BBA 65909

THE EFFECT OF CATIONS, g-STROPHANTHIN AND OLIGOMYCIN ON THE LABELING FROM [^{32}P]ATP OF THE ($Na^+ + K^+$)-ACTIVATED ENZYME SYSTEM AND THE EFFECT OF CATIONS AND g-STROPHANTHIN ON THE LABELING FROM [^{32}P]ITP AND $^{32}P_i$

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(Received January 24th, 1969)

SUMMARY

1. The particle which contained the ($Na^+ + K^+$)-activated enzyme system was labeled with ^{32}P from [^{32}P]ATP, [^{32}P]ITP and $^{32}P_i$. The labeling from these three different sources was influenced by Mg^{2+}, Na^+, K^+, and g-strophanthin.

2. With [^{32}P]ATP there were two kinds of labeling which differed in that one, the unstable, decreased when all [^{32}P]ATP was hydrolyzed, whereas the other, the stable, did not. With Mg^{2+}, the unstable labeling was low and the stable high. Na^+ increased the initial rate as well as the amount of unstable labeling and decreased the stable labeling; this effect was most pronounced with a low concentration of ATP. With $Mg^{2+} + Na^+ + K^+$, the unstable labeling was very low, and the stable labeling was lower than with $Mg^{2+} + Na^+$. When K^+ or unlabeled ATP was added to a pre-labeled particle, the unstable labeling rapidly decreased, whereas the stable labeling remained unaffected.

3. The enzyme system was also labeled from [^{32}P]ATP at 0°, and the effects of the cations at 0° and at 37° were similar. However, at 0°, the decrease in labeling due to K^+ was not accompanied by an increase in hydrolysis.

4. With [^{32}P]ITP, the effect of the cations on the labeling was similar to that found with [^{32}P]ATP, but the rate of labeling and the amount of ^{32}P incorporated per mg protein was lower.

5. The highest labeling from $^{32}P_i$ was found with Mg^{2+}, then followed by $Mg^{2+} + Na^+$, and the lowest labeling was found with $Mg^{2+} + Na^+ + K^+$. This is the same pattern as for the stable labeling from [^{32}P]ATP. From the experiments with $^{32}P_i$ it could be estimated that 5–30% of the stable labeling in the experiments with [^{32}P]ATP came from $^{32}P_i$ released in the test solution when [^{32}P]ATP was hydrolyzed.

6. g-Strophanthin with Mg^{2+} and [^{32}P]ATP or [^{32}P]ITP in the medium affected neither the labeling nor the hydrolysis. With [^{32}P]ATP and $Mg^{2+} + Na^+$, g-strophanthin decreased the unstable and increased the stable labeling. In concentrations of g-strophanthin giving a maximum effect, the labeling with $Mg^{2+} + Na^+ +$ g-strophanthin was identical with that of $Mg^{2+} + Na^+ + K^+ +$ g-strophanthin.

7. With [^{32}P]ATP and $Mg^{2+} + Na^+$ at 0°, g-strophanthin decreased the labeling

to the level found with $Mg^{2+} + Na^+ + K^+$ and decreased the hydrolysis. The concentration of g-strophanthin necessary to decrease the labeling was lower than that which was necessary to give the same relative decrease in hydrolysis. With $Mg^{2+} + Na^+ + K^+$, g-strophanthin had no effect on the labeling and caused only a very slight decrease in hydrolysis.

8. With [^{32}P]ITP, the effect of g-strophanthin was similar to that with [^{32}P]ATP at 37°. With $^{32}P_i$ and with $Mg^{2+} + Na^+ + K^+$, g-strophanthin increased the labeling to a level which was higher than with Mg^{2+} without g-strophanthin. This contrasted with the stable labeling from [^{32}P]ATP, which, with $Mg^{2+} + Na^+ + K^+$ + g-strophanthin, was between the labeling with Mg^{2+} and $Mg^{2+} + Na^+$.

9. At 37°, oligomycin, like g-strophanthin, had no effect on the labeling or on the hydrolysis with Mg^{2+}, but decreased the hydrolysis with $Mg^{2+} + Na^+$ and with $Mg^{2+} + Na^+ + K^+$. In contrast to the effect of g-strophanthin, it increased both the unstable and the stable labeling with $Mg^{2+} + Na^+$; it also increased both the unstable and the stable labeling with $Mg^{2+} + Na^+ + K^+$ to a level approximating that obtained with $Mg^{2+} + Na^+$ without oligomycin.

10. The unstable labeling showed that there was a phosphorylation followed by a dephosphorylation of the enzyme particle. The similarity between the effects of the cations and of g-strophanthin on the phosphorylation and on the hydrolysis of ATP suggested that the ($Na^+ + K^+$)-activated enzyme system was responsible for the phosphorylation. From the experiments it was not possible to tell if the stable labeling was also catalyzed by this enzyme system or was due to another enzyme which used the same substrate but had a different rate constant.

11. It is discussed whether the phosphorylation and dephosphorylation found with $Mg^{2+} + Na^+$ in the medium are part of the same hydrolysis pathway as when the system is activated by $Na^+ + K^+$, or whether they are due to a pathway of hydrolysis found under conditions where the system gives a Na^+ — Na^+ exchange.

INTRODUCTION

In order to understand how the ($Na^+ + K^+$)-activated enzyme system functions in transporting cations across the cell membrane, it is necessary to know the intermediate steps in the hydrolysis of ATP by the enzyme system.

Results of experiments on the ADP–ATP exchange reaction catalyzed by the enzyme system suggest that the hydrolysis of ATP leads to a phosphorylation of a compound in the microsomal particle which contains the enzyme system[1–4]. More direct evidence for this comes from experiments with [^{32}P]ATP as the substrate[2,5–18]. From these experiments it was concluded that there is a Na^+-dependent phosphorylation followed by a K^+-dependent dephosphorylation[5,7,11,19].

However a number of problems must be solved before a scheme can be set up for the intermediate steps in the hydrolysis of ATP by the enzyme system. One problem is the relationship between stable and unstable labeling found with [^{32}P]ATP as the substrate[13,15]. Another is the apparent discrepancy between experimental results concerning exchange and labeling. The highest exchange is found with Mg^{2+}, whereas Na^+ decreases the exchange[1]. Only when the Mg^{2+} concentration is very low or Mg^{2+} is omitted from the medium does Na^+ increase the exchange[3,4]. However, in the labeling

experiments, Mg^{2+} is required for the Na^+ effect. A third problem is the interpretation of the Na^+ and K^+ effects on labeling. It is sometimes overlooked that the conclusion that there is a Na^+-dependent phosphorylation followed by a K^+-dependent dephosphorylation is based on an assumption; namely that the phosphorylation found with Na^+ but not with K^+ in the medium is due to Na^+ at the "Na^+ site" and not to Na^+ at the "K^+ site" (for details, see DISCUSSION).

In this paper are given the experimental results of the labeling from [^{32}P]ATP in the concentrations of 25 μM and 3mM, from [^{32}P]ITP in the concentration of 25 μM and from $^{32}P_i$. Some of the results have been given in a preliminary form[12,13]. The purpose of the study was to elucidate some of the above-mentioned problems, but from that point of view we have not been successful, for the results raised more questions than gave answers.

MATERIALS AND METHODS

Enzyme preparation

The enzyme was prepared from ox brain by a slightly modified method previously reported[20]. 400 g of the gray matter of the brain were homogenized in 2 l of ice-cold 0.25 M sucrose, 30 mM histidine (pH 7.2, 2°) and 0.1% sodium deoxycholate. After centrifuging at 10 000 × *g* for 20 min, the sediment was discarded; the supernatant was centrifuged at 20 000 × *g* for 60 min, and the sediment was resuspended by homogenization in 780 ml of 0.25 M sucrose with 30 mM histidine (pH 7.2, 2°) and 5 mM EDTA (set to pH 7.2 (2°) with 2-amino-2-methyl-1,3-propanediol), and 0.05% sodium deoxycholate. The suspension was centrifuged at 10 000 × *g* for 20 min., and the sediment was discarded. The modification was that the incubation time with deoxycholate was increased from 20 min to several h by leaving the supernatant from this centrifugation overnight at 2°. This increased the $Na^+ + K^+$ activation. The following day the supernatant was centrifuged at 20 000 × *g* for 60 min; the sediment was discarded, and the supernatant was centrifuged at 100 000 × *g* for 30 min. This sediment was resuspended in 120 ml of 0.25 M sucrose, 30 mM histidine (pH 7.2, 2°) and was used as an enzyme source after a final wash. It contained 1–2 mg protein per ml. The specific activity was 160–200 μmoles P_i per mg protein per h (measured with 3 mM Mg^{2+}, 3 mM ATP, 120 mM Na^+, 30 mM K^+ (pH 7.4, 37°). The $(Mg^{2+} + Na^+ + K^+)/Mg^{2+}$ activity ratio was between 8 and 12. The enzyme was stored at −20°.

In the experiments comparing the labeling of the enzyme preparation with a low $(Mg^{2+} + Na^+ + K^+)/Mg^{2+}$ activity ratio and with one having a higher ratio, the enzyme was prepared from ox kidney. The cortex of the kidney was homogenized in ice-cold 30 mM histidine buffer (pH 7.2, 2°). After centrifuging twice at 10 000 × *g* for 30 min, the supernatant was centrifuged at 35 000 × *g* for 30 min; the sediment was washed once, resuspended in ice-cold 30 mM histidine buffer (pH 7.2, 2°) and used as an enzyme source. This preparation had an activity ratio of 1.5. A part of it was incubated for 30–60 min at 0° with 0.1% deoxycholate and 5 mM EDTA (pH 7.2, 2°). After incubation, the enzyme particles were precipitated by centrifugation, resuspended in ice-cold 30 mM histidine buffer (pH 7.2, 2°) and used as an enzyme source. The activity ratio by this treatment with deoxycholate increased to about 8, partly due to an increase in activity with $Mg^{2+} + Na^+ + K^+$ and partly to a decrease in activity with Mg^{2+}.

