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A STUDY ON THE INFLUENCE OF THE CONCENTRATION OF Mg^{2+}, P_i, K^+, Na^+, AND TRIS ON ($Mg^{2+}+P_i$)-SUPPORTED g-STROPHANTHIN BINDING TO ($Na^+ + K^+$)-ACTIVATED ATPase FROM OX BRAIN

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SUMMARY

1. The inhibitory effect of K^+, Na^+ and Tris on ($Mg^{2+}+P_i$)-supported g-[^{3}H]strophanthin binding to ATPase-containing plasma membrane fragments from ox brain has been studied.

2. Regardless of the concentration of Mg^{2+}, P_i, K^+, Na^+ and Tris, the overall process for the enzyme (E)–strophanthin (G) interaction might be described by the model

$$E + G \rightleftharpoons EG$$

but the apparent dissociation constant varied with the concentration of the factors mentioned.

3. The apparent dissociation constant for the overall process as a function of the concentration of each of the ions with facilitating or inhibitory effects on g-strophanthin binding was investigated. The relationship was used to construct some possible reaction schemes for ion–enzyme interactions, which may explain the respective effects of the ions on g-strophanthin binding.

4. g-Strophanthin binding data is compatible with the binding of one Mg^{2+} and one P_i per g-strophanthin binding unit, and with stepwise binding of more than one Na^+, K^+ or Tris per g-strophanthin binding unit.

5. Tentative binding constants for ion–enzyme interaction were calculated based on the proposed models. The dissociation constant was for enzyme–Mg 5.75 mM, enzyme–P_i 0.64 mM, enzyme–K 0.27 mM, enzyme $<^{K}_{K}$ 0.94 mM, combined enzyme–Na and enzymes $<^{Na}_{Na}$ 4.45 mM, combined enzyme–Tris and enzyme $<^{Tris}_{Tris}$ 19.4 mM.

INTRODUCTION

In the presence of Mg^{2+}, Na^+ and ATP the reaction of the ($Na^+ + K^+$)-activated ATPase enzyme system with g-strophanthin (ouabain) may be described simply as $E+G \rightleftharpoons EG$, where E denotes the enzyme system and G stands for g-strophanthin[1]. The affinity of g-strophanthin for the system is dependent on the

ionic composition of the incubation medium, but with favourable conditions it is very high. For the reaction of $(Na^+ + K^+)$-ATPase with g-strophanthin the medium must contain magnesium *plus* either ATP or P_i[2-6]. With magnesium *plus* ATP, sodium increases the binding, and with magnesium *plus* P_i, sodium decreases it. Potassium decreases it both with magnesium *plus* ATP and with magnesium *plus* P_i.

In this report an extension of the above overall reaction is attempted so as to make allowance for some of the components which facilitate or inhibit g-strophanthin binding. At the same time, an attempt has been made to use the influence of ionic conditions on g-strophanthin binding as a tool to obtain pertinent information on ion–enzyme interaction even when this interaction is not coupled to the trapping g-strophanthin complex.

The model for enzyme–strophanthin interaction was deduced for conditions where Mg^{2+}, Na^+ and ATP supported g-strophanthin binding; but, due to ATP hydrolysis, this means steady state and not equilibrium conditions. The ADP and P_i release, the Mg–ATP complex formation *etc.* complicate the analysis of the reaction. For these reasons, if binding supported by $Mg^{2+} + P_i$ is used instead of the $(Mg^{2+} + Na^+ + ATP)$-supported one, it is easier to control the factors which influence binding. This paper reports on results which show that the same overall reaction scheme as deduced for $(Mg^{2+} + Na^+ + ATP)$-supported binding[1] may be used to describe the g-strophanthin binding obtained with $Mg^{2+} + P_i$, independent of any inhibition of the $(Mg^{2+} + P_i)$-supported binding by K^+, Na^+ or Tris. The overall reaction scheme is expanded with models to account for the influence of Mg^{2+}, P_i, K^+, Na^+ and Tris on the equilibrium binding level of g-strophanthin. Tentative binding constants for ion–enzyme interaction are calculated based on the extended models.

MATERIALS AND METHODS

Microsomal fractions from ox brain were prepared as described by Skou and Hilberg[7] and g-strophanthin binding was carried out as described previously[1]. In these studies ^3H-labelled g-strophanthin ([^3H]ouabain, lot No. 184-244 N.E.N.) was used.

The parameter measured in these binding studies is the content of non-bound isotope; the difference between added and free isotope is taken to represent the amount of g-strophanthin bound to the membranes. The binding is measured as a function of time and all binding data given in this paper represent the values obtained at the plateau level of binding at equilibrium (see Fig. 1).

