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THE EFFECT OF MAGNESIUM, ATP, P_i, AND SODIUM ON THE INHIBITION OF THE ($Na^+ + K^+$)-ACTIVATED ENZYME SYSTEM BY g-STROPHANTHIN

J. C. SKOU, K. W. BUTLER* AND O. HANSEN
Institute of Physiology, University of Aarhus, Aarhus (Denmark)
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SUMMARY

The influence of magnesium, sodium, ATP, and P_i on the reaction of the ($Na^+ + K^+$)-activated enzyme system with g-strophanthin has been investigated.

Magnesium is required for the reaction, but with magnesium alone the rate of reaction with g-strophanthin is very low.

With magnesium, ATP has an effect both on the rate and on the steady-state level of inhibition. With ATP in concentrations which are low relative to the concentration of magnesium, it increases the rate of inhibition. With a non-limiting concentration of magnesium, the concentration of ATP for half-maximum inhibition by 1 μM g-strophanthin is about 1 μM. When the ATP concentration is increased above the magnesium concentration, *i.e.* with decreasing concentrations of uncomplexed magnesium (Mg^{2+}), both the rate and the steady-state level of inhibition is decreased.

With magnesium, P_i increases the rate of inhibition just as ATP, but the concentration of P_i to obtain a certain inhibition by 1 μM g-strophanthin is higher than the concentration of ATP. The maximum rate of inhibition obtained with magnesium and P_i is higher than with magnesium and ATP. P_i in concentrations higher than the concentration of magnesium (3 mM with 2.5 mM magnesium and 2 mM EDTA) did not decrease the rate and the steady-state level of inhibition as did ATP.

With ATP, sodium decreases the requirement for magnesium for the inhibition. With 1 μM g-strophanthin the concentration of Mg^{2+} for 50 % inhibition and the $MgATP/ATP_f \times K$ ratio is decreased by a factor of 10^3–10^4 by an increase in the sodium concentration from 0 to 10 mM (Mg^{2+} decreased from 4 μM to 0.7 nM). Sodium increases the rate of inhibition and with sub-optimal concentrations of magnesium it also increases the steady-state level of inhibition. The maximal rate of inhibition with magnesium, ATP, and sodium is faster than with magnesium and P_i, and also than with magnesium and ATP; but even under optimal conditions for the reaction the rate of inhibition with 1 μM g-strophanthin is a relatively slow process.

Without ATP and with or without P_i, sodium has the opposite effect. It decreases the requirement for Mg^{2+} for a given inhibition. With 1.5 mM P_i, the concentration of Mg^{2+} necessary for 50 % inhibition by 1 μM g-strophanthin is increased from 5 μM to 3 mM when the sodium concentration is increased from 0 to 50 mM.

* Present address: National Research Council of Canada, 100 Sussex Drive Ottawa 7, Canada.

The concentration of g-strophanthin to give 50 % inhibition under steady-state conditions with 3 mM ATP and 100 mM sodium decreases when the Mg^{2+} concentration is increased and goes towards a minimum value of 10 nM. With non-limiting concentrations of magnesium and P_i for inhibition, the value was 11 nM and with magnesium, sodium and P_i 440 nM. The results suggest that with ATP, sodium increases the affinity for Mg^{2+} while with P_i, sodium decreases the affinity for g-strophanthin.

INTRODUCTION

g-Strophanthin inhibits the $(Na^+ + K^+)$-activated enzyme system; this has been one of the arguments that the system is involved in the active transport of sodium and potassium (for literature see ref. 1). The inhibitory effect can partly be prevented by an increase in the concentration of potassium (ref. 2). This is not due to a direct competition between g-strophanthin and potassium and seems to depend not only on the concentration of potassium, but also on the ratio between the concentrations of sodium and potassium in the external solution[3]. The rate of reaction of the enzyme system with g-strophanthin is markedly influenced by magnesium, ATP, sodium and potassium in the medium[4–8] and also by P_i (refs. 5 and 8). The effect of magnesium ATP, P_i, sodium and potassium on the rate of inhibition seems to be parallel with their effect on the binding of the cardiac glycosides to the enzyme system[4,5,8].

Thus, the inhibitory effect of g-strophanthin depends on an effect of the substrate on the system and of the parameters which activate the reaction of the system with the substrate. This suggests that it may be possible to obtain information on the effect of these parameters on some of the steps in the reaction from an investigation of the conditions under which g-strophanthin inhibits. For this it is necessary to have detailed information on the effect of the cations, ATP, P_i and ADP on the inhibition by g-strophanthin. In the present paper are given the results of experiments with magnesium, sodium, ATP and P_i. The results of experiments with potassium will be given in a following paper. Some of the results have been given in a preliminary form[6].

MATERIAL AND METHODS

The enzyme was prepared from beef brain[9]. The specific activity was 160–220 μM P_i per mg protein per h (Mg^{2+} 3 mM; ATP, 3 mM; Na^+, 120 mM; K^+, 30 mM, pH 7.4, 37°). In the text and in the figures Mg is used for total magnesium content, ATP for total ATP content, while Mg^{2+} and ATP_f are used for non-complexed magnesium and ATP, respectively. The g-strophanthin-sensitive/g-strophanthin-insensitive activity ratio was between 8 and 12.

In the pre-incubation experiments 100–150 μg of enzyme protein was pre-incubated with g-strophanthin and with varying concentrations of cations, with and without ATP and/or EDTA in 30 mM Tris–HCl buffer (pH 7.4) 37° in a volume of 8.0 or 9.7 ml. After the end of pre-incubation, ATP was added to a final concentration of 3 mM, sodium to 120 mM, potassium to 30 mM, and magnesium to the sum of the ATP and EDTA concentrations, in a final volume of 10 ml. The 8.0 ml volume was used in the experiments where the cations and ATP added after the end of pre-incubation could not be contained in 0.3 ml; g-strophanthin was also added to obtain the same

concentration in the final 10 ml as during pre-incubation. The added solution was pre-heated to 37°. From the time of addition the activity of the enzyme was measured by sampling 1 ml of the 10 ml at different times for the next 16 min; the first sample was taken after 30 sec of reaction. The reaction was stopped by the addition of the 1-ml sample to 0.1 ml 50 % trichloroacetic acid and the P_i was measured by the method of FISKE AND SUBBAROW[10], with the modification that amidole was used as reducing agent.

The test was carried out on the same concentration of g-strophanthin as during pre-incubation in order to be able to run the pre-incubation with a low concentration of enzyme so as to maintain the hydrolysis of ATP during pre-incubation at a low level and furthermore, in order to minimize the effect of reversibility of the g-strophanthin effect on the slopes of the curves. The control reaction for the effect of g-strophanthin on the enzyme during pre-incubation was to use enzyme pre-incubated under the same conditions without g-strophanthin in the pre-incubation medium, but with g-strophanthin added with ATP, magnesium, EDTA, sodium and potassium at zero time for the measurement of activity.

In order to decrease the P_i background in the test in the experiments with P_i instead of ATP, the enzyme was pre-incubated in a smaller volume, 2–5 ml dependent on the P_i concentration; after the end of preincubation the volume was increased to 10 ml as given above. The control reaction consisted of enzyme pre-incubated under the same conditions without g-strophanthin, but tested with g-strophanthin at the same concentration as used in the pre-incubation experiments.

The pyruvate kinase was obtained from Boehringer. The $(NH_4)_2SO_4$ added with the kinase did not influence the activity of the enzyme under the conditions used for the test. For the calculation of Mg^{2+} a stability constant of $MgEDTA^{2-} = 10^{-9}\ M^{-1}$ was used as well as a dissociation constant of $EDTA^{4-} = 10^{-10}$ M (ref. 11), and a stability constant of $MgATP^{2-} = 10^{-4}\ M^{-1}$ (ref. 12).

The data presented are representative of the results obtained and are based on three to five experiments of each type.

RESULTS

Fig. 1 shows the hydrolysis of ATP as a function of time with and without added 1 μM g-strophanthin. The test solution contained 5 mM magnesium, 2 mM EDTA, 120 mM sodium, 30 mM potassium and 3 mM ATP. For comparison is shown the hydrolysis without sodium, potassium and g-strophanthin. The reaction was started by the addition of enzyme at zero time for the measurement of activity, which means that the enzyme in these experiments was not pre-incubated with g-strophanthin. The figure also shows the result of parallel experiments where 1 mM phosphoenol pyruvate and pyruvate kinase have been added in order to maintain the concentration of ATP constant and that of ADP constant at a low level during the test.