Labeling of ATP and ITP

ATP was labeled with ^{32}P in the γ-position according to the method of PFLEIDERER[21]. [^{32}P]ITP was prepared from [^{32}P]ATP according to the method of KALCHAR[22], and the procedure was controlled by chromatography[23]. Both [^{32}P]ATP and [^{32}P]ITP were separated from $^{32}P_i$ and organic phosphate compounds by elution with Tris–HCl from a Dowex 1 column. The activity of [^{32}P]ATP was 3–4 mC/μmole ATP. $^{32}P_i$ was obtained from the Radiochemical Centre, Amersham, England.

Assay

In the labeling experiments, 3 ml of a test solution contained 0.6 ml of enzyme solution (1–2 mg protein per ml), 30 mM Tris–HCl buffer (pH 7.4) and cations as the chloride salt in the concentrations given in the figures. In the experiments with [^{32}P]ATP and [^{32}P]ITP, the solution also contained 0.5 mM P_i to dilute $^{32}P_i$ released by the hydrolysis of the triphosphates. The temperature was either 37° or 0°; the reaction was started by the addition of the labeled substrate while the solution was continuously stirred. The reaction time is given in the figures.

The reaction was stopped by adding 3 ml of 8% ice-cold $HClO_4$, and the protein was precipitated by centrifugation. In the experiments with [^{32}P]ATP and [^{32}P]ITP, the amount of $^{32}P_i$ and [^{32}P]ATP or [^{32}P]ITP in the supernatant after the first centrifugation was determined according to the method of LINDBERG AND ERNSTER[24], and the hydrolysis of ATP or ITP was calculated.

The precipitate was washed 5 times with ice-cold 0.1% trichloroacetic acid and with 10 mM P_i, and, in the experiments with [^{32}P]ATP and [^{32}P]ITP, 0.1 mM of the respective triphosphates was added. After the final wash the precipitate was dissolved in 1 ml 1 M NaOH. A part of this solution was used for protein determination by the method of LOWRY *et al.*[25], and a suitable part was plated for $^{32}P_i$ counting in a Frieseke–Hoepfner gas flow counter.

The results of the labeling are given as nmoles or pmoles P_i bound per mg protein and are calculated on the basis of the counts/min per mg protein and on the specific activity of the added [^{32}P]ATP, [^{32}P]ITP or $^{32}P_i$.

The figures show single experimental results, but all experiments were repeated from 3 to 5 or more times with the same result.

RESULTS

Stable and unstable labeling

In agreement with what has been found by others[2,5–18], the microsomal particle which contains the ($Na^+ + K^+$)-activated enzyme system is labeled with ^{32}P when [^{32}P]ATP is used as the substrate. This is shown in Fig. 1 for an experiment with a low (25 μM) concentration of ATP and in Fig. 2 with a higher (3 mM) concentration of ATP.

It is seen from Figs. 1 and 2 that there are two different kinds of labeling: one decreases when all [^{32}P]ATP is hydrolyzed to ADP and P_i and another either does not decrease or decreases very slowly. They are named unstable and stable labeling, respectively, in the following (*cf.* ref. 15).

Furthermore, Figs. 1 and 2 show that the labeling is highly influenced by the cations in the medium and that the differences in the cation effect are influenced by the ATP concentration.

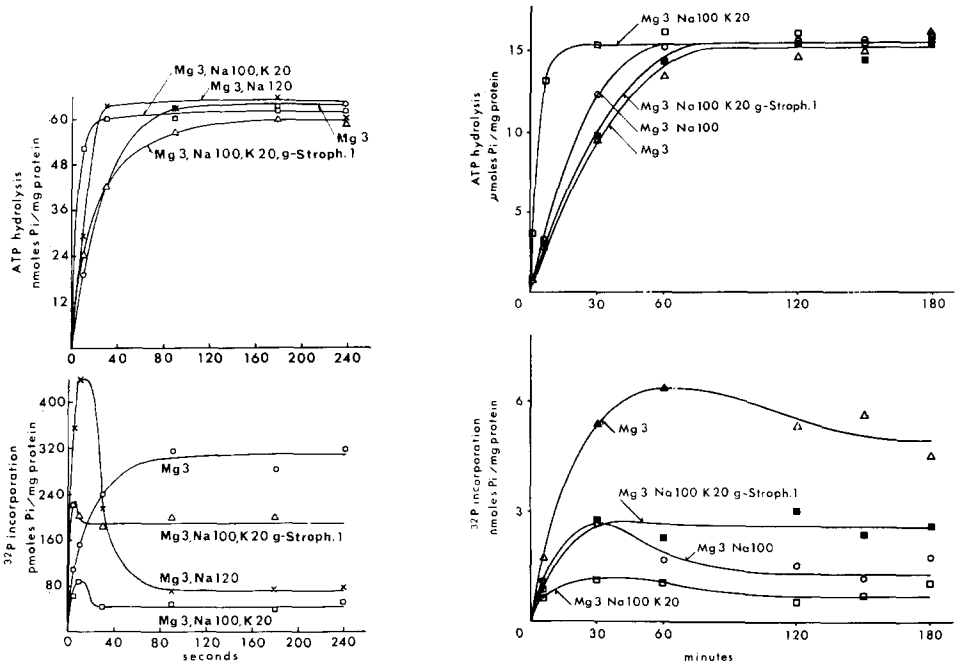

Fig. 1. The effects of cations and of g-strophanthin on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle (lower figure) and on the hydrolysis of ATP (upper figure). ATP concn. 25 μM; temp., 37°. The concentrations (mM) of the cations and of g-strophanthin are shown in the figure. The ^{32}P incorporation is given in this and the following figures apart from Figs. 9, 16, 17, 21, and 22 as nmoles or pmoles P_i bound per mg microsomal protein. The hydrolysis of ATP is given as μmoles or nmoles of P_i released from ATP per mg of microsomal protein.

Fig. 2. The effects of cations and of g-strophanthin on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle (lower figure) and on the hydrolysis of ATP (upper figure). ATP concn., 3 mM; temp., 37°. The concentrations (mM) of the cations and of g-strophanthin are shown in the figure.

Effect of Mg^{2+}

With Mg^{2+} the labeling increases to a high level of stable labeling. In some of the experiments, it increases to a maximum from which there is a low decrease to the stable level, Fig. 2. In others there is a steady increase to the level of stable labeling, Fig. 1. This difference is independent of the ATP concentration (*cf.* Figs. 1 and 8).

The concentration of Mg^{2+} needed to give maximum labeling with the low ATP concentration is about 3 mM, Fig. 3.

Effect of Na^+

Na^+ in addition to Mg^{2+} has three effects on the labeling: (a) it increases the initial rate of labeling, (b) changes the maximum labeling obtained and (c) decreases the stable labeling.

For this Na^+ effect, Mg^{2+} is required in the medium. At the low ATP concen-

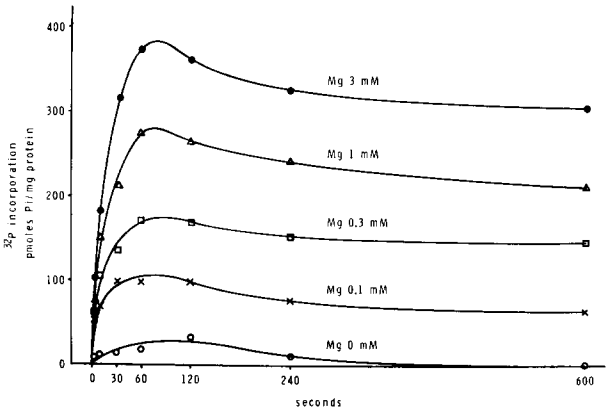

Fig. 3. The effect of varying concentrations of Mg^{2+} on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle. ATP concn., 25 μM; temp., 37°. The concentration (mM) of Mg^{2+} is shown in the figure.

tration, the effect is found without the addition of Mg^{2+}, Fig. 4; however, because the effect of Na^+ disappears when EDTA is added and reappears on the addition of Mg^{2+}, it seems to be due to Mg^{2+} in the test solution. This Mg^{2+} comes partly from the added enzyme and partly from the added ATP, which contains a small amount of Mg^{2+}.

Both the maximum labeling and the level of stable labeling increase with the Mg^{2+} concentration up to 3 mM, the highest measured. The differences between the

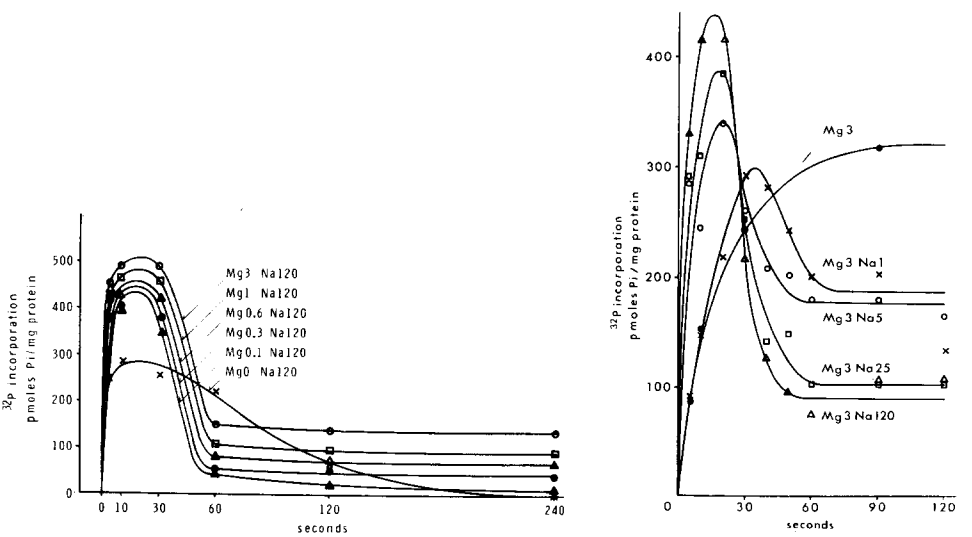

Fig. 4. The effects of 120 mM Na^+ and of varying Mg^{2+} concentrations on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle. ATP concn., 25 μM; temp., 37°. The concentration (mM) of Mg^{2+} is shown in the figure.