Impurities of the ^3H-labelled g-strophanthin were taken into account in the calculations. The purity was estimated in the following way. Enzyme, *i.e.* microsomes containing $(Na^+ + K^+)$-activated ATPase, was incubated with a low g-[^3H]strophanthin concentration (approx. 10^{-8} M) of high specific activity. At binding equilibrium, about 90% of the isotope was bound to the enzyme. The content of the ultrafiltrate (deprived of enzyme) diluted with unlabelled g-strophanthin ending with the same concentration as the first step (thus decreasing the specific activity) was incubated with fresh enzyme *etc.* The percentage uptake of the isotope decreased as compared to the first extraction, the last 5% of the isotope not being extractable by $(Na^+ + K^+)$-activated ATPase preparations. The purity of the labelled g-strophanthin batch was thus assumed to be only 95%.

The basic incubation medium for g-strophanthin binding was 3 mM Mg^{2+}, 3 mM P_i (buffered with 5.5 mM propandiol) and 40 mM Tris–HCl (pH 7.25, 37 °C). Small variations in Tris–HCl or $Tris^+$ concentration did not affect g-strophanthin binding to a measurable extent but if the Tris concentration was increased considerably, the equilibrium level of bound g-strophanthin decreased (see below). As discussed later, it is not yet settled whether this is due to a specific effect of $Tris^+$ mimicking the effect of alkali metal ions or due to the increasing ionic strength. When Mg^{2+}, P_i and K^+ concentrations were varied, the ionic strength was kept constant by varying the Tris concentration (but in the range of Tris concentration where g-strophanthin binding was very little affected). In the experiments with varying Na^+ concentrations, no attempt was made to keep the ionic strength constant due to the high concentration of Na^+ needed to obtain an effect on g-strophanthin binding. If the basic Tris concentration had to be so high that Tris might have been replaced by Na^+, the initial Tris concentration would have considerably influenced g-strophanthin binding.

The contamination of the enzyme preparation with K^+ and Na^+ was determined by flame photometry (Eppendorf) on the supernatant after centrifugation of the stock enzyme solutions at 100 000 × g for 30 min. The concentration of K^+ in the supernatant was 50–200 μM, and that of Na^+ was 150–400 μM *i.e.* an insignificant concentration of these ions considering the dilution of the enzyme preparation in the binding experiments (usually 1 : 10).

RESULTS

The g-strophanthin-binding model previously presented[1] was based on binding data obtained with 5 mM Mg^{2+}, 2 mM EDTA, 10 mM Na^+, 3 mM ATP, 60 mM Tris (pH 7.4, 37 °C) and with varying g-strophanthin concentrations. Further experiments at varying Na^+ concentrations (all other things being equal) revealed the same overall reaction scheme for enzyme–strophanthin interaction. However, the apparent dissociation constant for the g-strophanthin-binding process decreased with increasing Na^+ concentration. From a value of about 25 nM with 10 mM Na^+ it decreased to 4–6 nM at 120 mM Na^+ (data not shown).

At a given g-strophanthin concentration, nearly identical binding levels were reached when the enzyme was incubated either with 3 mM Mg^{2+} + 3 mM P_i or with 3 mM Mg^{2+} + 3 mM ATP + 120 mM Na^+, as seen from Fig. 1. Equilibrium binding data for free and bound g-strophanthin, G_f and EG, respectively, obtained with Mg^{2+} + P_i at different initial g-strophanthin concentrations, fitted the same model as (Mg^{2+} + Na^+ + ATP)-binding data. The apparent dissociation constant was close to that calculated with 120 mM Na^+ in the ATP-facilitated system. The omission of EDTA and the equivalent concentration of Mg^{2+} was without effect on g-strophanthin binding.

In the following, the effects of variations in Mg^{2+}, P_i, K^+, Na^+, and Tris concentrations on the g-strophanthin binding has been measured. For each set of parameters the equilibrium values of bound and free g-strophanthin have been determined for a number of different concentrations of added g-strophanthin. A Scatchard-type plot has been applied to the equilibrium binding data, which, according to the model, should give straight lines when EG is plotted *versus* EG/G_f.

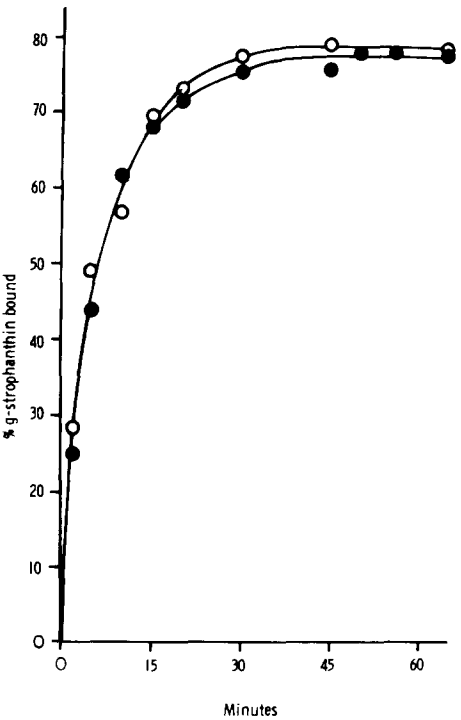

Fig. 1. Comparisons between ATP- and P_i-supported g-strophanthin binding. ○—○, incubation with 5 mM Mg^{2+}, 2 mM EDTA, 3 mM ATP, 30 mM Tris and $5 \cdot 10^{-5}$ mM g-strophanthin; ●—●, incubation with 5 mM Mg^{2+}, 2 mM EDTA, 3 mM P_i, 30 mM Tris and $5 \cdot 10^{-5}$ mM g-strophanthin. Enzyme B_{35} 14/3, 0.245 mg protein/ml, g-strophanthin-sensitive activity 141 μmoles P_i/mg protein per h.