It is seen that the slope of the curve for the reaction with sodium *plus* potassium with g-strophanthin is lower and decreases more with time than the slope of the curve for the reaction with sodium *plus* potassium without g-strophanthin. With added phosphoenol pyruvate and pyruvate kinase, the rate by which the slope of the curves decreases is slightly lower both with and without g-strophanthin in the medium, but the relative effect of g-strophanthin is the same as without phosphoenol pyruvate and kinase.

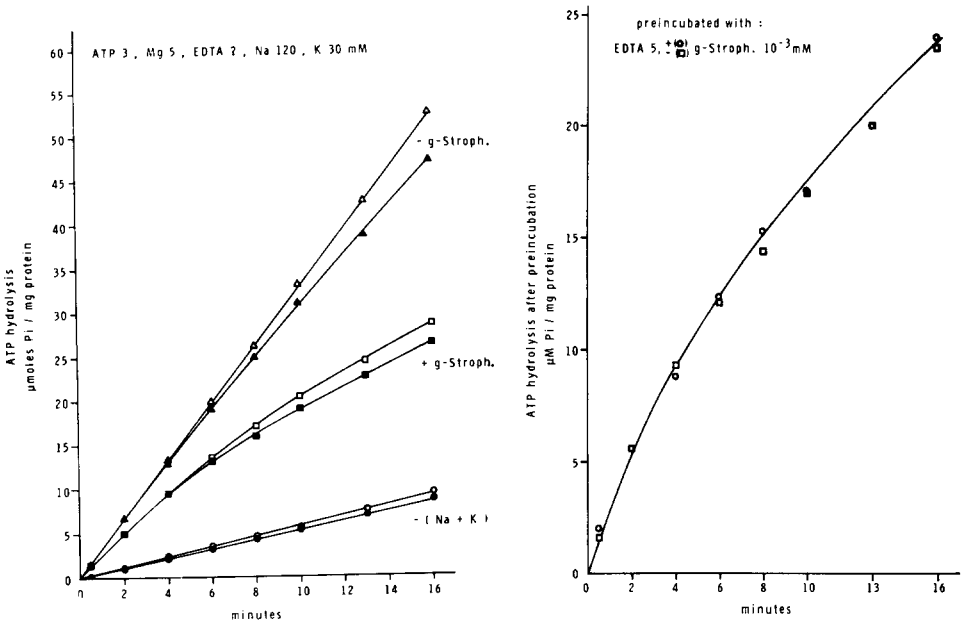

Fig. 1. The effect of 1 μM g-strophanthin on the hydrolysis of ATP by the ($Na^+ + K^+$)-activated enzyme system. For comparison is shown the hydrolysis without g-strophanthin with and without sodium *plus* potassium. In the experiments with sodium *plus* potassium and with g-strophanthin the solution contained 5 mM magnesium, 3 mM ATP, 2 mM EDTA, 120 mM sodium, 30 mM potassium and 30 mM Tris–HCl buffer (pH 7.4). Without sodium *plus* potassium the composition was the same except that sodium and potassium was omitted. In the figures represented by the open symbols the solution also contained 1 mM phosphoenol pyruvate and pyruvate kinase. Temp. 37°.

Fig. 2. The hydrolysis of ATP as a function of time after pre-incubation of the enzyme system for 20 min at 37° in 30 mM Tris–HCl buffer (pH 7.4) with 5 mM EDTA without and with 1 μM g-strophanthin. The activity in this and the experiments shown in the following figures was tested in a solution containing 30 mM Tris–HCl (pH 7.4), 3 mM ATP, 120 mM sodium, 30 mM potassium, 1 μM g-strophanthin and magnesium in a concentration which equals the concentration of ATP *plus* EDTA, temp. 37° (see MATERIALS AND METHOD).

From the experiment shown in Fig. 1 it is seen that the rate by which g-strophanthin inhibits is low; it takes 16 min or more to reach a steady-state. It is seen furthermore that the increase in the inhibition by g-strophanthin with time is not due to an increase in the ADP concentration or to a decrease in the ATP concentration during the hydrolysis of ATP. An effect of the increase in the P_i concentration, however, cannot be excluded from this experiment (see below).

The effect of magnesium and ATP

In Fig. 2 is shown the hydrolysis of ATP as a function of time by enzyme which has been pre-incubated for 20 min at 37° in a 30 mM Tris buffer (pH 7.4), with 5 mM EDTA, with and without 1 μM g-strophanthin. EDTA was added to complex the small amount of Mg^{2+} which contaminates the enzyme preparation. The concentrations given on Fig. 2 and the following figures refer to the composition of the pre-incubation medium. The activity was in this and the following experiments tested in a solution which contained 120 mM sodium, 30 mM potassium, 3 mM ATP, magnesium equal to

EDTA + ATP, and apart from Fig. 19, 1 μM g-strophanthin (see MATERIALS AND METHODS).

It is seen from Fig. 2 that the activity is the same whether or not there has been g-strophanthin in the pre-incubation medium. The same result is obtained when 3 mM ATP or P_i was added to the pre-incubation medium (not shown). In view of the results shown in Fig. 1, the observation that the activity of the enzyme is the same whether it has been pre-incubated with or without g-strophanthin indicates that the enzyme has not reacted with g-strophanthin during the pre-incubation.

With magnesium in the pre-incubation medium the enzyme system reacts with g-strophanthin. This is seen from Fig. 3, which shows that the slopes of the curves for the hydrolysis of ATP as a function of time after the pre-incubation is lower for the enzyme system which has been pre-incubated with magnesium and g-strophanthin than without g-strophanthin. The figure also shows that pre-incubation with magnesium and without g-strophanthin leads to a decrease in the activity, but not to the same extent as with g-strophanthin. The reaction with g-strophanthin increases slowly with the time of pre-incubation.

With magnesium in the pre-incubation medium the addition of ATP has two effects. One is to prevent the decrease in activity during pre-incubation with magnesium but without g-strophanthin. The other is to increase the rate by which g-strophanthin reacts with the enzyme system; this is seen from a comparison between Figs. 3 and 4, which shows that the time taken of pre-incubation to give a certain decrease in the initial slope is lower with ATP than without.

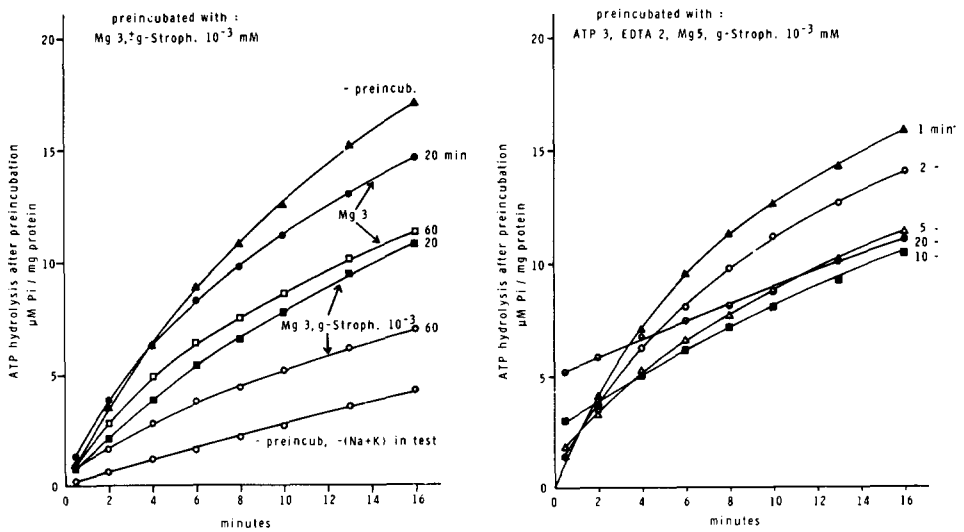

Fig. 3. The effect of magnesium on inhibition by g-strophanthin. The enzyme was pre-incubated with 3 mM of magnesium with and without 1 μM g-strophanthin for 20 and 60 min. The pre-incubation medium contained as in Fig. 2 and as in the experiments shown on the following figures: 30 mM Tris–HCl buffer (pH 7.4) and the temperature was 37°. In the figure is shown the hydrolysis of ATP as a function of time by the enzyme system after the pre-incubation.

Fig. 4. The activity of the enzyme after varying times of pre-incubation with 3 mM ATP, 5 mM magnesium, 2 mM EDTA and 1 μM g-strophanthin. The pre-incubation time is shown in the figure. The enzyme activity was tested as in Fig. 2.