Fig. 5. The effect of 3 mM Mg and of varying Na^+ concentrations on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle. ATP concn., 25 μM; temp., 37°. The concentrations (mM) of Na^+ are shown in the figure.

maximum and the level of stable labeling which may represent the unstable labeling are nearly independent of the Mg^{2+} concentration from 0.1 to 3 mM (Fig. 4).

At the low ATP concentration, Na^+ increases both the initial rate of labeling and the maximum obtained, and there is a decrease in the stable labeling (Fig. 5). Thus, the unstable labeling increases. The effect increases with increasing Na^+ concentration up to 120 mM, the highest concentration used.

At the high concentration of ATP, Na^+ increases the initial rate of labeling (Fig. 6), but the maximum labeling is decreased simultaneously with a decrease in stable labeling (Figs. 2 and 7). In some experiments Na^+ decreases the stable labeling more than the maximum labeling, *i.e.* there seems to be an increase in unstable labeling, while in others there is none. The increase in the rate above that with Mg^{2+} is higher with 10 mM Na^+ and lasts longer than with 120 mM (Fig. 7). Na^+ increases the hydrolysis, but there seem to be no differences in the effects of 10 mM Na^+ and 120 mM Na^+ (Fig. 7).

The effect of Na^+ on the labeling is also found if Na^+ is added to the medium a certain time after the reaction has been started only with Mg^{2+} (Fig. 8). The addition of Na^+ after 10 sec affects the unstable labeling similarly to Na^+ added at the start of the reaction. However, the effect on the stable labeling is much lower. It appears that

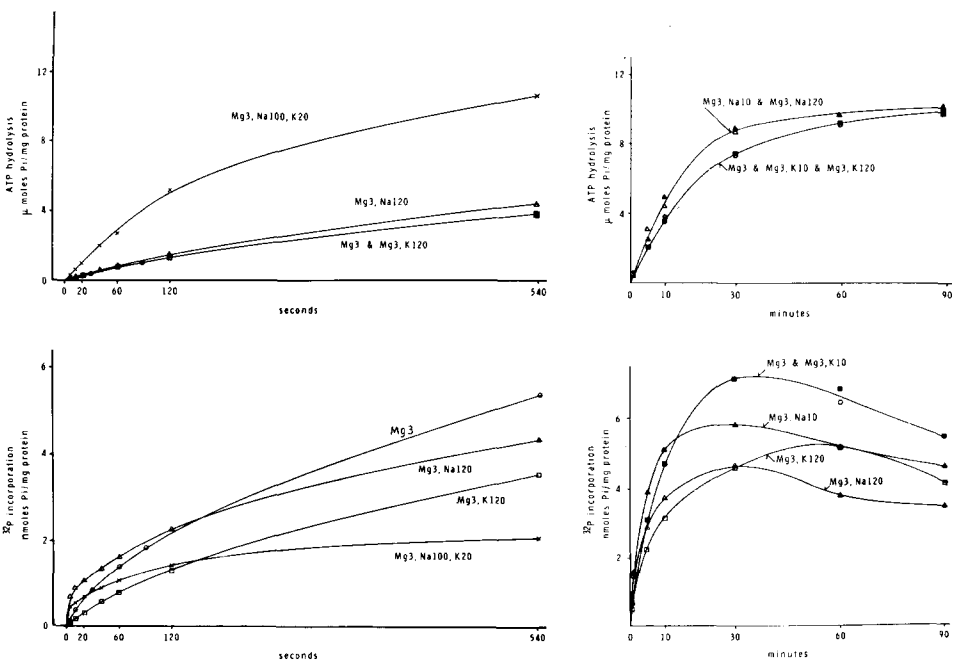

Fig. 6. The effects of 3 mM Mg^{2+}; 3 mM Mg^{2+}, 120 mM Na^+; 3 mM Mg^{2+}, 120 mM K^+; and of 3 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+ ,respectively, on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle (lower figure). The upper figure shows the effects on hydrolysis. ATP concn., 3 mM; temp., 37°.

Fig. 7. The effect of 3 mM Mg^{2+}; 3 mM Mg^{2+}, 10 mM Na^+; 3 mM Mg^{2+}, 120 mM Na^+; 3 mM Mg^{2+}, 10 mM K^+; and 3 mM Mg^{2+}, 120 mM K^+, respectively, on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle (lower figure). The upper figure shows the effects on hydrolysis. ATP concn., 3 mM; temp., 37°.

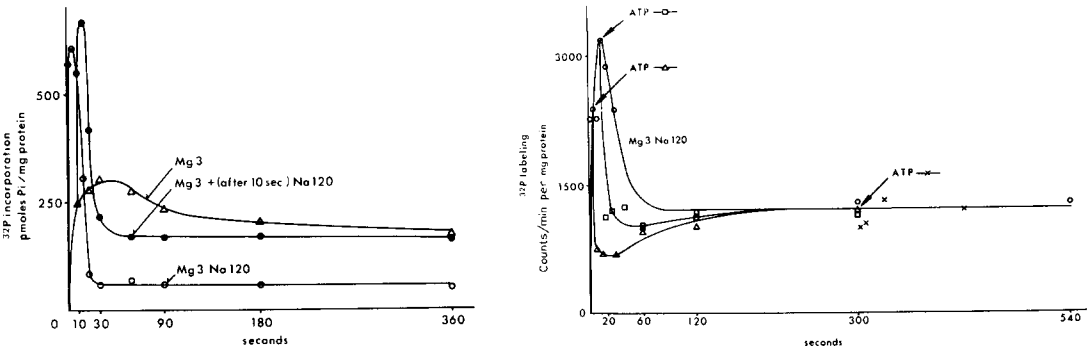

Fig. 8. The effect of Na^+ on the incorporation of ^{32}P from [^{32}P]ATP when Na^+ is added 10 sec after the reaction has been started with 3 mM Mg^{2+}. ATP concn., 25 μM; temp., 37°. 100 μl of a 3.6 M solution of NaCl was added to 2.9 ml of test solution to bring the concentration of Na^+ to 120 mM. For comparison is shown the incorporation with 3 mM Mg^{2+} and with 3 mM Mg^{2+}, 120 mM Na^+, respectively.

Fig. 9. The effects on the labeling with ^{32}P from [^{32}P]ATP of the addition of unlabeled ATP at 3 different times after the start of the reaction with 3 mM Mg^{2+}, 120 mM Na^+. ATP concn., 25 μM; temp., 37°. Unlabeled ATP was added to a concentration of 500 μM after 10, 20 and 300 sec, respectively, in 3 parallel experiments. For comparison is shown the labeling with 3 mM Mg^{2+}, 120 mM Na^+ without addition of unlabeled ATP. Abscissa: time (sec). Ordinate: labeling (counts/min per mg protein).

the stable labeling has reached a certain level during the 10-sec run with only Mg^{2+}, and that the addition of Na^+ cannot decrease the stable labeling but prevents a further increase. In accordance with this, it is found that the addition of Na^+ after complete hydrolysis of ATP has no effect on the stable labeling.

Effect of unlabeled ATP on prelabeled particles

Unlabeled ATP added to the reaction mixture at different times after the reaction has been started with $Mg^{2+} + Na^+$ immediately decreases the counts/min per mg protein as long as the initial [^{32}P]ATP has not been completely hydrolyzed (Fig. 9). The labeling decreases to a lower level the earlier the unlabeled ATP is added in the reaction. Thereafter, the labeling increases again to a level identical to the stable labeling obtained when unlabeled ATP has not been added.

Unlabeled ATP added after complete hydrolysis of the [^{32}P]ATP has no effect on the stable labeling (*cf.* ref. 17).

Effect of K^+

When there is Mg^{2+} in the medium, the effect of Li^+ is similar to, although lower than, that of Na^+ (not shown). K^+ has no effect at a low ATP concentration. At a high ATP concentration, 10 mM K^+ has no effect, whereas 120 mM K^+ decreases the labeling. K^+ does not increase the initial rate of labeling as Na^+ but rather decreases it (Figs. 6 and 7). K^+ has no effect on the hydrolysis.

With $Mg^{2+} + Na^+$ in the medium, K^+ decreases the labeling both at low and high ATP concentrations (Figs. 1 and 2). It is not clear from the present experiments whether the initial rate of labeling with $Mg^{2+} + Na^+ + K^+$ is identical to that with $Mg^{2+} + Na^+$, but after a few seconds the rate is lower (Figs. 1 and 6). The maximum labeling obtained with $Mg^{2+} + Na^+ + K^+$ is also lower than with $Mg^{2+} + Na^+$.

