$ form into the ${}^{0}\text{K}_m^+/{}^{i}\text{K}_n^+$ form. As the ${}^{0}\text{K}_m^+/{}^{i}\text{K}_n^+$ form has no activity and the ${}^{0}\text{K}_m^+/{}^{i}\text{Na}_n^+$ form in the presence of the low ATP concentration has an activity which is only slightly higher than the activity of the ${}^{0}\text{Na}_m^+/{}^{i}\text{Na}_n^+$ form only a minor fraction of the enzyme molecules need to be transformed from the ${}^{0}\text{K}_m^+/{}^{i}\text{Na}_n^+$ form into the ${}^{0}\text{K}_m^+/{}^{i}\text{K}_n^+$ form before the total activity is lower than when all the molecules are on the ${}^{0}\text{Na}_m^+/{}^{i}\text{Na}_n^+$ form.

## ACKNOWLEDGEMENT

The author is indebted to Mrs Hanne Ouertani and Miss Bitten Holm for excellent technical assistance.

## REFERENCES

1 Skou, J. C. (1974) Biochim. Biophys. Acta, 339, 246–257

2 Lindberg, O. and Ernster, L. (1956) in Methods in Biochemical Analysis, Vol. 3, pp. 1–22, Interscience, New York

3 Clausen, T., Gliemann, J., Vinten, J. and Kohn, P. G. (1970) Biochim. Biophys. Acta 211, 233–243

4 Nørby, J. G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116

5 Fiske, C. H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400

6 Nørby, J. G. (1970) Acta Chem. Scand. 24, 3276–3286

7 Sillén, G. G. and Martell, A. E. (1964) Stability Constants of Metal-Ion Complexes, Special publication No. 17, p. 652, The Chemical Society, London

8 Glynn, I. M. (1962) J. Physiol. London 160, 18P

9 Laris, P. C. and Letchworth, P. E. (1962) J. Cell. Comp. Physiol. 60, 229–234

10 Whittam, R. (1962) Biochem. J. 84, 110–118

11 Fahn, S., Koval, G. J. and Albers, R. W. (1966) J. Biol. Chem. 241, 1882–1889

12 Hegyvary, C. and Post, R. L. (1971) J. Biol. Chem. 246, 5234–5240

13 Czerwinski, A., Gittelman, H. J. and Welt, L. G. (1967) Am. J. Physiol. 213, 786–792

14 Neufeld, A. H. and Levy, H. M. (1969) J. Biol. Chem. 244, 6493–6497

15 Blostein, R. (1970) J. Biol. Chem. 245, 270–275

16 Post, R. L., Hegyvary. C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540