The ordinate intercept corresponds to EG_{max}, *i.e.* the total number of binding sites present, and the slope corresponds to the apparent dissociation constant.

Fig. 2 shows the effect of a variation in the P_i concentration under conditions where the Mg^{2+} concentration is kept constant at 3 mM, and with no sodium or potassium added. In Fig. 3 is shown the effect of a variation in the Mg^{2+} concentration, when the P_i concentration is kept constant at 3 mM.

In Fig. 4 is shown the effect of a variation in the K^+ concentration with 3 mM Mg^{2+}, 3 mM P_i, and with a Tris concentration which with 0 mM K^+ was 40 mM, and with a higher concentration of K^+ decreased with the increase in the K^+ concentration.

In Fig. 5 the concentration of Na^+ has been varied with 3 mM Mg^{2+}, 3 mM P_i, and 40 mM Tris, which means that the ionic strength of the solution varied with the Na^+ concentration. Note the enzyme preparation is different from that used in the experiments presented in Figs 2, 3, 4 and 6, and this fact explains the change in ordinate intercept.

In Fig. 6 it is the concentration of Tris that has been varied with 3 mM Mg^{2+} and 3 mM P_i, which means that in this experiment also the ionic strength varied with the $Tris^+$ concentration.

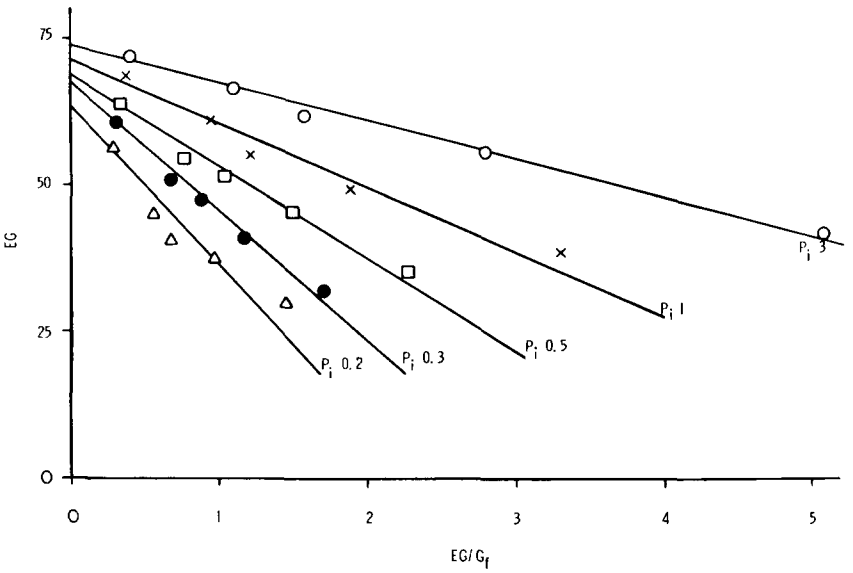

Fig. 2. Bound g-strophanthin (*EG*) in nM *versus* bound g-strophanthin/free g-strophanthin (EG/G_f) at equilibrium with the P_i concentration varied. Incubation medium: 3 mM Mg^{2+}, 0.2–3 mM P_i, 40–48 mM Tris and $5 \cdot 10^{-5}$–$2.5 \cdot 10^{-4}$ mM g-strophanthin. Enzyme B_{35} 31/7, 0.166 mg protein/ml, g-strophanthin-sensitive activity 227 μmoles P_i/mg protein per h.