When the hydrolysis of ATP is nearly complete, the labeling with $Mg^{2+} + Na^+ +$

K^+ slightly decreases to a level of stable labeling, suggesting that part of the labeling with $Mg^{2+} + Na^+ + K^+$ is of the unstable nature although it is much lower than with $Mg^{2+} + Na^+$. The stable labeling with $Mg^{2+} + Na^+ + K^+$ is lower than with $Mg^{2+} + Na^+$ (Figs. 1 and 2).

The addition of K^+ to the medium with $Mg^{2+} + Na^+$ increases the ATP hydrolysis and decreases the labeling simultaneously. As seen from the comparison between Figs. 1 and 2, the increase in hydrolysis due to K^+ is relatively higher at a high ATP concentration than at a low one.

The K^+ concentration which gives an effect on the labeling depends on the Na^+ concentration in the medium in the same manner as the K^+ concentration which gives an effect on the hydrolysis depends on the Na^+ concentration (Fig. 10). The higher the Na^+ concentration, the higher must the K^+ concentration be to give a certain effect. The K^+ concentration giving a certain decrease in the labeling and an increase in the hydrolysis is low compared to the Na^+ concentration. This suggests that both for the

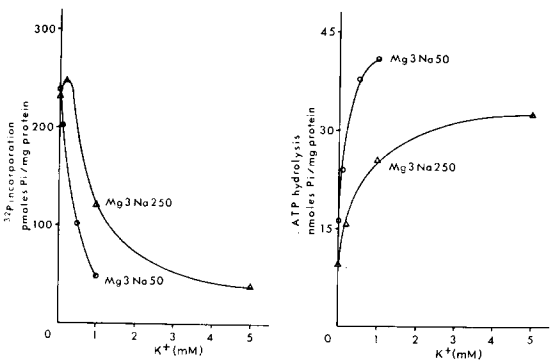

Fig. 10. The effects of varying concentrations of K^+ on the ^{32}P incorporation from [^{32}P]ATP and on the ATP hydrolysis with 3 mM Mg^{2+}, 50 mM Na^+ and with 3 mM Mg^{2+}, 250 mM Na^+, respectively. ATP concn., 25 μM; temp., 37°. The time of reaction was 10 sec (*cf.* Fig. 1). Abscissa: K^+ concn. (mM). Ordinate: on the left figure, ^{32}P incorporation in pmoles P_i incorporated per mg protein; on the right figure, ATP hydrolysis in nmoles P_i hydrolyzed from ATP per mg protein.

effect on the labeling and on the hydrolysis, K^+ competes with Na^+ at a site having greater affinity for K^+ than for Na^+; this is the "K-site".

Effect of K^+ on prelabeled particles

When the enzyme particle has been prelabeled with $Mg^{2+} + Na^+$ in the medium, the addition of K^+ decreases the labeling, and the rate by which the labeling decreases is high as long as [^{32}P]ATP is not completely hydrolyzed. However, there is no effect of K^+ when it is added after complete hydrolysis of [^{32}P]ATP. The addition of K^+ leads to a simultaneous increase in hydrolysis (Figs. 11 and 12).

At the low concentration of ATP, Fig. 11, K^+ decreases the labeling to the level of stable labeling found when K^+ has been added from the start of the reaction. This is the case when K^+ is added during the rising phase of the curve for the labeling with $Mg^{2+} + Na^+$. When added later, K^+ decreases the labeling to a slightly higher level which is inbetween the stable labeling with $Mg^{2+} + Na^+ + K^+$ and with $Mg^{2+} + Na^+$.

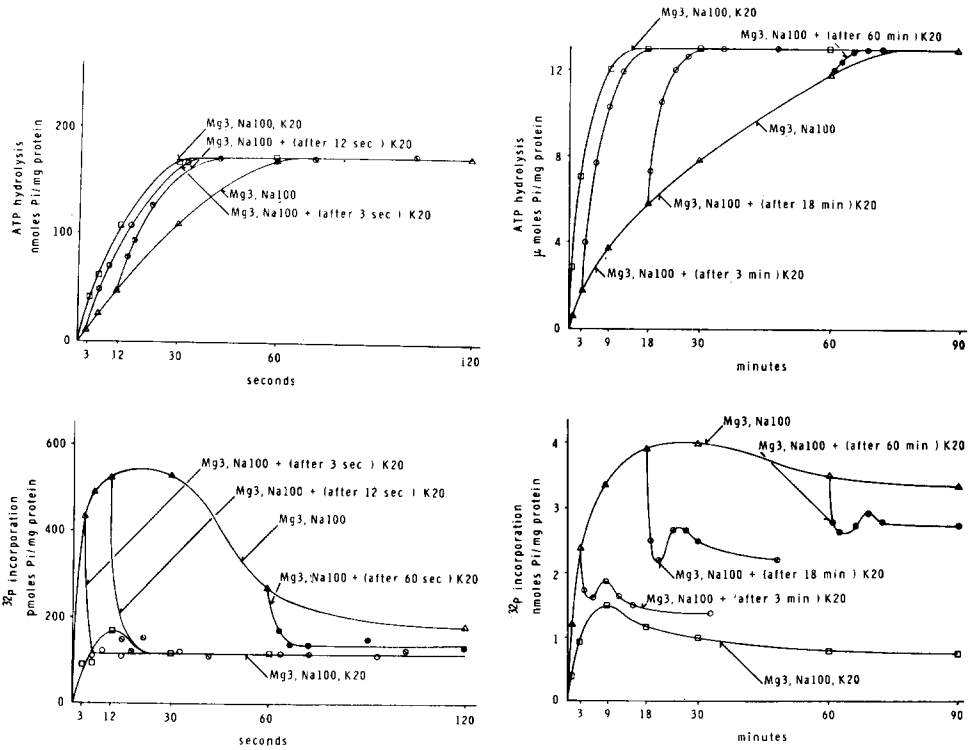

Fig. 11. The effect on the incorporation of ^{32}P from [^{32}P]ATP and on the hydrolysis of ATP of the addition of K^+ at different times after the reaction has been started with 3 mM Mg^{2+}, 100 mM Na^+. ATP concn., 25 μM; temp., 37°. 50 μl of a K^+ solution was added after 3, 12 and 60 sec, respectively, to the 3 ml test solution to give a final concentration of 20 mM. For comparison is shown the ^{32}P incorporation with 3 mM Mg^{2+}, 100 mM Na^+ with and without K^+ in the medium.

Fig. 12. The effect on the incorporation of ^{32}P from [^{32}P]ATP and on the hydrolysis of ATP of the addition of K^+ at different times after the reaction had been started with 3 mM Mg^{2+}, 100 mM Na^+. ATP concn., 3 mM; temp., 37°. 3, 18 and 60 min after the reaction was started, 50 μl of a KCl solution was added to 3 ml test solution to give a final K^+ concentration of 20 mM.

At the high ATP concentration (Fig. 12) K^+ gives a relatively lower decrease in labeling when added during the rising phase of the curve for the labeling with $Mg^{2+} + Na^+$ (*cf.* Figs. 11 and 12). The level to which the labeling decreases is higher the later K^+ is added. The decrease in labeling is followed by a small increase, and after complete hydrolysis of ATP, the labeling decreases to a stable labeling level similar to that to which the labeling primarily decreased after the addition of K^+. The shape of the curve with a small rise and fall in the labeling when it has been decreased after the addition of K^+ is similar to the shape of the curve with $Mg^{2+} + Na^+ + K^+$ where K^+ was initially present.

The increase in the level to which the labeling decreases upon the addition of K^+ shows that there is a steady increase in stable labeling during the run with $Mg^{2+} + Na^+$; this is most pronounced at the high ATP concentration. This also shows what fraction of the labeling is unstable with $Mg^{2+} + Na^+$. This fraction is lower at the high ATP concentration than at the low one. Furthermore, from the experiment in Fig. 12 it is

seen that it is not possible to determine from the slight decrease in labeling found with $Mg^{2+} + Na^+$, when all ATP has been hydrolyzed, what fraction of the labeling is unstable during the run.

Effect of g-strophanthin and oligomycin

g-Strophanthin affects the labeling both with $Mg^{2+} + Na^+$ and with $Mg^{2+} + Na^+ + K^+$, whereas there is no effect with Mg^{2+} alone (Figs. 1 and 2).

In concentrations giving a maximum effect, g-strophanthin gives the same labeling whether there is $Mg^{2+} + Na^+$ or $Mg^{2+} + Na^+ + K^+$ in the medium (Fig. 13).

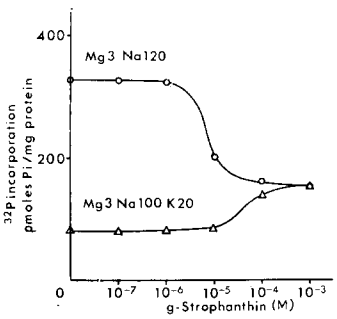

Fig. 13. The effects of varying concentrations of g-strophanthin with 3 mM Mg^{2+}, 120 mM Na^+ and with 3 mM Mg^{2+}, 100 mM Na^+, 20 mM K^+, respectively, on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle and on the ATP hydrolysis. ATP concn., 25 μM; temp., 37°. The time of reaction was 10 sec (*cf.* Fig. 1).

The labeling with g-strophanthin present is higher than that with $Mg^{2+} + Na^+ + K^+$ (Figs. 1 and 2). It is lower than with $Mg^{2+} + Na^+$ in that part of the reaction where the labeling with $Mg^{2+} + Na^+$ increases, whereas later in the reaction the labeling with g-strophanthin is higher. This is found both at high and low ATP concentrations. It is not possible from the experiments to tell how much of the labeling with g-strophanthin is unstable, but it seems as if the labeling is mainly stable. The final level with g-strophanthin is between the stable labeling with $Mg^{2+} + Na^+$ and with Mg^{2+}.