There is a certain hydrolysis of ATP during the pre-incubation. The activity of the enzyme preparation with magnesium and without sodium *plus* potassium is, however, relatively low and as the pre-incubation is performed with an enzyme concentration which is only slightly higher than the concentration during the test, the hydrolysis of ATP is relatively low even after 20 min of pre-incubation. This is seen from Fig. 4 where the P_i concentration after 20 min of pre-incubation has increased to 0.05 mM (5 μM per mg protein on the figure; the concentration of protein was 10 μg/ml). This means that the ATP concentration during the pre-incubation has decreased from 3 to 2.95 mM and the P_i and ADP concentrations have increased to 0.05 mM. These low concentrations of ADP and P_i did not give a measurable effect on the rate of hydrolysis under the conditions used for the test.

The pyruvate kinase requires monovalent cations for activation and as the cations have an effect on the inhibition by g-strophanthin (see below), phosphoenol pyruvate and pyruvate kinase cannot be used to regenerate ATP during the pre-incubation.

With magnesium, the concentration of ATP necessary to give an effect on the inhibition by g-strophanthin is very low (Fig. 5). (For convenience the values in Fig. 5 and in the following figures are corrected for the hydrolysis during pre-incubation.) After 20 min of pre-incubation with 3 mM magnesium, 2 mM EDTA and 1 μM g-stro-

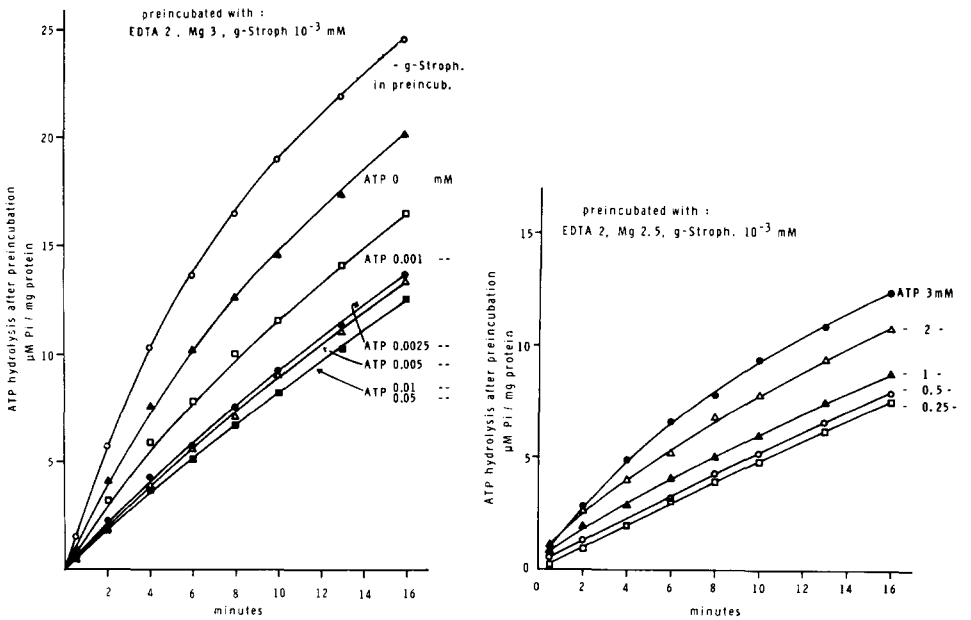

Fig. 5. The effect of varying concentrations of ATP on the inhibition by g-strophanthin. The enzyme was pre-incubated for 20 min with 3 mM magnesium, 2 mM EDTA, 1 μM g-strophanthin and a varying concentration of ATP (pH 7.4), 37°. The control is the enzyme which has been pre-incubated for 20 min with 3 mM magnesium, 2 mM EDTA, and 1 mM ATP without g-strophanthin and with 1 μM g-strophanthin but without ATP. The enzyme activity was tested as in Fig. 2.

Fig. 6. The effect of varying concentrations of ATP on the inhibition by g-strophanthin. The enzyme was pre-incubated for 20 min with 2.5 mM magnesium, 2 mM EDTA, 1 μM g-strophanthin and concentrations of ATP varying from 3 to 0.25 mM (pH 7.4), 37°. The activity was tested as in Fig. 2.

phanthin, the maximum effect on the activity is obtained with 10 μM ATP added to the pre-incubation medium; the concentration of ATP necessary to give a half-maximum decrease of the initial slope of the curves is about 1 μM. Considering that there is a certain hydrolysis of ATP during the preincubation, the concentration of ATP to give a half-maximum effect must be lower than the 1 μM added (for the effect of the released P_i, seen below).

The effect of magnesium *plus* ATP on the inhibition by g-strophanthin depends on their ratio; this is seen from a comparison between Figs. 6 and 7. When pre-incubated for 20 min with 2.5 mM magnesium, 2 mM EDTA, and 1 μM g-strophanthin (Fig. 6) the inhibition increases when the ATP concentration in the pre-incubation medium is decreased from 3 to 0.5 mM; with a 0.25 mM concentration, the same inhibition is obtained as with a 0.5 mM concentration. On the other hand, if the ATP concentration in the pre-incubation medium is kept constant at 3 mM and the concentration of magnesium is increased above the 2.5 mM used in Fig. 6, the inhibition increases with the magnesium concentration up to 5 mM (EDTA, 2 mM) (Fig. 7).

In Fig. 8 is shown the percent inhibition after varying times of pre-incubation with 1 μM g-strophanthin, 3 mM ATP, 2 mM EDTA, and varying concentrations of magnesium. After pre-incubation the hydrolysis of ATP as a function of time has been tested as in the previous experiments. The initial slopes of the curves have been used

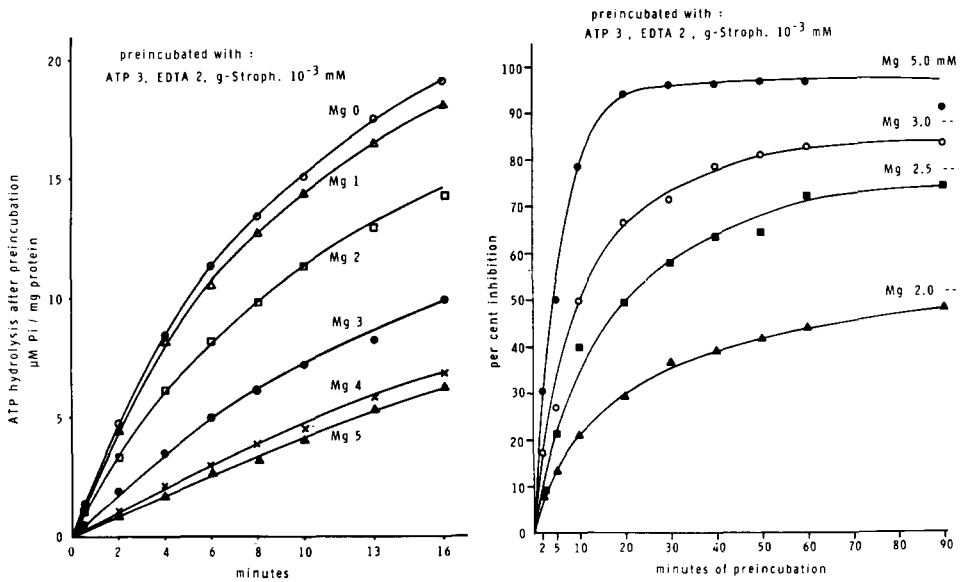

Fig. 7. The effect of varying concentrations of magnesium on the inhibition by g-strophanthin. The enzyme system was pre-incubated for 20 min with 3 mM ATP, 2 mM EDTA, 1 μM g-strophanthin and magnesium in concentrations varying from 0 to 5 mM (pH 7.4), 37°. The activity was tested as in Fig. 2.

Fig. 8. The effect of varying concentrations of magnesium on the inhibition by g-strophanthin. The enzyme system was pre-incubated as shown on the abscissa with 3 mM ATP, 2 mM EDTA, 1 μM g-strophanthin and varying concentrations of magnesium (pH 7.4), 37°. The activity was tested as in Fig. 2 and the initial slopes of the curves for the hydrolysis as a function of time have been used to express the activity after pre-incubation. The inhibition is given in percent of the activity of control *minus* the activity with magnesium without sodium *plus* potassium.

to express the activity of the enzyme system after pre-incubation and after correction for the magnesium-activated activity the percentage inhibition of the $(Na^+ + K^+)$-activated activity during the pre-incubation has been calculated. The control reaction was the enzyme pre-incubated without g-strophanthin, but tested with g-strophanthin.

The inhibition has not reached a steady-state level after 90 min of pre-incubation with lower magnesium concentrations, but it seems as if it approaches a steady-state level, which is lower the lower the magnesium concentration. This means that the values obtained after 20 min of pre-incubation (Figs. 6 and 7) are not steady-state values.