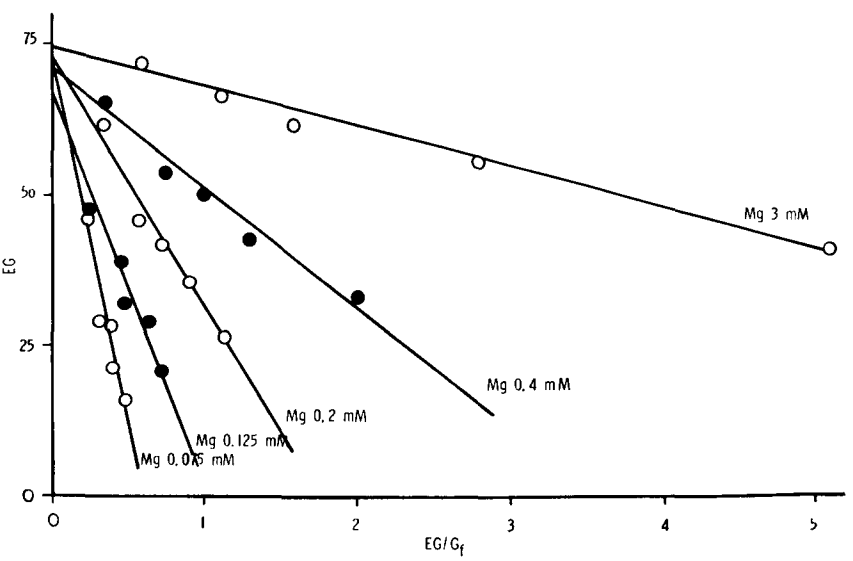

Fig. 3. As Fig. 2 except that the Mg^{2+} concentration varied from 0.075 to 3 mM, and Tris from 40 to 51 mM.

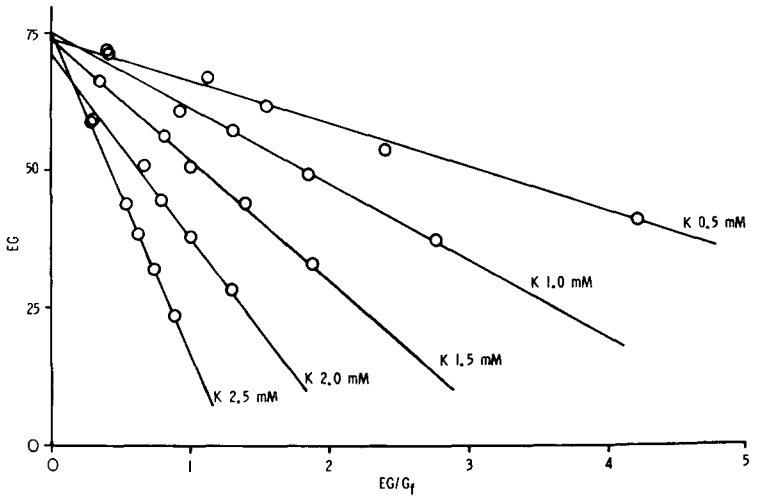

Fig. 4. As Fig. 2 except that the K^+ concentration varied from 0 to 2.5 mM, and Tris from 37 to 40 mM.

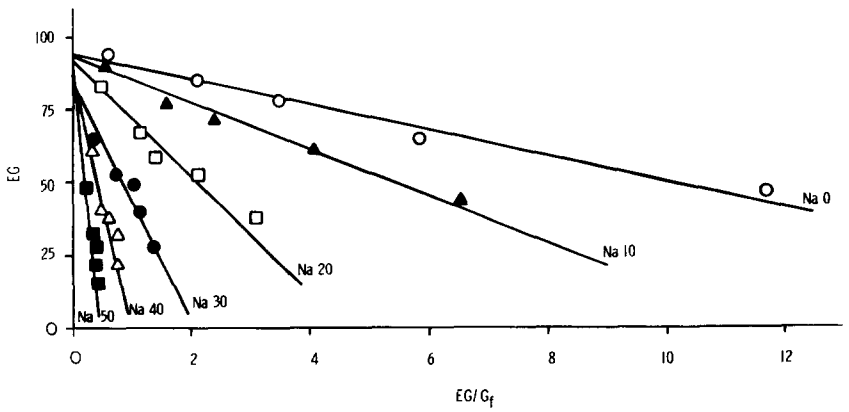

Fig. 5. Bound g-strophanthin (EG) in nM *versus* bound g-strophanthin/free g-strophanthin (EG/G_f) at equilibrium with the Na^+ concentration varied. Incubation medium: 3 mM Mg^{2+}, 3 mM P_i, 40 mM Tris, 0–50 mM Na^+, $5 \cdot 10^{-5}$–$2.5 \cdot 10^{-4}$ mM g-strophanthin. Enzyme B_{35} 1/12, 0.279 mg protein/ml, g-strophanthin-sensitive activity 168 μmoles P_i/mg protein per h.

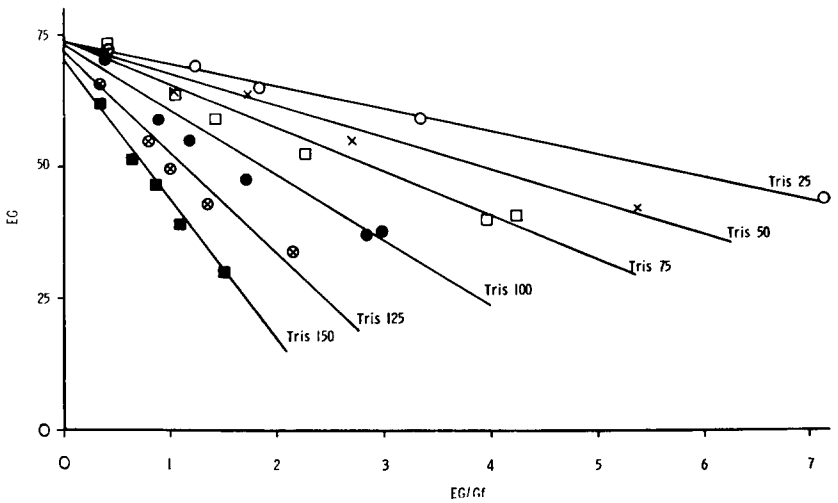

Fig. 6. As Fig. 2 except that the Tris concentration varied from 25 to 150 mM.