The concentration of g-strophanthin giving a certain effect is higher with the low ATP concentration than with the high one. This may not be due to the differences in ATP concentrations. Because the reaction with g-strophanthin is time-dependent[26,27], it may be due to the different times the reaction is followed. At a given ATP concentration, the concentration of g-strophanthin needed to obtain a certain effect is higher with $Mg^{2+} + Na^+ + K^+$ than with $Mg^{2+} + Na^+$ (Fig. 13).

The effect of oligomycin on ATP hydrolysis is similar to that of g-strophanthin in that it is not effective with Mg^{2+}, while it decreases the hydrolysis with $Mg^{2+} + Na^+$ and with $Mg^{2+} + Na^+ + K^+$ (*cf.* ref. 26). 10 μg/ml oligomycin inhibits the hydrolysis relatively more at high than at low ATP concentrations (Figs. 14 and 15), which may indicate that oligomycin, like g-strophanthin, is more effective at high ATP concentrations (see above). Oligomycin has, just like g-strophanthin, no effect on the labeling with Mg^{2+}. In contrast to g-strophanthin, oligomycin increases the labeling both with $Mg^{2+} + Na^+$ and with $Mg^{2+} + Na^+ + K^+$.

With $Mg^{2+} + Na^+$, 10 μg/ml of oligomycin (20–30 μg/mg of protein) increases the labeling with 3 mM ATP to a level approximating that with only Mg^{2+}, and it

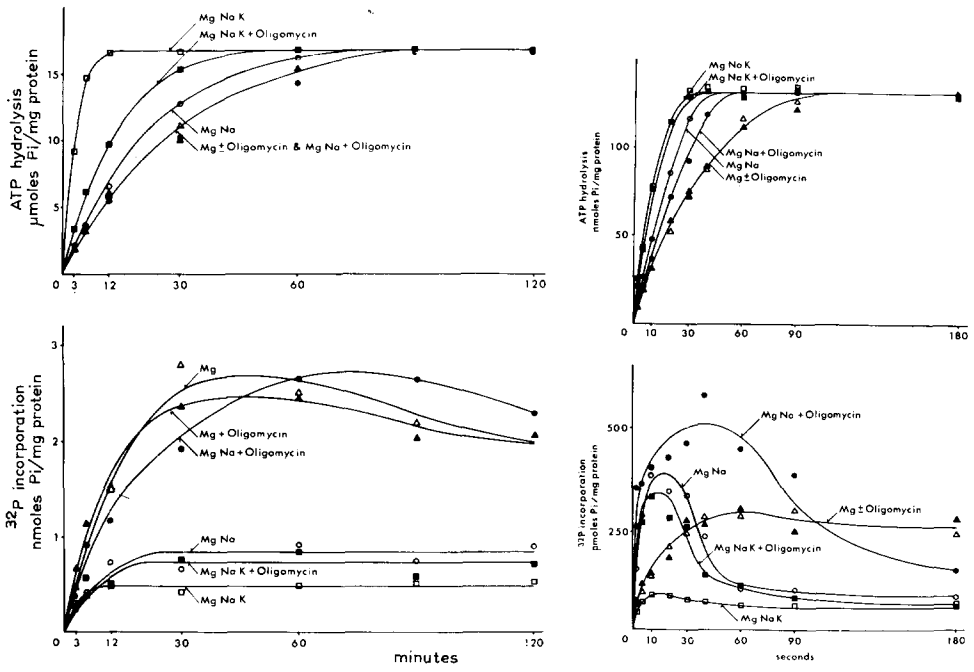

Fig. 14. The effect of 10 μg/ml oligomycin on the incorporation of ^{32}P from[^{32}P]ATP into the microsomal particle, and on the hydrolysis of ATP. ATP concn., 3 mM; temp., 37°. △—△, 3 mM Mg^{2+}; ▲—▲, 3 mM Mg^{2+} + oligomycin; ○—○, 3 mM Mg^{2+} + 120 mM Na^+; ●—●, 3 mM Mg^{2+} + 120 mM Na^+ + oligomycin; □—□, 3 mM Mg^{2+} + 100 mM Na^+ + 20 mM K^+; ■—■, 3 mM Mg^{2+} + 100 mM Na^+ + 20 mM K^+ + oligomycin.

Fig. 15. The effect of 10 μg/ml oligomycin on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle, and on the hydrolysis of ATP. ATP concn., 25 μM; temp., 37°. △—△, 3 mM Mg^{2+}; ▲—▲, 3 mM Mg^{2+} + oligomycin; ○—○, 3 mM Mg^{2+} + 120 mM Na^+; ●—●, 3 mM Mg^{2+} + 120 mM Na^+ + oligomycin; □—□, 3 mM Mg^{2+} + 100 mM Na^+ + 20 mM K^+; ■—■, 3 mM Mg^{2+} + 100 mM Na^+ + 20 mM K^+ + oligomycin.

decreases the hydrolysis to the Mg^{2+} level (Fig. 14). With 25 μM ATP, oligomycin increases the labeling to a level further from the Mg^{2+} level than is the labeling with Mg^{2+} + Na^+ without oligomycin; it decreases the hydrolysis but not as low as the Mg^{2+} level (Fig. 15).

With Mg^{2+} + Na^+ + K^+, oligomycin both at the high and the low ATP concentration increases the labeling to a level approximating but not higher than that with Mg^{2+} + Na^+ without oligomycin. The hydrolysis of ATP decreases but does not reach the Mg^{2+} + Na^+ level (Figs. 14 and 15).

Labeling at different (Mg^{2+} + Na^+ + K^+)/Mg^{2+} activity ratios

The effect of the cations on the labeling is influenced by the (Mg^{2+} + Na^+ + K^+)/Mg^{2+} activity ratio of the enzyme preparation. This is shown in Fig. 16 with 25 μM ATP and in Fig. 17 with 3 mM ATP. In the left-hand part of Figs. 16 and 17 is shown the ^{32}P incorporation into the microsomal particles of an enzyme preparation with an activity ratio of 1.5. In the right-hand part of Figs. 16 and 17 is shown the

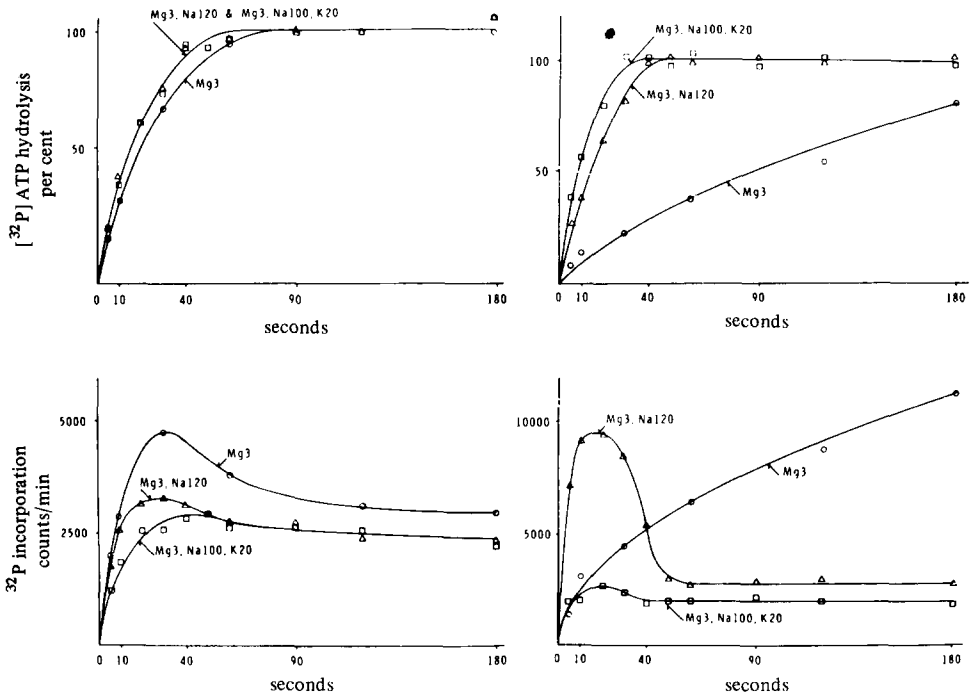

Fig. 16. The effects of cations on the incorporation of ^{32}P from [^{32}P]ATP into a microsomal particle preparation with a low ($Mg^{2+} + Na^+ + K^+$)/Mg^{2+} activity ratio (1.5) (left figure) and into the same microsomal particle fraction after the activity ratio has been increased to about 8 by treatment with deoxycholate (right figure). The upper figures show the hydrolysis of ATP. ATP concn., 25 μM; temp., 37°. The concentrations (mM) of the cations are shown in the figure. The microsomal particles were prepared from ox kidney.

^{32}P incorporation into the microsomal particles of the same enzyme preparation after the activity ratio has been increased to about 8. It is seen how the differences in the cation effect on the labeling increase with an increase in the activity ratio.