After 90 min of pre-incubation the concentration of Mg^{2+} for half-maximum inhibition with 1 μM g-strophanthin is of the order of 4 μM (that of ATP_f of 2885 μM and of MgATP 115 μM).

The experiments shown in Figs. 6, 7, and 8 either suggest that Mg^{2+} is necessary for the effect of g-strophanthin, or that ATP_f inhibits an effect of MgATP.

ADP has an effect on the inhibition similar to the effect of ATP, but under identical conditions the effect was found to be lower. The enzyme preparation has an adenylate kinase activity which is about 5 % of the activity of the $(Na^+ + K^+)$-activated ATPase. It was possible by washing to remove about half of this activity, but not to remove it all. As we have not been able to exclude the possibility that the results found with ADP were due to the ATP formed during the pre-incubation, no experiments are reported with ADP as substrate.

The effect of P_i

Without magnesium, pre-incubation with P_i has no effect on the inhibition by g-strophanthin (see above). With magnesium, it increases the rate of reaction in the same manner as ATP. As seen from Fig. 9, the rate of reaction with non-limiting concentrations of magnesium is faster with 1.5 mM P_i than with 3 mM ATP, but not as fast as with 3 mM ATP and 100 mM sodium (see below).

The concentration of P_i which is necessary to obtain a given effect is higher than the concentration of ATP. With 2.5 mM magnesium and 2 mM EDTA, a half-maximum effect is obtained with a P_i concentration of the order of 50 μM. while a maximum effect is obtained with 250 μM (Fig. 10, *cf.* Fig. 5). P_i in high concentrations up to 3 mM does not lead to a decrease in the inhibition, as in the case with ATP (*cf.* Fig. 5).

About 3 mM magnesium concentration is required to give a maximum effect with 3 mM P_i, 2 mM EDTA, and 1 μM g-strophanthin (Fig. 11). The concentration of Mg^{2+} at half-maximum inhibition is about 5 μM.

As shown in Fig. 9, the rate of inhibition with 1.5 mM P_i was higher than with 3 mM ATP without sodium. The addition of 1.0 mM P_i to a pre-incubation medium with 3 mM ATP, 5 mM magnesium, and 1 μM g-strophanthin did not increase the rate of inhibition (Fig. 12). This seems to exclude that the effect of ATP is due to the P_i released by the magnesium-dependent hydrolysis and it shows, in agreement with the above given results for the half-maximum values of ATP aud P_i, that the affinity for ATP is higher than for P_i.

The effect of sodium

With magnesium in the pre-incubation medium, the addition of sodium prevents both the decrease in the activity found with and without g-strophanthin. The

concentration of sodium necessary to prevent the decrease in activity increases with the magnesium concentration. After 60 min of pre-incubation with 1 μM g-strophanthin and 3 mM magnesium, 18 mM sodium is needed to lower the inhibition to half of the inhibition found without sodium in the pre-incubation medium; with 1 mM magnesium, 8 mM sodium is needed (not shown).

Without magnesium, the addition of ATP to the pre-incubation medium did not lead to an inhibition by g-strophanthin (see above). If, however, sodium is added with ATP, there is an inhibition by g-strophanthin without added magnesium (Fig. 13). The pre-incubation medium contained 3 mM ATP, 120 mM sodium 1 μM g-strophanthin and varying concentrations of EDTA. It is seen that without EDTA added, there is an inhibition by g-strophanthin when the medium contains sodium *plus* ATP, and that the effect decreases when the EDTA concentration is increased. But even with 5 mM EDTA, the highest concentration used, the effect of g-strophanthin is not completely prevented.

There was no magnesium added to the pre-incubation medium in the experiment shown in Fig. 13, but a small amount of magnesium is bound to the enzyme preparation. The effect of EDTA may indicate that these small amounts of magnesium

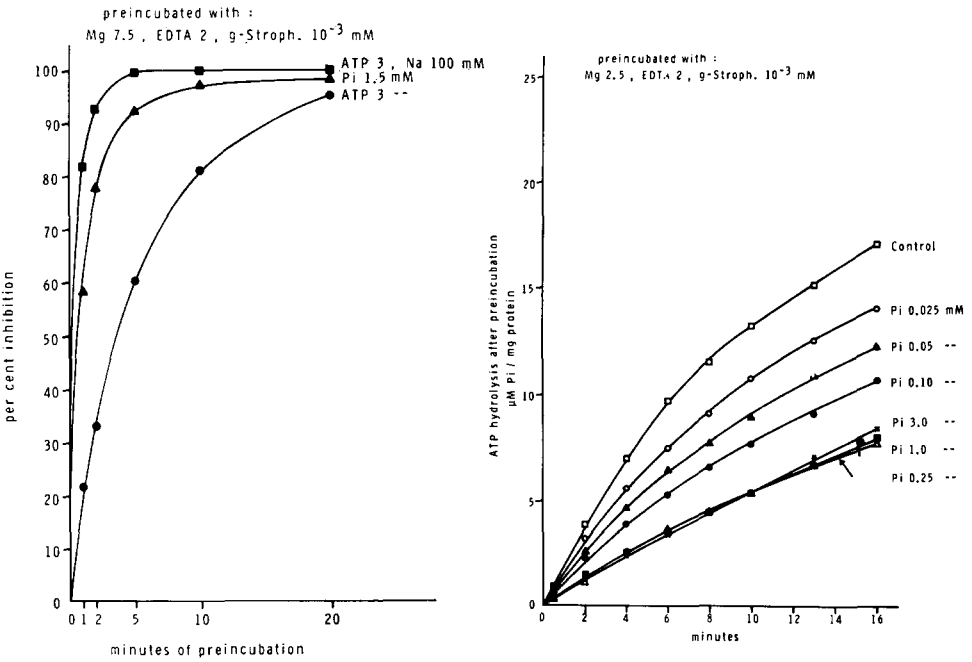

Fig. 9. The effect of ATP, ATP *plus* sodium and of P_i on the rate of inhibition by 1 μM g-strophanthin. The enzyme system was pre-incubated for the time shown on the abscissa. In all the experiments there was 7.5 mM magnesium, 2 mM EDTA, 1 μM g-strophanthin. Besides this there was in the one experiment 3 mM ATP, in the other 3 mM ATP and 100 mM sodium and in the third 1.5 mM P_i (pH 7.4), 37°. The activity was tested as in Fig. 2, and the percent inhibition was calculated as in Fig. 8.

Fig. 10. The effect of varying concentrations of P_i on the inhibition by g-strophanthin. The enzyme system was pre-incubated for 20 min with 2.5 mM magnesium, 2 mM EDTA, 1 μM g-strophanthin and P_i from 0 to 3 mM. The activity was tested as in Fig. 2.

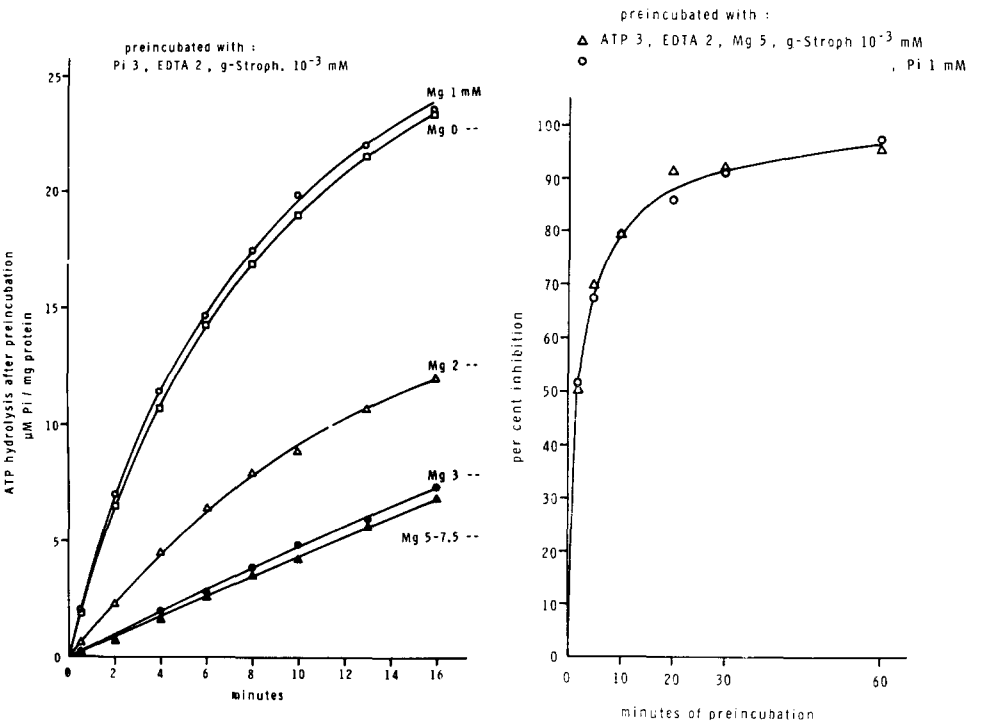

Fig. 11. The effect of varying concentrations of magnesium with 3 mM P_i on the inhibition by g-strophanthin. The enzyme system was pre-incubated for 20 min (pH 7.4), 37°, with 2 mM EDTA, 3 mM P_i, 1 μM g-strophanthin and magnesium in concentrations from 0 to 7.5 mM. The activity was tested as in Fig. 2.