It is seen that at fixed concentrations of Mg^{2+}, P_i, Na^+, K^+ and Tris, *i.e.* for a given set of parameters, the equilibrium binding values obtained at different initial g-strophanthin concentrations all seem to fall on straight lines. This means that, for a given set of parameters, the reaction can be described by the same simple scheme as for the reaction with $Mg^{2+}+ATP+Na^+$, namely $E+G \rightleftharpoons EG$.

When the concentration of all the ions except one were kept constant, the equilibrium binding data at varying initial concentrations of g-strophanthin still gave straight lines and the same ordinate intercept (*i.e.* the same total number of binding sites) but the slopes of the straight lines varied (*i.e.* the apparent dissociation constant varied) with the variation of the parameter. With Mg^{2+} and P_i (Figs 2 and 3) a decrease in one of the parameters leads to an increase in the slope, while with Na^+, K^+, and Tris (Figs 4, 5 and 6) an increase in the concentration leads to an increase in the slope. The effect of K^+ is much more pronounced than that of sodium, which again has a much more pronounced effect than Tris.

The concept of straight lines with nearly constant ordinate intercepts is only disturbed in experiments with varying P_i concentrations. The explanation for the decrease in ordinate intercept at the lower P_i concentrations (Fig. 2) seems to be that the ($Na^+ + K^+$)-activated ATPase activity decreases during the incubation period when Mg^{2+} is present in high concentration relative to the P_i concentration. This denaturing effect of Mg excess on the ATPase is 15–20% during 1 h of incubation and is thus comparable to the decrease in ordinate intercept at lower P_i concentrations, whereas the ATPase activity is stable during 1 h of incubation under other ionic conditions. The slope of the lines, however, may still represent the apparent dissociation constant for the remaining activity. Due to the faster g-strophanthin binding rates at higher g-strophanthin concentrations, a protective effect against Mg-dependent denaturation might dominate at these higher g-strophanthin concentrations and explain a tendency towards curved P_i lines.

Apart from the special effect of Mg^{2+} excess on the P_i binding curves, it

is concluded from the straight lines with nearly constant ordinate intercept in Figs 2–6 that the overall reaction scheme

$$E + G \rightleftharpoons EG$$

may be valid for all ionic conditions.

Extension of the binding model

Since the equilibrium binding level at a given g-strophanthin concentration increased with increasing concentration of Mg^{2+} and P_i, while it decreased with increasing concentration of Na^+, K^+ and $Tris^+$ (*i.e.* Mg^{2+} and P_i promote whereas Na^+, K^+ and $Tris^+$ inhibit g-strophanthin binding) it was assumed that the following detailed reactions might describe the sequence of events:

$$E + Mg \rightleftharpoons EMg \quad \text{(I)}$$

$$EMg + P_i \rightleftharpoons EMgP_i \quad \text{(II)}$$

$$E + X \rightleftharpoons EX \quad \text{(III)}$$

where X denotes Na^+, K^+ or Tris but only

$$EMgP_i + G \rightleftharpoons EMgP_iG \quad \text{(IV)}$$

Since

$$E_{total} = E + EMg + EMgP_i + EX + EMgP_iG \quad \text{(V)}$$

the following equation may be deduced for the equilibrium situation:

$$\frac{[E_{total} - EMgP_iG]\cdot[G]}{[EMgP_iG]} = K^{EMgP_iG}\left\{\frac{K^{EMgP_i}}{[P_i]}\left(+ \frac{K^{EMg}}{[Mg]}\left[1 + \frac{[X]}{K^{EX}}\right]\right) + 1\right\} \quad \text{(VI)}$$

where K^{EMg}, K^{EMgPi}, K^{EX} and K^{EMgPiG} denote the dissociation constants for processes I–IV, respectively.

The left hand side of Eqn VI denotes the apparent dissociation constant for the overall reaction. With fixed concentrations of three of the parameters, and variation of the fourth, plots of this variable (*i.e.* $1/P_i$, 1/Mg, Na or K) against the apparent dissociation constant should give straight lines according to Eqn VI in order to be consistent with the reaction schemes. From the slopes of the straight lines the ion–enzyme dissociation constants may be obtained.

Figs 7, 8, 9, and 10 represent plots of the apparent dissociation constants *versus* the variables. It is seen that only Mg^{2+} and P_i seem to fulfill the requirements, whereas the effect of K^+, Na^+ and Tris on the apparent dissociation constant is seen to increase non-linearly with their increasing concentrations. According to Eqns I–IV, the same stoichiometric relationship between enzyme units defined as g-strophanthin-binding units and each of the ions should exist. If this condition is not fulfilled, no linear relationship between the variable and the dissociation constant should be obtained. By means of Hill plot, *i.e.* log–log plots of the effects of increasing concentrations of the ions on g-strophanthin binding, possible interaction between activators or inhibitors on the parameter measured may be tested.