Labeling at 0°

There is a labeling of the microsomal particle from [^{32}P]ATP when the experiments are run at 0° (Fig. 18). As at 37°, the labeling rate with Mg^{2+} is low. Na^+ increases both the initial rate and the maximum obtained, whereas K^+ decreases the labeling found with $Mg^{2+} + Na^+$. Na^+ increases the hydrolysis, whereas K^+ in some experiments gives a slight increase in the hydrolysis with $Mg^{2+} + Na^+$; in others it has no effect. But in all the experiments, K^+ decreases the labeling.

g-Strophanthin decreases the labeling with $Mg^{2+} + Na^+$ to the level with $Mg^{2+} + Na^+ + K^+$ (Figs. 18 and 19); and corroborating the observation that at higher temperatures g-strophanthin gives the same labeling with $Mg^{2+} + Na^+$ and with $Mg^{2+} + Na^+ + K^+$ in the medium, g-strophanthin practically has no effect on the labeling with $Mg^{2+} + Na^+ + K^+$ at 0°.

g-Strophanthin decreases the hydrolysis with $Mg^{2+} + Na^+ + K^+$ to the Mg^{2+} level, whereas in concentrations up to 10^{-3} M, only a very slight decrease in hydrolysis with $Mg^{2+} + Na^+ + K^+$ is effected (Fig. 19). K^+, which has no effect on the hydrolysis

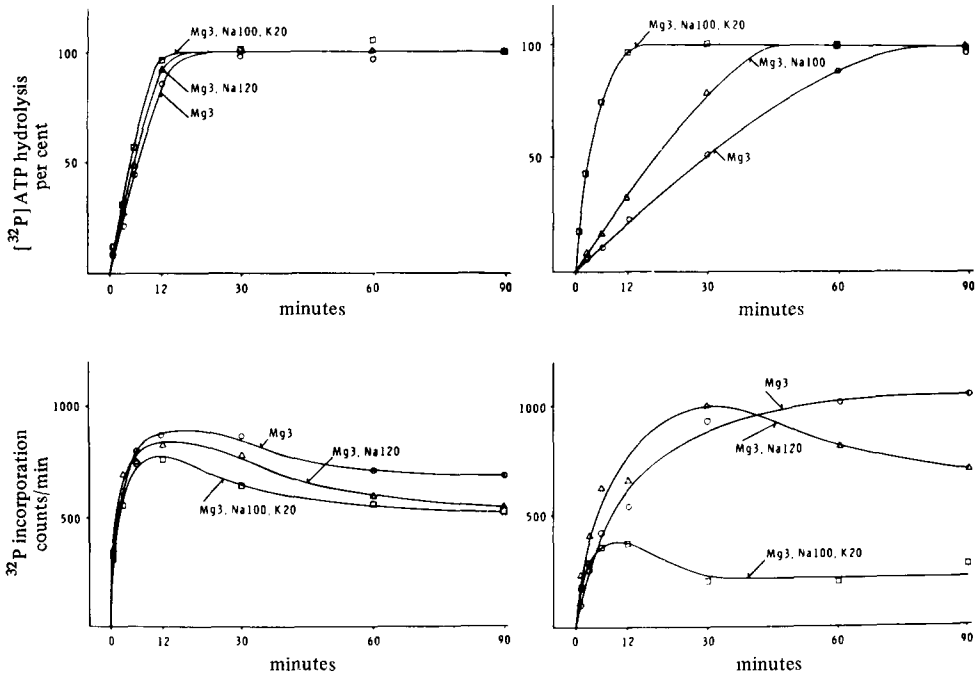

Fig. 17. The effects of cations on the incorporation of ^{32}P from [^{32}P]ATP into a microsomal particle preparation with a low $(Mg^{2+} + Na^+ + K^+)/Mg^{2+}$ activity ratio (1.5) (left figure) and into the same microsomal particle preparation after the activity ratio has been increased to about 8 by treatment with deoxycholate (right figure). The upper figures show the hydrolysis of ATP. ATP concn., 3 mM; temp., 37°. The concentrations (mM) of the cations are shown in the figure. The microsomal particles were prepared from ox kidney.

with $Mg^{2+} + Na^+$, thus protects almost completely against the effect of g-strophanthin on the hydrolysis at 0°.

It is seen from Fig. 19 that the concentration of g-strophanthin necessary to give a certain decrease in labeling is lower than the concentration necessary to give the same relative decrease in hydrolysis.

ITP as substrate

When [^{32}P]ITP is used in place of [^{32}P]ATP as a substrate, the microsomal particle is labeled with ^{32}P (Fig. 20). The concentration of ITP was 25 μM.

The pattern for the effect of the cations and of g-strophanthin on the [^{32}P]ITP labeling are the same as with [^{32}P]ATP as the substrate (*cf.* Fig. 1). The rate of labeling and the amount of P_i incorporated per mg protein is lower than with ATP as the substrate.

The labeling with $Mg^{2+} + Na^+$ compared to that with Mg^{2+} seems to be relatively higher with [^{32}P]ITP than with [^{32}P]ATP (*cf.* Figs. 20 and Fig. 1), but this may be due to the fact that a steady state of labeling is not obtained with $Mg^{2+} + Na^+$ and with ATP in contrast to the situation with ITP.

K^+ decreases the labeling both with [^{32}P]ITP and with [^{32}P]ATP. However, with [^{32}P]ITP, this K^+ effect is accompanied by a decrease in hydrolysis.

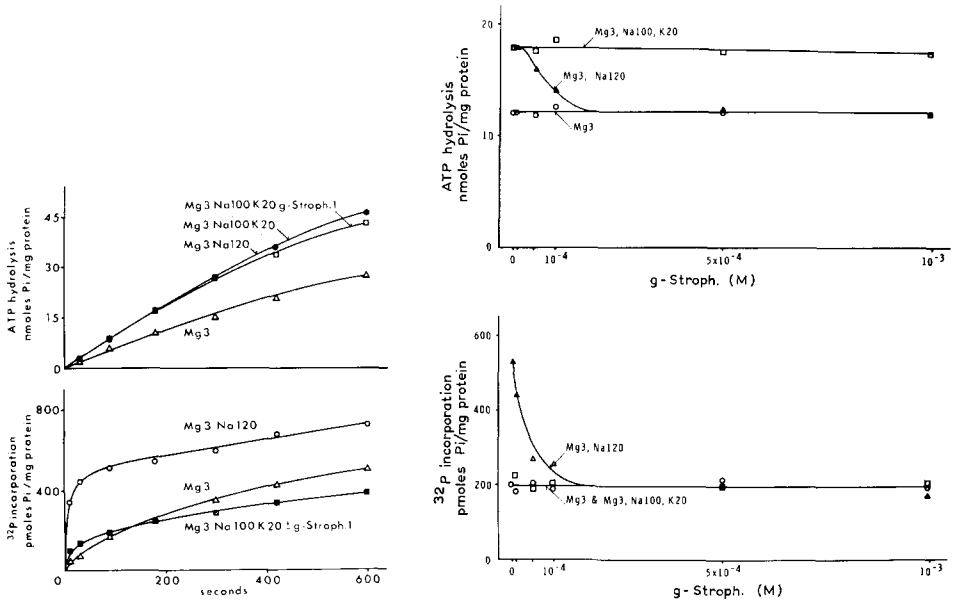

Fig. 18. The effects of cations and of g-strophanthin on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle at 0°. The upper figure shows the effect on ATP hydrolysis. ATP concn., 25 μM. The concentrations (mM) of the cations and g-strophanthin are shown in the figure.

Fig. 19. The effects of g-strophanthin at 0° on the incorporation of ^{32}P from [^{32}P]ATP into the microsomal particle with 3 mM Mg^{2+}, 3 mM Mg^{2+} + 120 mM Na^+ and with 3 mM Mg^{2+} + 100 mM Na^+ + 20 mM K^+, respectively. The upper figure shows the effect on the hydrolysis of ATP. ATP concn., 25 μM. Abscissa: concentration of g-strophanthin (M).

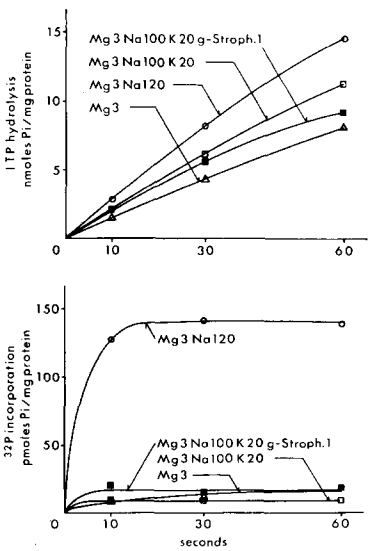

Fig. 20. The effects of cations and of g-strophanthin on the incorporation of ^{32}P from [^{32}P]ITP into the microsomal particles and on the hydrolysis of ITP. ITP concn., 25 μM; temp., 37°. The concentrations (mM) of the cations and g-strophanthin are shown in the figure.

Labeling from $^{32}P_i$

When [^{32}P]ATP is hydrolyzed by the enzyme system, there is a continuous release of $^{32}P_i$ in the test solution. To see whether this $^{32}P_i$ could give a labeling and could explain the stable labeling, a series of experiments were run where $^{32}P_i$ was added instead of [^{32}P]ATP.

It was found that added $^{32}P_i$ labeled the microsomal particle. The labeling was at a maximum in less than 2 sec, the shortest time measured. When the $^{32}P_i$ counts/min per ml test solution was kept constant and when the P_i concentration increased, labeling decreased with the P_i concentration to a certain level where it stayed constant (Fig. 21).