Fig. 12. The effect of 1 mM P_i on the rate of inhibition by 1 μM g-strophanthin with 3 mM ATP, 2 mM EDTA, and 5 mM of magnesium. The activity was tested as in Fig. 2. △—△, preincubated with 3 mM ATP, 2 mM EDTA, 5 mM magnesium and 10^{-3} mM g-strophanthin; ○—○, preincubated with the same medium *plus* 1 mM P_i.

influence the inhibitory effect of g-strophanthin when the enzyme is incubated with sodium *plus* ATP in the medium. In agreement with this it is found that the concentrations of magnesium necessary to increase the inhibition are much smaller in experiments where the enzyme is pre-incubated with 3 mM ATP, 2 mM EDTA, 1 μM g-strophanthin, and 120 mM sodium than in a parallel experiment without sodium, (Fig. 14, *cf.* Fig. 7).

At a given magnesium concentration an increase in the sodium concentration increases the steady-state level of inhibition by g-strophanthin and also the rate by which it is obtained. This is seen from Fig. 15, where the magnesium concentration was 0.05 mM with 2 mM EDTA, 3 mM ATP, and 1 μM g-strophanthin in the pre-incubation medium, and where the sodium concentration was varied from 2 to 50 mM. It is seen that maximum effect is obtained by a concentration of sodium of the order of 50 mM, and that this gives an inhibition of about 70 % of the control activity.

In Fig. 16 is shown how an increase in the magnesium concentration at a given sodium concentration increases both the steady-state level and the rate of inhibition.

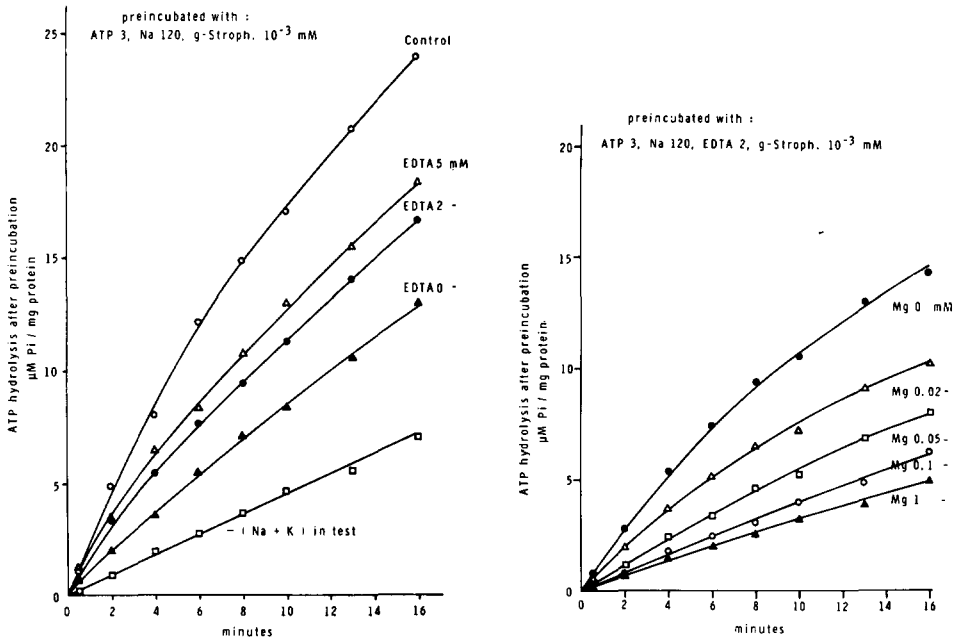

Fig. 13. The effect of ATP *plus* sodium on the inhibition by g-strophanthin with varying concentrations of EDTA. The enzyme system was pre-incubated for 20 min (pH 7.4), 37°, with 3 mM ATP, 120 mM sodium, 1 µM g-strophanthin and from 0 to 5 mM EDTA. The activity was tested as in Fig. 2.

Fig. 14. The effect of ATP *plus* sodium and varying concentrations of magnesium on the inhibition by 1 µM g-strophanthin. The enzyme system was pre-incubated for 20 min (pH 7.4), 37°, with 3 mM ATP, 120 mM sodium, 2 mM EDTA, 1 µM g-strophanthin, and from 0 to 1 mM magnesium. The activity was tested as in Fig. 2.

The concentration of sodium was 10 mM, with 2 mM EDTA, 3 mM ATP, and 1 µM g-strophanthin in the medium and varying concentrations of magnesium.

As seen from a comparison between Figs. 15 and 16, an increase in the sodium concentration at a low magnesium concentration cannot bring the inhibition to the same level of inhibition as is obtained by a further increase in the magnesium concentration. It is apparently the magnesium concentration which sets the upper limit for the effect of sodium. On the other hand, the degree of inhibition which can be obtained by an increase in the magnesium concentration is independent of sodium (*cf.* Figs. 16 and 8) but with sodium the concentration of magnesium necessary is lower than without. This indicates that sodium cannot substitute for magnesium in the effect on the inhibition by g-strophanthin; magnesium seems to be necessary for the effect of sodium.

In a series of experiments the concentration of magnesium for 50 % inhibition with 3 mM ATP, 2 mM EDTA, 1 µM g-strophanthin and varying concentrations of sodium was determined. The time of pre-incubation was 60 min, which according to Figs. 15 and 16 should give a steady-state value for the inhibition with sodium in the medium. The value for the magnesium concentration at 50 % inhibition without sodium has been calculated from the 90-min values in Fig. 8. The results are given in

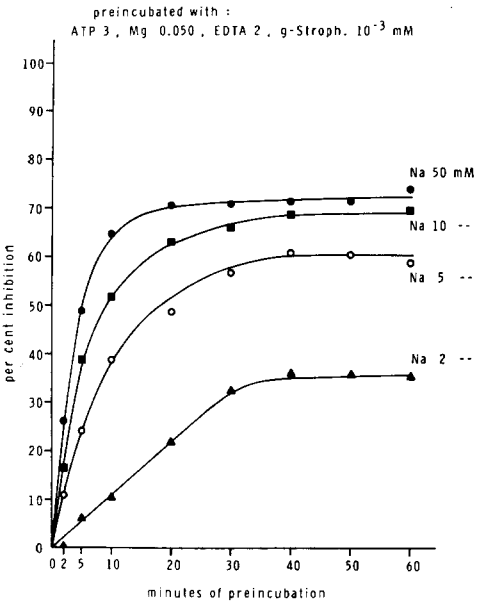

Fig. 15. The effect of varying concentrations of sodium on the inhibition by g-strophanthin after varying times of pre-incubation. The enzyme system was pre-incubated for the times shown on the abscissa (pH 7.4), 37°, with 3 mM ATP, 0.05 mM magnesium, 2 mM EDTA, and 1 μM g-strophanthin, and with 2, 5, 10, and 50 mM sodium, respectively. The activity after pre-incubation was tested as in Fig. 2 and the percent inhibition was calculated as in Fig. 8.

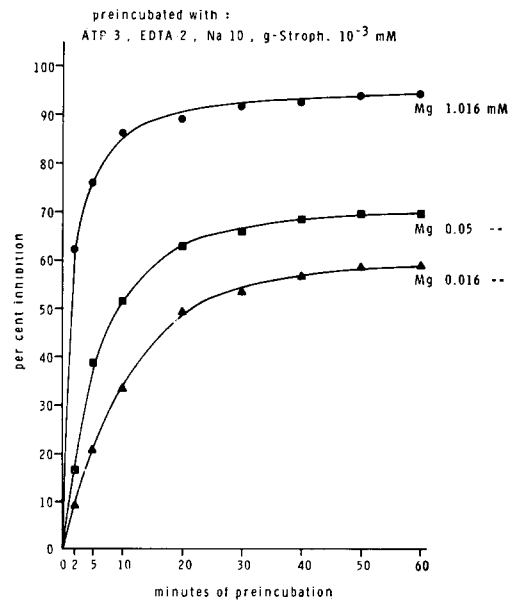

Fig. 16. The effect of varying concentrations of magnesium on the percent inhibition by g-strophanthin after varying times of pre-incubation. The enzyme system was pre-incubated for the times shown on the abscissa with 3 mM ATP, 2 mM EDTA, 10 mM sodium, 1 μM g-strophanthin and with 0.016, 0.05 and 1.016 mM magnesium respectively. The activity was tested as in Fig. 2, and the percent inhibition was calculated as in Fig. 8.