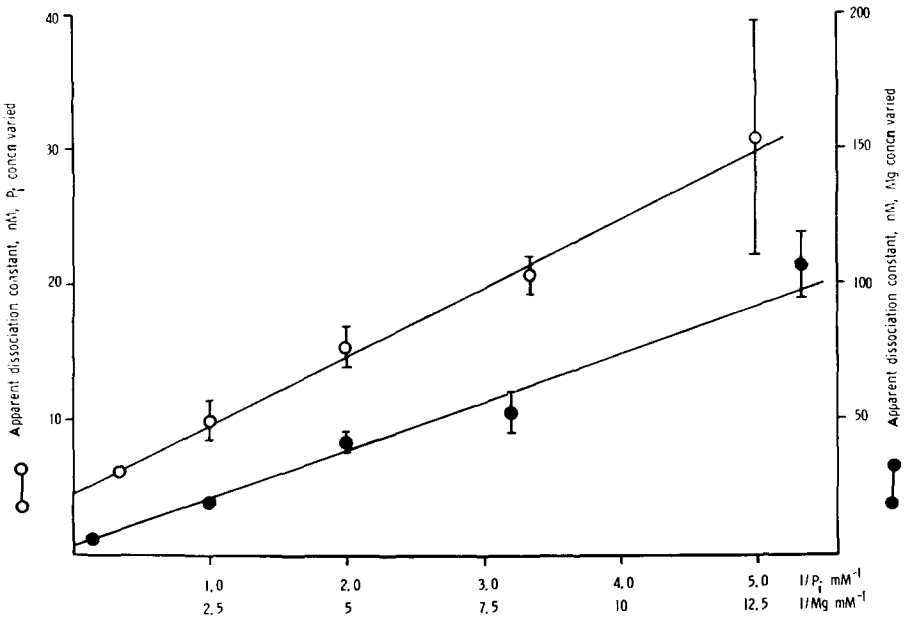

Fig. 7. Apparent dissociation constants in nM of the overall process for enzyme–strophanthin interaction calculated from the slopes of the lines in Figs 2 and 3 *versus* the reciprocal Mg^{2+} and P_i concentration, $1/Mg^{2+}$ and $1/P_i$ mM^{-1}.

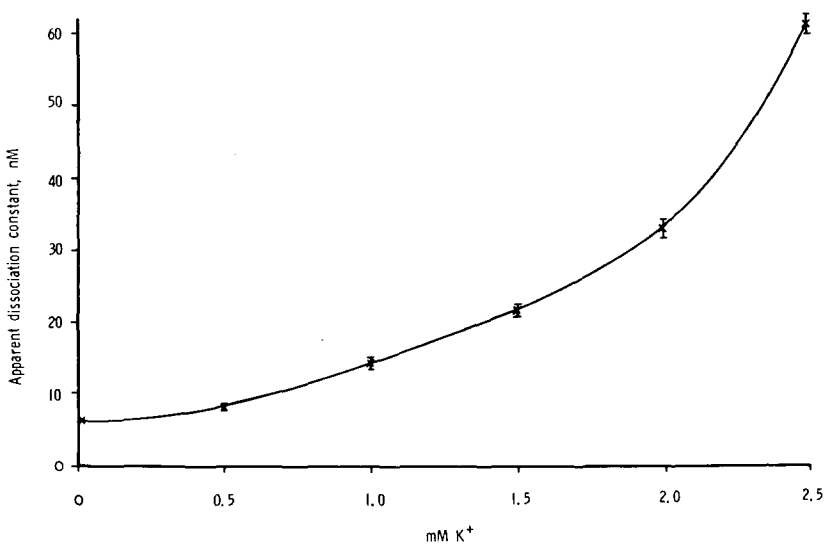

Fig. 8. Apparent dissociation constant in nM of the overall process for enzyme–strophanthin interaction calculated from the slopes of the lines in Fig. 4 *versus* the K^+ concentration in mM.