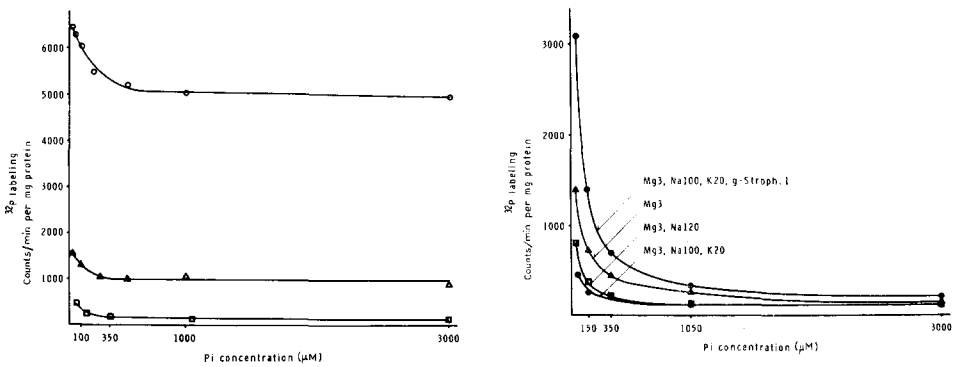

Fig. 21. The effect of the P_i concentration on the ^{32}P labeling of the microsomal particle from three different batches of $^{32}P_i$. Temp., 37°; time of incubation, 30 min. The $^{32}P_i$ counts/min per ml test solution was kept constant, while the P_i concentration varied from 50 to 3000 μM, *i.e.* the specific activity decreased with the increase in P_i concentration. The counts/min per ml test solution was the same in all three experiments. The solution contained 3 mM Mg^{2+}, 120 mM Na^+, 30 mM K^+. Abscissa: concentration of P_i (μM). Ordinate: ^{32}P labeling (counts/min per mg protein).

Fig. 22. The effects of cations and of g-strophanthin on the ^{32}P-labeling of the microsomal particles from $^{32}P_i$ at different of P_i concentrations. The $^{32}P_i$ solution was washed twice with microsomal particles (see text) before it was used in the experiment. Temp., 37°; time of incubation, 30 min. The $^{32}P_i$ counts/min per ml test solution was the same in all experiments, but the P_i concentration varied from 50 to 3000 μM. Abscissa: P_i concentration (μM). Ordinate: ^{32}P labeling (counts/min per mg protein). The concentrations (mM) of cations and of g-strophanthin are shown in the figure.

The level to which the labeling decreased when unlabeled P_i was added varied for different batches of $^{32}P_i$. One way to explain this was that the $^{32}P_i$ was contaminated with another isotope which was bound to the microsomal particle and the uptake of which was independent of the added unlabeled P_i.

To test this hypothesis the following experiment was made. A portion of the microsomal particle was added to a solution of $^{32}P_i$ with 3.5 mM P_i, and after a 20-min incubation at 37°, the microsomal particles were sedimented by ultracentrifugation. The sediment was washed 5 times with trichloroacetic acid as usual, and after the final wash the number of counts/min per mg protein was determined. After the first centrifugation, a new portion of microsomal particles was added to the supernatant, and the procedure was repeated. The results are shown in Table I. The counts/min per ml solution and the concentration of P_i were 20% lower during the second incubation due to the dilution by adding the second portion of the microsomal particle; the specific

TABLE I

LABELING OF TWO EQUAL PORTIONS OF ENZYME PARTICLE INCUBATED SUCCESSIVELY IN THE SAME SOLUTION OF $^{32}P_i$

0.6 ml of enzyme solution (1 mg protein per ml) was incubated in a total volume of 3 ml containing 3.5 mM $^{32}P_i$, 3 mM Mg^{2+}, 30 mM Tris buffer (pH 7.3). After a 30-min incubation at 37°, the enzyme particles were sedimented by ultracentrifugation (260 000 × *g* for 30 min). The sediment was resuspended in 30 mM Tris buffer (pH 7.3), was precipitated with $HClO_4$ and was washed 5 times as described in MATERIALS AND METHODS. After the final wash, the counts/min per mg protein was determined. To 2.4 ml of the supernatant after the ultracentrifugation was added a new 0.6-ml portion of enzyme solution, and the procedure was repeated. The addition of the enzyme solution diluted the $^{32}P_i$ concentration from the 3.5 mM in the first experiments to 2.8 mM in the second; the specific activity was the same in both experiments.

Expt. No.	$^{32}P_i$ *concn. (mM)*	^{32}P *(counts/ min per μmole P_i)*	^{32}P *(counts/ min per mg protein)*	*Counts bound to total protein (% of total counts in soln.)*
1	3.5	141 000	1491	0.112
2	2.8	141 000	66	0.006

activity of P_i was the same during both incubations. It is seen in Table I that the labeling of the microsomal particle after the second incubation is only about 1/20th of the labeling after the first incubation. Furthermore, the number of counts removed by the microsomal particle during the first incubation is 0.112% of the total number of counts in the solution, whereas after the second incubation it is only 0.006%.

The results shown in Table I suggest that $^{32}P_i$ is contaminated with a small amount, about 0.1–0.2% in counts/min, of another isotope which is bound to the microsomal particle. The nature of the contamination has not be discovered. It is eluted with $^{32}P_i$ from the Dowex column in the procedure for the separation of [^{32}P]ATP and $^{32}P_i$ and does not, or only to a very slight extent, contaminate [^{32}P]ATP. If there is a slight contamination of the [^{32}P]ATP solution, it is measured as the [^{32}P]ATP blank and is subtracted from the labeling found with [^{32}P]ATP as the substrate.

In Fig. 22 is shown the labeling of the microsomal particle as a function of the $^{32}P_i$ concentration where the $^{32}P_i$ solution has been washed twice with microsomal particles to remove the contamination. Fig. 22 shows that the labeling decreases asymptotically toward the x-axis as the P_i concentration increases, and that the cations and g-strophanthin affect the labeling from $^{32}P_i$. With Mg^{2+}, $Mg^{2+} + Na^+$, and $Mg^{2+} + Na^+ + K^+$, the labeling follows the pattern for the cation effect on the stable labeling from [^{32}P]ATP. With $Mg^{2+} + Na^+ + K^+$ + g-strophanthin, the labeling from $^{32}P_i$ is higher than with Mg^{2+} in contrast to the stable labeling from [^{32}P]ATP, which with $Mg^{2+} + Na^+ + K^+$ + g-strophanthin is lower than with Mg^{2+}.

Assuming that the $^{32}P_i$ released from [^{32}P]ATP gives a labeling in the same manner as does the added $^{32}P_i$, it is possible from the results shown in Fig. 22 to estimate the labeling which should come from the $^{32}P_i$ released. Such a calculation shows that with 25 μM ATP, the estimated labeling from released $^{32}P_i$ after complete hydrolysis of [^{32}P]ATP is about 5% of the measured stable labeling. With 3 mM ATP the estimated labeling is about 30% of the measured stable labeling with $Mg^{2+} + Na^+ + K^+$ in the medium and about 12% with Mg^{2+}, whereas with $Mg^{2+} + Na^+$ and with $Mg^{2+} + Na^+ + K^+$ + g-strophanthin, it is between these two values.

DISCUSSION

Relationship between unstable and stable labeling

There appears to be at least two different kinds of labeling, an unstable and a stable one (*cf.* ref. 15). From some of the experiments it is obvious that a large part of the labeling is unstable, as in the experiment with $Mg^{2+} + Na^+$ and with the low ATP concentration. However, in others it is not possible from the decrease in labeling when all ATP is hydrolyzed to determine what the ratio is between the two different kinds of labeling. This is most clearly seen in Fig. 12 where K^+ is added to $Mg^{2+} + Na^+$ at different times. With $Mg^{2+} + Na^+$, the amount of labeling which decreases when all ATP is hydrolyzed is low. The addition of K^+ shows that during the run there is a relatively larger part of the labeling which can be decreased by K^+, *i.e.* unstable.

The unstable labeling decreases when all [^{32}P]ATP is hydrolyzed. That there is no labeling from [^{14}C]ATP (ref. 7) suggests that the unstable labeling is due to a transfer of phosphate from [^{32}P]ATP to the microsomal particle, and that this phosphorylation is followed by a dephosphorylation.

It is not possible from the present experiments to tell from where the stable labeling comes. There is a labeling from added $^{32}P_i$ implying that there must be a labeling from $^{32}P_i$ released from [^{32}P]ATP. This labeling is influenced by cations but not by g-strophanthin in a manner similar to the stable labeling and can only count for a fraction of the measured stable labeling.

Unlabeled ATP does not decrease the stable labeling when added after the hydrolysis of [^{32}P]ATP is complete; nor does unlabeled ATP added before the hydrolysis of [^{32}P]ATP is complete decrease the final level of the stable labeling. This implies that the stable labeling cannot be due to an isotope exchange between the phosphorylated site and another phosphate group on the particle but rather suggests that the stable labeling is proportional to the ATP concentration.

Apparently the stable labeling is related in some way to the unstable one; both are influenced by the cations and by g-strophanthin, and, as seen in Fig. 5 from the experiments with a low ATP concentration where Na^+ is increased, the decrease in stable labeling parallels an increase in the unstable one. This may suggest that it is the same enzyme which catalyzes two different kinds of labeling. However, as suggested by RODNIGHT *et al.*[15], it may be two different enzymes which use the same substrate and catalyze two different labelings; thus, the relationship between the two labelings must be apparent and due to different rate constants for the two enzymes.

Exchange reaction and labeling

In the ADP–ATP exchange experiments where 3 mM Mg^{2+} was equal to the ATP concentration, the highest exchange was found with Mg^{2+}; Na^+ decreased the exchange, and with $Mg^{2+} + Na^+$, K^+ gave a further decrease[1,3]. This is the same pattern as the cation effect on the stable labeling. But as the single rate constants in the process are not known, it is impossible to tell whether this means that the exchange reaction is related to the stable labeling. On the other hand, when the Mg^{2+} concentration compared to the ATP concentration is low, Na^+ increases the exchange reaction[3,4], suggesting that at least part of the exchange reaction is related to the unstable labeling.