Fig. 17, and the concentrations of magnesium are given at the ordinate as the concentration of Mg^{2+}. As Mg^{2+} equals $MgATP/ATP_f \times K$, the ordinate also represents the $MgATP/ATP_f \times K$ ratio, It is seen that by increasing sodium from 0 to 10 mM, the concentration of Mg^{2+} for 50 % inhibition and the $MgATP/ATP_f \times K$ ratio is decreased by a factor of 10^3–10^4.

The concentration of ATP necessary to give an effect is very low with magnesium *plus* sodium in the pre-incubation medium. With 0.2 mM magnesium, 2 mM EDTA, 120 mM sodium and 1 μM g-strophanthin, the concentration which gives a maximum effect is of the order of added 2.5 μM ATP (Fig. 18). A half-maximum effect on the initial slope of the curve is obtained with a concentration of less than added 1 μM ATP. It is a lower concentration of ATP than that found to give half-maximum effect without sodium in the medium (*cf.* Fig. 5). It must, however, be emphasized that with the lower concentration of magnesium used in the experiments with sodium in the medium, the hydrolysis of ATP during pre-incubation is lower than in the experiments without sodium, shown in Fig. 5. The experiments are thus not comparable.

In Fig. 19 is shown how the concentration of g-strophanthin for half-maximum

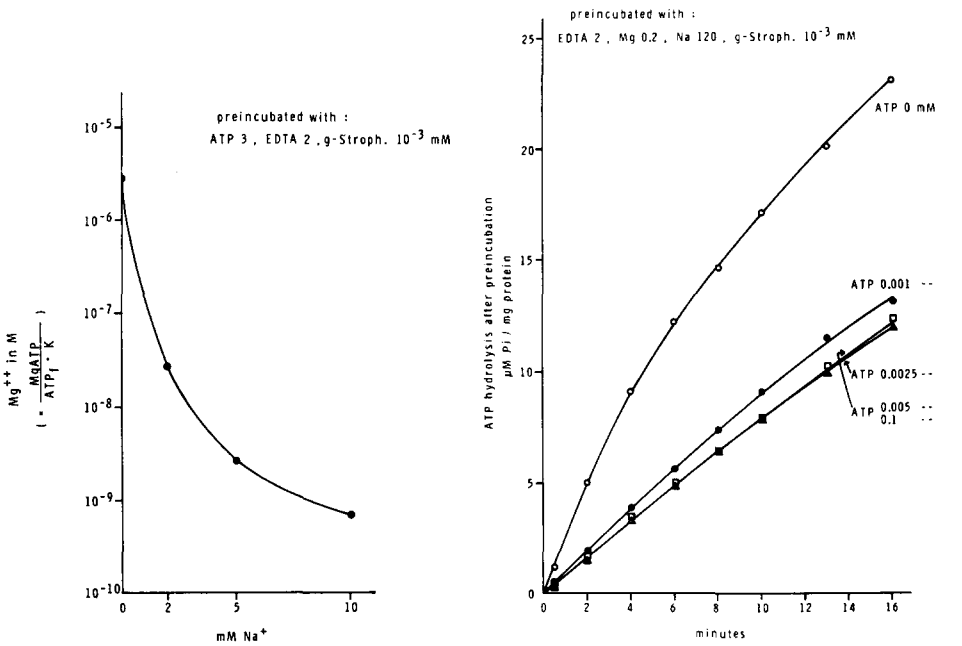

Fig. 17. The relationship between the concentrations of sodium and the concentrations of Mg^{2+} (or the $MgATP/(ATP_f \times K)$ ratio) which gives 50% inhibition of the enzyme system by 1 μM g-strophanthin. The enzyme system was pre-incubated for 60 min with 3 mM ATP, 2 mM EDTA, 1 μM g-strophanthin (pH 7.4), 37°, and with 2, 5, and 10 mM of sodium, respectively and for each sodium concentration with a number of magnesium concentrations. After pre-incubation, the enzyme activity was tested as in Fig. 2, and the percent inhibition calculated as in Fig. 8. From curves for the percent inhibition as a function of the magnesium concentration at each of the sodium concentrations, the magnesium values for 50 % inhibition were obtained and the concentration of Mg^{2+} was calculated.

Fig. 18. The activity of the enzyme system after pre-incubation for 20 min with 2 mM EDTA, 0.2 mM magnesium, 120 mM sodium, 1 μM g-strophanthin, and varying concentrations of ATP (pH 7.4), 37°. The activity was tested as in Fig. 2.

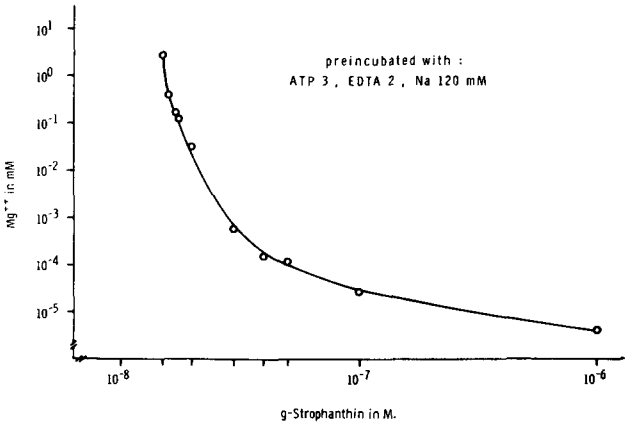

Fig. 19. The effect of the concentration of Mg^{2+} on the concentration of g-strophanthin to give 50% inhibition of the enzyme activity. The enzyme system was pre-incubated for 60 min with 3 mM ATP, 120 mM sodium, the concentrations of Mg^{2+} shown on the figure, and for each Mg^{2+} concentration varying concentrations of g-strophanthin. After the end of pre-incubation the activity was tested; the test solution contained the concentration of g-strophanthin used for the pre-incubation and the control was run with the same concentration of g-strophanthin. The percent inhibition was calculated as in Fig. 8. From the curves for the percent inhibition as a function of the g-strophanthin concentration for each Mg^{2+}, the values for 50% inhibition were determined.

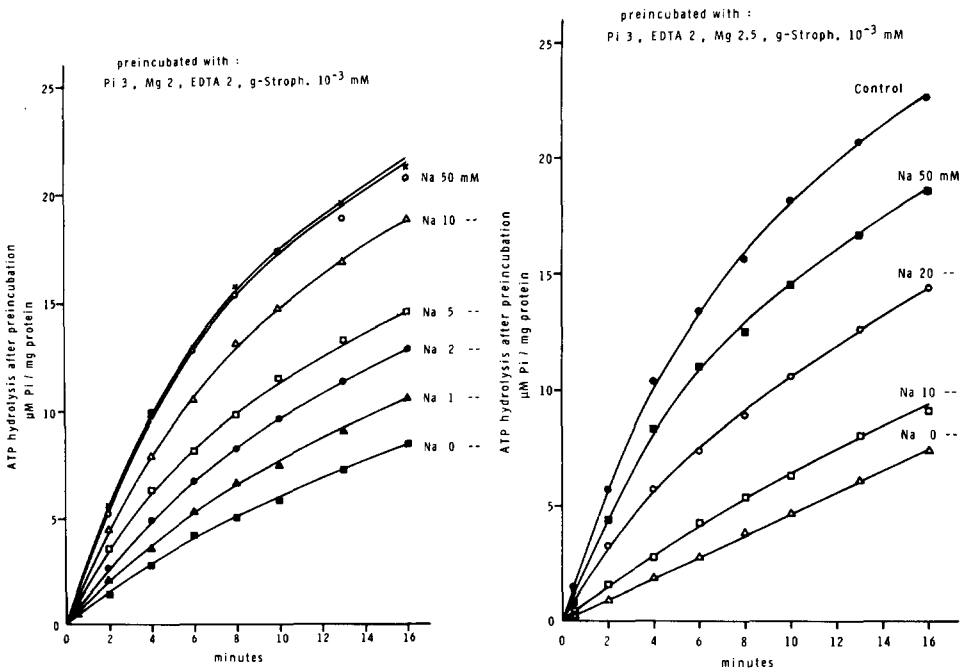

Fig. 20. The activity of the enzyme system after pre-incubation for 20 min with 3 mM P_i, 2 mM EDTA, 2.0 mM magnesium, 1 µM g-strophanthin and varying concentrations of sodium (pH 7.4), 37°. The activity was tested as in Fig. 2.