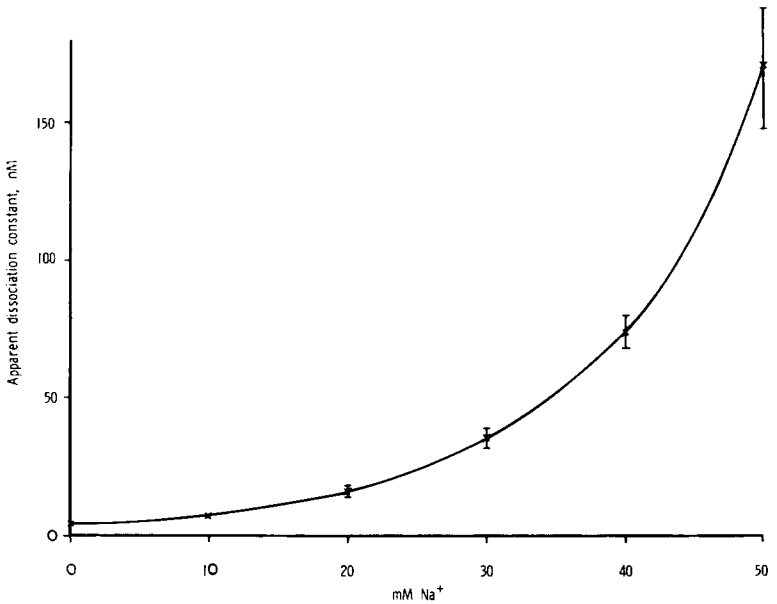

Fig. 9. Apparent dissociation constant in nM of the overall process for enzyme–strophanthin interaction calculated from the slopes of the lines in Fig. 5 *versus* the Na^+ concentration in mM.

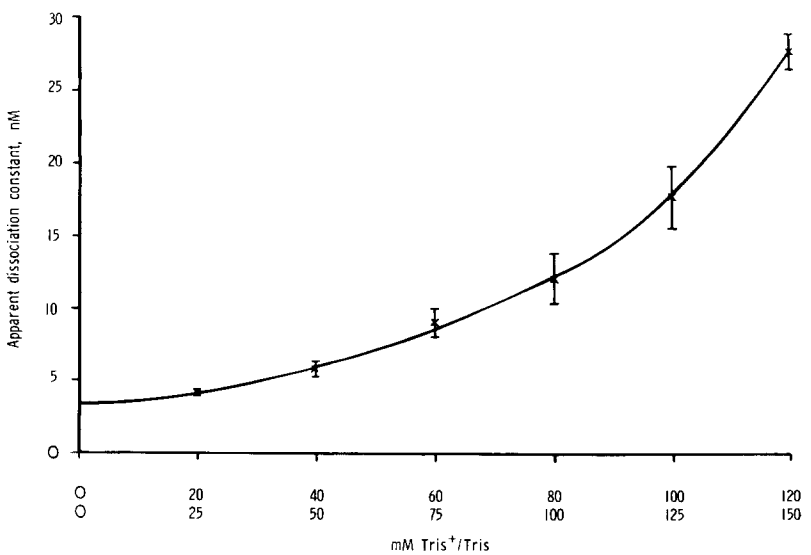

Fig. 10. Apparent dissociation constant in nM calculated from the slopes of the lines in Fig. 6 *versus* the Tris and $Tris^+$ concentration in mM.

The application of Hill plots to the binding values at one initial g-strophanthin concentration but with varying ion concentrations gives slopes just around unity for Mg^{2+} and P_i, and around 2 for K^+ and Na^+. This could indicate a 1:1 relationship between enzyme units and Mg^{2+} and P_i, or at least a lack of co-operativity, but binding of more than one K^+ and Na^+ per g-strophanthin site and co-operativity among the ions bound to one g-strophanthin unit. If each g-strophanthin-binding unit reacts with two monovalent cations, and only the enzyme which has not reacted with the monovalent cation (or more correctly $E\mathrm{MgP_i}$) can react with g-strophanthin, then Eqn III should be extended to

$$E< \; + X \rightleftharpoons E<^{X} \tag{IIIa}$$

$$E<^{X} + X \rightleftharpoons E<^{X}_{X} \tag{IIIb}$$

whereas binding of g-strophanthin may occur only according to

$$E< \mathrm{MgP_i} + \mathrm{G} \rightleftharpoons E< \mathrm{MgP_iG}$$

< denoting non-occupied binding sites for monovalent cations. in this case the compound Eqn VI, instead of containing the term $[X]/K^{EX}$, should contain the terms

$$\frac{[X]}{K^{E<x}} + \frac{[X]^2}{K^{E<_x} \cdot K^{E<^{x}_{x}}}$$

$$\frac{[E_{\mathrm{total}} - E\mathrm{MgP_iG}]\cdot[\mathrm{G}]}{[E\mathrm{MgP_iG}]} =$$

$$K^{E\mathrm{MgP_iG}} \left\{ \frac{K^{E\mathrm{MgP_i}}}{[\mathrm{P_i}]} \left(1 + \frac{K^{E\mathrm{Mg}}}{[\mathrm{Mg}]} \left[1 + \frac{[X]}{K^{E<x}} + \frac{[X]^2}{K^{E<_x} \cdot K^{E<^{x}_{x}}} \right] \right) + 1 \right\} \tag{VIa}$$

which can explain the accelerating effect of K^+, Na^+ or Tris on the apparent dissociation constant for the overall process. However, no simple plot giving the relationship between ionic concentration and the apparent dissociation constant may be divised if cations on one binding site affect the affinity for cations at the other site. Likewise, no simple dissociation constants exist, but only stepwise equilibrium constants[8].