High and low ATP concentration

With 3 mM ATP the P_i incorporation from [^{32}P]ATP is higher than with 25 μM, but the relative effect of Mg^{2+}, $Mg^{2+} + Na^+ + K^+$, and $Mg^{2+} + Na^+ + K^+$ + g-strophanthin is about the same at both concentrations. The relative effect of Na^+ differs, however, at the two concentrations, and the effect of Na^+ relative to the effect of the other cations also differs. With 25 μM ATP, Na^+ increases the initial rate in concentrations up to 120 mM; with 3 mM ATP, the maximum effect of Na^+ is obtained with a lower Na^+ concentration, whereas at 120 mM Na^+, the rate again decreases (*cf.* Figs. 5 and 7). At the low ATP concentration, it is obvious that Na^+ increases the unstable labeling and that a large proportion of the labeling is unstable. At the high ATP concentration, the proportion of unstable labeling with $Mg^{2+} + Na^+$ is much lower (*cf.* Figs. 1, 2 and 7), and it is difficult to tell if Na^+ increases the unstable labeling; this is mainly because no experiments exist which show how much of the labeling with Mg^{2+} is unstable. A problem in this connection is whether part of the labeling found with Mg^{2+} is due to Na^+ bound to the enzyme. 120 mM K^+ decreases the labeling with Mg^{2+}, which could be due to a displacement of Na^+. However, 10 mM K^+ should also be able to displace Na^+ in the amount which can come from the enzyme preparation (*cf.* Fig. 10). As 10 mM K^+ has no effect on the labeling with Mg^{2+}, it seems unlikely that a larger part of the labeling found with Mg^{2+} is due to Na^+ contamination.

What is the significance of the influence of the ATP concentration on the Na^+ effect on unstable labeling? Is there a difference in the way ATP is hydrolyzed with $Mg^{2+} + Na^+$ at low and high ATP concentrations? Or is the ATP effect only apparent? A difficulty is that it is not known if there are one or two enzymes, a Mg^{2+}-activated and ($Mg^{2+} + Na^+ + K^+$)-activated, which catalyze the labeling and which may differ in their affinities for ATP. The labeling due to the one may, at a high ATP concentration, more or less cover the labeling due to the other.

Relationship between labeling and enzyme activity

The similarity between the effects of monovalent cations and of g-strophanthin on the ATP hydrolysis and on the unstable labeling suggests, as previously pointed out[2,5–17], that the same enzyme system is responsible for both. This is corroborated by the observation that an increase in the ($Na^+ + K^+$)-activation of the enzyme simultaneously increases the effects of Na^+ and of $Na^+ + K^+$ on the labeling. There is thus reasonably good evidence that the phosphorylation followed by a dephosphorylation found with $Mg^{2+} + Na^+$ in the medium is catalyzed by the ($Na^+ + K^+$)-activated enzyme system.

With $Mg^{2+} + Na^+ + K^+$ in the medium, the labeling is low and consists mainly of the stable form. When K^+ is added to a particle which has been prelabeled with $Mg^{2+} + Na^+$ in the medium, the rate of dephosphorylation increases[7,11] (*cf.* Figs. 11 and 12); Mg^{2+} is required for this effect[11]. This suggests that the effect of K^+ is to increase the rate of dephosphorylation, and this has led to the conclusion that the hydrolysis of ATP is a two-step reaction in the sense that there is a Na^+-dependent phosphorylation of a compound in the microsomal particle followed by a K^+-dependent dephosphorylation[5,7,11].

The enzyme system has two sites with affinities for monovalent cations. A "Na^+ site" which, in the cell membrane, faces the inside of the cell and has a higher affinity for Na^+ than for K^+, and a "K^+ site", which faces the outside of the cell and has a

higher affinity for K^+ than for Na^+ (for literature, see ref. 12). It may be two different sites or the same site in two different forms.

The scheme of the Na^+-dependent phosphorylation followed by the K^+-dependent dephosphorylation is based on the assumption that it is an effect of Na^+ at the "Na^+ site" which leads to the phosphorylation and an effect of K^+ at the "K^+ site" which leads to the dephosphorylation. With Na^+ but no K^+ in the medium, there is Na^+ at both the "Na^+ site" and the "K^+ site". The possibility exists that under these conditions the ATP hydrolysis leads to phosphorylation not because there is Na^+ at the "Na^+ site" but because there isNa^+ and not K^+ at the "K^+ site". Na^+ at the "Na^+ site" may be required for the effect, or if it is the same site in two different forms, the hydrolysis may lead to a phosphorylation because Na^+ is exchanged for Na^+ and not for K^+ when the Na^+ form is converted into the K^+ form; the conversion must then be due to an effect of ATP as such and not to a phosphorylation (see later).

If this view is correct, it means that the displacement of Na^+ by K^+ from the "K^+site" decreases the phosphorylation and that the low labeling found with $Na^+ + K^+$ in the medium is due to a decreased phosphorylation and not to an increase in the rate of dephosphorylation. The increased rate of dephosphorylation by K^+ of a prephosphorylated particle may be an indication that a bond between a phosphate and the system cannot exist when the system is in the Na^+–K^+ form, *i.e.* cannot be formed when the bond between the γ- and β-phosphates of ATP is hydrolyzed (*cf.* that the labeling from $^{32}P_i$ is lower with K^+ in the medium).

According to this view, phosphorylation is due to an ATP hydrolysis found when the system is in the Na^+–Na^+ form, *i.e.* a form in which it give a Na^+–Na^+ exchange[27]. In the Na^+–K^+ form in which the system gives a Na^+ efflux coupled to a K^+ influx, ATP is hydrolyzed in a slightly different way which does not lead to phosphorylation. In the cell membrane and also in the test tube, the transport system exists both in the Na^+–Na^+ and Na^+–K^+ forms, and the ratio of these forms depends on the ratio between the Na^+ and K^+ concentrations in the solutions in contact with the two sides of the system. This may explain that a small amount of unstable labeling is found in the experiments with 100 mM Na^+ + 20 mM K^+.

There are no experiments to determine whether the effect of K^+ is to increase the rate of dephosphorylation or to decrease phosphorylation. The correlation found between the rate of phosphorylation with $Mg^{2+} + Na^+$ and the rate of hydrolysis with $Mg^{2+} + Na^+ + K^+$ supports the view that phosphorylation is part of the hydrolysis with $Mg^{2+} + Na^+ + K^+$ (refs. 7, 11). It is, however, easy to set up a scheme for the hydrolysis of ATP by the enzyme system which leads to phosphorylation with $Mg^{2+} + Na^+$ and to no phosphorylation with $Mg^{2+} + Na^+ + K^+$, and in which the hydrolysis of the bond between the γ- and β-phosphates of ATP is rate-limiting for the phosphorylation found with $Mg^{2+} + Na^+$ and for the hydrolysis with $Mg^{2+} + Na^+ + K^+$. The same rate of hydrolysis of this bond in both cases will give such a correlation although there is no phosphorylation with $Mg^{2+} + Na^+ + K^+$, and although the rate of hydrolysis with $Mg^{2+} + Na^+$ is lower than with $Mg^{2+} + Na^+ + K^+$.

K^+ at 0° decreases the labeling without affecting the hydrolysis. This has been taken to indicate that the effect of K^+ cannot be to increase the rate of dephosphorylation; K^+ must decrease the phosphorylation[12]. Another explanation may be that there is a phosphorylation with $Mg^{2+} + Na^+ + K^+$ in the medium, that K^+ increases the rate of dephosphorylation, but that there is a step which proceeds the phosphory-

lation (*e.g.* a change in configuration) which is rate-limiting at 0° and the rate of which is the same with $Mg^{2+} + Na^+$ and with $Mg^{2+} + Na^+ + K^+$ in the medium.

g-Strophanthin increases the labeling with $Mg^{2+} + Na^+ + K^+$, but it is not possible from the present experiments to tell whether there is an increase in the unstable labeling or only in the stable labeling. But even if the increased labeling in part is due to an increased unstable labeling, it does not reach the level achieved with $Mg^{2+} + Na^+$, implying that the effect of g-strophanthin cannot be due solely to a decrease in the dephosphorylation rate. With $Mg^{2+} + Na^+$, g-strophanthin decreases both the unstable labeling and the hydrolysis, showing that g-strophanthin, either decreases the phosphorylation or decreases it more than the dephosphorylation (*cf.* ref. 7).

The effect of g-strophanthin requires Mg^{2+} and is increased by ATP and Na^+ (ref. 28 and J. C. SKOU, unpublished observations); g-strophanthin is effective only when applied to the outside of the membrane[29]. If there is only one site in two forms, it is reasonable to assume that g-strophanthin reacts with the enzyme system only when the Na^+ form has been converted to the K^+ form. The decrease in phosphorylation by g-strophanthin then suggests that it cannot be the phosphorylation which leads to the transformation of the Na^+ form to the K^+ form; the phosphorylation must follow the transformation. This may support the hypothesis that it is because there is Na^+ at the "K^+ site" that the hydrolysis leads to a phosphorylation.

At present it thus does not seem possible to decide whether the phosphorylation found with $Mg^{2+} + Na^+$ and with no K^+ in the medium is part of the hydrolysis with $Mg^{2+} + Na^+ + K^+$, or whether the phosphorylation is due to a slightly different ATP hydrolysis found under conditions where the transport of Na^+ is not coupled with a transport of K^+. This problem, as well as the problems about the relationships between unstable and stable labeling and between the results from the labeling and the exchange experiments, must be solved before a scheme for the intermediary steps in the ATP hydrolysis by the enzyme system can be constructed.

ACKNOWLEDGMENTS

This investigation was supported by grants from the National Institute of Health (NB 3253-01-04), Den Danske Stats Almindelige Videnskabsfond and Aarhus Universitets Forskningsfond.

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