Fig. 21. The activity of the enzyme system after pre-incubation for 20 min with 3 mM P_i, 2 mM EDTA, 2.5 mM magnesium, 1 µM g-strophanthin and varying concentrations of sodium (pH 7.4), 37°. The activity was tested as in Fig. 2.

inhibition varies with the concentration of Mg^{2+} with 3 mM ATP and 100 mM sodium. The pre-incubation time was 60 min, which was found to give steady-state values. The g-strophanthin concentration decreases as the Mg^{2+} concentration increases and it approaches a minimum value of 15 nM when the Mg^{2+} concentration is increased. The figure gives the added g-strophanthin concentrations. The enzyme system, however, binds g-strophanthin, which means that the equilibrium concentration is lower than the added concentration of g-strophanthin. The concentration of g-strophanthin binding sites in the pre-incubation medium was 10 pM; this was calculated from the specific activity of the enzyme preparation used and a molecular activity of 9500 (ref. 13). At 50 % inhibition half of the sites have bound g-strophanthin, and the concentration on the figure must be corrected for the amount bound. This means that the minimum value of g-strophanthin for 50 % inhibition found with the high concentration of Mg^{2+} is 10 pM.

The effect of sodium with P_i

The enhancing effect of sodium on the inhibition by g-strophanthin is not found when ATP is replaced by P_i; on the contrary, with magnesium *plus* P_i the addition of sodium leads to a decrease in the inhibition by g-strophanthin (Fig. 20). The impeding effect of sodium depends on the magnesium concentration in such a way that it de-

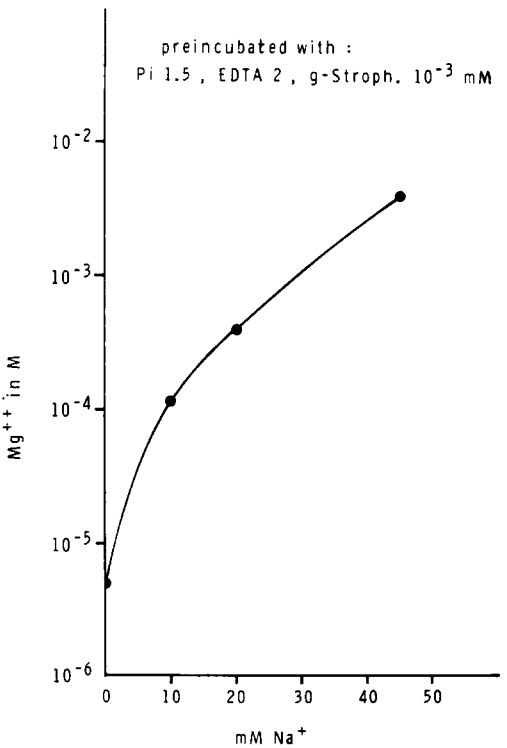

Fig. 22. The relationship between the concentrations of sodium and Mg^{2+} necessary for 50 % inhibition by 1 μM g-strophanthin with 1.5 mM P_i, and 2 mM EDTA in the pre-incubation medium. The pre-incubation was for 20 min. The experiments were performed as described in Fig. 17, but with 1.5 mM P_i instead of 3 mM ATP. The pre-incubation time was 20 min.

creases when the magnesium concentration is increased. This is seen from a comparison between Fig. 20 where the magnesium concentration was 2.0 mM and Fig. 21 where it was 2.5 mM, in both cases with 2 mM EDTA.

In Fig. 22 is shown the effect of sodium on the requirement for magnesium for 50 % inhibition by 1 μM g-strophanthin with 1.5 mM P_i. The incubation time was 20 min (*cf.* Fig. 9). It is seen how the concentration of Mg^{2+} necessary increases with the sodium concentration; with 50 mM of sodium, it is about 10^3 times higher than without sodium.

Without sodium, the concentration of g-strophanthin for 50 % inhibition decreases with an increase in the concentrations of Mg^{2+} and P_i up till 2 mM P_i and 10 mM Mg^{2+}. An increase above these concentrations did not give a further decrease in the concentration of g-strophanthin for 50 % inhibition. The pre-incubation time used was 90 min, *cf.* Fig. 9. After correction for the amount of g-strophanthin bound to the enzyme system, the minimum value was found to be 11 nM, which is similar to the value found with magnesium, ATP and sodium, namely 10 nM.

When these experiments were repeated, but with sodium in the medium and with non-limiting concentrations of sodium, magnesium and P_i, the 50 % value was found to be 440 nM.

DISCUSSION

The experiments show that the enzyme system does not react with g-strophanthin without magnesium in the medium. In agreement with observations by others it is found that with magnesium it does, and that the reactivity is increased by ATP[4–8] and P_i[5,8] and that sodium decreases the reactivity with magnesium with an without P_i[5,8], while it increases the reactivity with magnesium *plus* ATP[4–8].

The experiments furthermore show that the reaction with g-strophanthin goes to a state of equilibrium.

In the intact cell g-strophanthin only inhibits the transport system when applied to the outside of the membrane[14]. It seems likely that the effect of magnesium on the reactivity is on the inside of the membrane, which seems to exclude the possibility that magnesium takes part in the binding of g-strophanthin and suggests that magnesium in some way changes the system from a state in which it cannot react with g-strophanthin to a state in which it can. It suggests that with magnesium alone the reaction with g-strophanthin involves at least two steps, a reaction of the system with magnesium followed by the reaction with g-strophanthin. (TS for transport system and G for g-strophanthin).

$$TS + Mg^{2+} \leftrightharpoons Mg\text{–}TS$$

$$Mg\text{–}TS + G \leftrightharpoons Mg\text{–}TS\text{–}G$$

As discussed under RESULTS the effect of magnesium *plus* ATP on the inhibition by g-strophanthin cannot be due to the P_i released by the magnesium-dependent hydrolysis of ATP.

Without sodium, ATP impedes the inhibition by g-strophanthin when the concentration is high relative to the concentration of magnesium. As the concentration of Mg^{2+} is equal to $MgATP/ATP_f \times K$, it is not possible to design an experiment with magnesium and ATP which can indicate whether the impeding effect of ATP is due to

a decrease in the concentration of Mg^{2+} or to a decrease in the MgATP/ATP_f ratio or to both. If it is due to a decrease in the MgATP/ATP_f ratio, it means that ATP inhibits an effect of MgATP. In other words the question arises as to whether Mg^{2+} is necessary for the reaction with magnesium and ATP in the medium, or whether the reaction is due to an effect of MgATP? If Mg^{2+} is necessary, one may ask whether the increase in the reactivity towards g-strophanthin with ATP in the medium is due to an effect of MgATP or of ATP_f?

The effect of magnesium with P_i as a substrate suggests that there is an effect of Mg^{2+}; this may suggest that also with ATP as substrate the effect of magnesium on the reaction with g-strophanthin is due to an effect of uncomplexed magnesium, Mg^{2+}, on the enzyme system.

With magnesium, P_i and g-strophanthin there is a phosphorylation of the system which seems to be identical with the phosphorylation found with sodium, magnesium and ATP in the medium[7,15,16]. This may mean that with magnesium and ATP the effect of sodium on the rate of reaction with g-strophanthin is due to the formation of the phosphorylated intermediate and that a phosphorylation is necessary for the reaction with g-strophanthin.

The reaction with g-strophanthin, with only magnesium added, may suggest that a phosphorylation is not necessary. It is, however, difficult to exclude that the enzyme preparation contains a small amount of P_i. The phosphorylation with P_i requires a reaction with g-strophanthin. This may mean that the system reacts with g-strophanthin prior to the phosphorylation, but on the other hand it need not be the case. The system reacts with g-strophanthin with magnesium *plus* ATP without sodium; there is, however, always a very low amount of sodium in the enzyme preparations and due to this there may be a slow rate of phosphorylation from ATP. With magnesium, ATP and sodium, g-strophanthin decreases the rate of dephosphorylation, which means that it reacts with the phospho-enzyme[7,17]. It also decreases, however, the labelling from [^{32}P]ATP[9,17,18,19]; this suggests that g-strophanthin reacts with the system prior to the phosphorylation, which means with the system in a non-phosphorylated form.

Thus, magnesium seems to be necessary for the reaction with g-strophanthin, but apparently not the phosphorylation.

There is a pronounced effect of sodium on the requirement for magnesium for the reaction of g-strophanthin. But why is it that the effect goes in the opposite direction with P_i and with ATP?

With P_i, sodium apparently increases the K_i value for g-strophanthin (compare the minimum value for 50 % inhibition increases from 11 to 440 nM when sodium is added in non-limiting concentrations). This may explain why the requirement for Mg^{2+} for 50 % inhibition by 1 μM g-strophanthin is increased when sodium is added. With a g-strophanthin concentration which is below the saturation concentration for the sodium–magnesium form of the system, the conversion of the system from the magnesium to the sodium–magnesium form will lead to a decrease in the percent inhibition. With a Mg^{2+} concentration which is lower than the saturation concentration, an increase in the Mg^{2+} concentration will increase the amount of the system on the magnesium and the sodium–magnesium form, which means on the form which can react with g-strophanthin. This will increase the percent inhibition and Mg^{2+} will thereby counteract the decrease in inhibition due to sodium.

With ATP, sodium decreases the requirement for Mg^{2+}; this is apparently not secondary to an effect on the affinity for g-strophanthin as the affinity seems to be the same with magnesium, ATP and sodium and with magnesium and P_i. One way to explain this is to suggest that sodium removes an inhibitory effect of free ATP. Another is that ATP removes competition between sodium and magnesium or increases the affinity for magnesium when the system is on the sodium form.

An increase in the affinity for magnesium due to sodium and ATP would agree with the view put forward by FAHN *et al.*[20], that there is a shift in the affinity for magnesium during the reaction with ATP. The reaction with ATP *plus* sodium and magnesium leads to the formation of two phosphorylated intermediates, E_1–P and E_2–P, and the requirement for magnesium for the formation of E_1–P is lower than for the formation of E_2–P (refs. 20 and 21). This suggests that it is the system before the phosphorylation which has the high affinity for magnesium and according to the experiments with g-strophanthin this would mean that it is ATP as such which gives the system on the sodium form a high affinity for magnesium. When the γ-β phosphate bond in ATP is cleaved, the affinity is decreased. An increase in the affinity of the system for magnesium when it reacts with sodium and ATP must mean that ATP and sodium in some way changes the system, TS_1 to TS_1^* in the following scheme.

$$TS_1 + n\mathrm{Na} \leftrightharpoons \mathrm{Na}_n\text{–}TS_1$$
$$\mathrm{Na}_n\text{–}TS_1 + \mathrm{ATP} \leftrightharpoons \mathrm{Na}_n\text{–ATP–}TS_1^*$$
$$\mathrm{Na}_n\text{–ATP–}TS_1^* + Mg^{2+} \leftrightharpoons \mathrm{Na}_n\text{–Mg–ATP–}TS_1^*$$

Na_n–Mg–ATP–TS_1^* may react with g-strophanthin (see above).

$$\mathrm{Na}_n\text{–Mg–ATP–}TS_1^* + G \leftrightharpoons \mathrm{Na}_n\text{–Mg–ATP–}TS_1^*\text{–G}$$

The fraction which does not react with g-strophanthin is phosphorylated from ATP with the formation of two phospho-enzymes; the one is formed when the magnesium concentration is low relative to the ATP concentration and it does not react with g-strophanthin[21]. As magnesium is necessary for the reaction with g-strophanthin, it may mean that it is a form which has lost its magnesium due to the change in the affinity for magnesium when ATP is cleaved.

"Low" magnesium:

$$\mathrm{Na}_n\text{–Mg–ATP–}TS_1^* \leftrightharpoons \mathrm{Na}_n\text{–}TS_1\text{–P} + \mathrm{ADP} + Mg^{2+}$$

The other phospho-enzyme is formed with a higher concentration of magnesium and it reacts with g-strophanthin[21], which may mean that it is a magnesium form of the system. The formation of this form may involve a change in the conformation of the system[21–23], TS_1^* to TS_2.

"High" magnesium:

$$\mathrm{Na}_n\text{–Mg–ATP–}TS_1^* \rightleftharpoons \mathrm{Na}_n\text{–Mg–}TS_2\text{–P} + \mathrm{ADP}$$
$$\mathrm{Na}_n\text{–Mg–P–}TS_2 + G \leftrightharpoons \mathrm{Na}_n\text{–Mg–}TS_2\text{–P–G}$$

In both phospho-enzymes the phosphate seems to be bound to the same group on the system[21], in the form of a high-energy bond[24–26].

In the scheme given by FAHN *et al.*[20] and by POST *et al.*[21] the two phospho-enzymes represent two consecutive steps in the reaction. Another possibility is as

shown above that either the one or the other is formed. The decrease in the affinity for Mg^{2+} when the γ–β phosphate bond of ATP is cleaved may be due to a change in the way Mg^{2+} is bound to the system and this may be of importance for the transformation from the TS_1 to the TS_2 state. The change in the affinity may so to say happen while the reaction proceeds; when the magnesium concentration is too low, magnesium may be lost from the system during the reaction, which means that under these conditions the reaction cannot proceed to the formation of the TS_2 state. The result is instead an abortive reaction which does not lead to a change in the conformation with formation of TS_2, but to phosphorylation of TS_1.

REFERENCES

1 J. C. SKOU, *Physiol. Rev.*, 45 (1965) 596.
2 I. M. GLYNN, *Pharmacol. Rev.*, 16 (1964) 381.
3 H. J. SCHATZMANN, *Biochim. Biophys. Acta*, 94 (1965) 89.
4 H. MATSUII AND A. SCHWARTZ, *Biochim. Biophys. Acta*, 151 (1968) 655.
5 R. W. ALBERS, G. J. KOVAL AND G. J. SIEGEL, *Mol. Pharmacol.*, 4 (1968) 324.
6 J. C. SKOU, in D. C. TOSTESON, *The Molecular Basis of Membrane Function*, The Society of General Physiologists, Prentice Hall, 1969, p. 455.
7 A. K. SEN, T. TOBIN AND R. L. POST, *J. Biol. Chem.*, 2 (1969) 6595.
8 T. TOBIN AND A. K. SEN, *Biochim. Biophys. Acta*, 198 (1970) 120.
9 J. C. SKOU AND C. HILBERG, *Biochim. Biophys. Acta*, 185 (1969) 198.
10 C. H. FISKE AND J. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
11 G. G. SILLEN AND A. E. MARTELL, *Stability Constants of Metal-ion Complexes*, Special publication No. 17, The Chemical Society, London, 1964.
12 J. NØRBY, *Acta Chem. Scand.*, 24 (1970) 3276.
13 O. HANSEN, *Biochim. Biophys. Acta*, 233 (1971) 122.
14 P. C. CALDWELL AND R. D. KEYNES, *J. Physiol.*, 148 (1959) 8P.
15 G. E. LINDENMAYER, A. K. LAUGHFER AND A. SCHWARTZ, *Arch. Biochem. Bivphys.*, 127 (1968) 187.
16 G. J. SIEGEL, G. J. KOVAL AND R. W. ALBERS, *J. Biol. Chem.*, 244 (1969) 3264.
17 J. S. CHARNOCK AND K. A. POTTER, *Arch. Biochem. Biophys.*, 134 (1969) 42.
18 R. L. POST, A. K. SEN AND A. S. ROSENTHAL, *J. Biol. Chem.*, 240 (1965) 1437.
19 S. FAHN, G. J. KOVAL AND R. W. ALBERS, *J. Biol. Chem.*, 243 (1968) 1993.
20 S. FAHN, G. J. KOVAL AND R. W. ALBERS, *J. Biol. Chem.*, 241 (1966) 1882.
21 R. L. POST, S. KUME, T. TOBIN, B. ORCUTT AND A. K. SEN, *J. Gen. Physiol.*, 54 (1969) 306s.
22 R. BLOSTEIN, *J. Biol. Chem.*, 245 (1970) 270.
23 J. C. SKOU, in D. R. SANADI, *Current Topics in Bioenergetics*, Vol. IV, Academic Press, 1971, in the press.
24 L. E. HOKIN, P. S. SASTRY, P. R. GALSWORTHY AND A. YODA, *Proc. Natl. Acad. Sci. U.S.*, 54 (1965) 177.
25 K. NAGANO, I. KANAZAWA, N. MIZUNO, Y. TASHINA, T. NAKAO, AND M. NAKAO, *Biochem. Biophys. Res. Commun.*, 19 (1965) 759.
26 H. BADER, A. K. SEN AND R. L. POST, *Biochim. Biophys. Acta*, 118 (1966) 106.