Another objection to the model represented by Eqns I–IV is that according to this only non-potassium- and non-sodium-bound enzyme may react with g-strophanthin. However, it is not possible to overcome the g-strophanthin effect by increasing the K^+ concentration, *i.e.* the binding never reaches zero but levels off at a low, but nevertheless significant plateau level (Fig. 11). For this reason, and because Mg^{2+} and P_i are necessary to obtain any binding with K^+, processes of the following types should also be included:

$$E< \mathrm{Mg} \; + \mathrm{K} \rightleftharpoons E<^{\mathrm{K}} \mathrm{Mg} \tag{VIIa}$$

$$E<^{\mathrm{K}} \mathrm{Mg} \; + \mathrm{K} \rightleftharpoons E<^{\mathrm{K}}_{\mathrm{K}} \mathrm{Mg} \tag{VIIb}$$

$$E< \mathrm{MgP_i} + \mathrm{K} \rightleftharpoons E<^{\mathrm{K}} \mathrm{MgP_i} \tag{VIIIa}$$

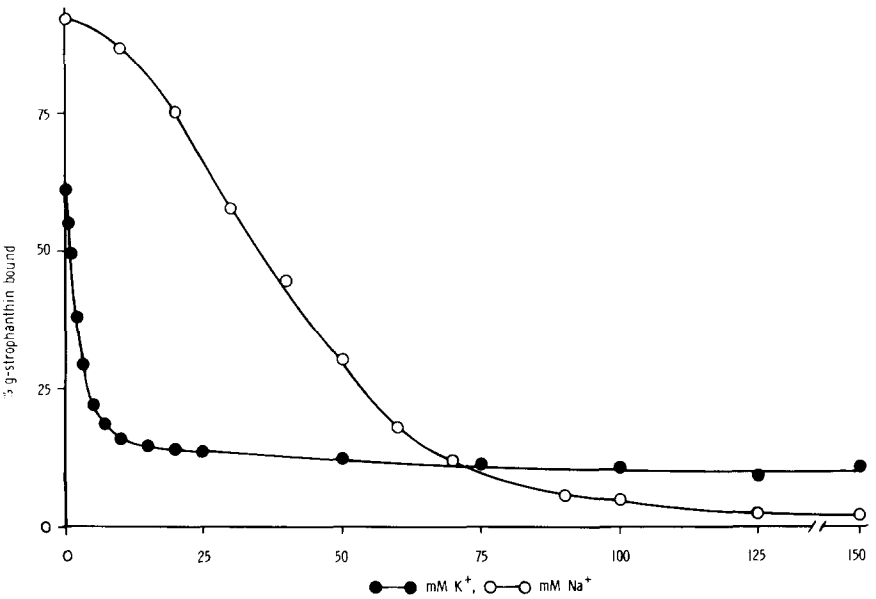

Fig. 11. Percentage of g-strophanthin binding as a function of increasing Na^+ and K^+ concentration. ○—○, enzyme B_{35} 1/12, 0.279 mg protein/ml, g-strophanthin-sensitive activity 168 μmoles P_i/mg protein per h. Incubation medium 3 mM Mg^{2+}, 3 mM P_i, 40 mM Tris, 0–150 mM Na^+, $5 \cdot 10^{-5}$ mM g-strophanthin. ●—●, enzyme B_{16} 31/7, 0.125 mg protein/ml, g-strophanthin-sensitive activity 193 μmoles P_i/mg protein per h. Incubation medium 3 mM Mg^{2+}, 3 mM P_i, 40 mM Tris, 0–150 mM K^+, and $5 \cdot 10^{-5}$ mM g-strophanthin.

$$E{<}^{K}MgP_i + K \rightleftharpoons E{<}^{K}_{K}MgP_i \qquad \text{(VIIIb)}$$

and in analogy with Eqn IV

$$E{<}^{K}MgP_i + G \rightleftharpoons E{<}^{K}MgP_iG \qquad \text{(IXa)}$$

$$E{<}^{K}_{K}MgP_i + G \rightleftharpoons E{<}^{K}_{K}MgP_iG \qquad \text{(IXb)}$$

and/or other sequences of reactions resulting in the same products but with one or more steps with lower affinity than the basic reactions I–IV.

Calculation of dissociation constants

Figs 7, 8, 9 and 10 may be explained by means of equations I, II, IIIa and IIIb. However, since it seems impossible to overcome g-strophanthin binding totally by increasing the K^+ concentration, perhaps contrary to the effect of increasing the Na^+ concentration (Fig. 11), these equations may not cover all possibilities. Reactions of the types shown in Eqns VII–IX may also be possible, but the affinity of g-strophanthin for the enzyme-potassium form (Eqn IX) must be much lower than for non-potassium-bound enzyme (see Fig. 11). Because the quantitative effects of K^+ were studied only with concentrations ranging from 0 to 2.5 mM, it may be possible to calculate ion–enzyme binding constants by the use of Eqn VIa and disregard Eqns VII–IX.

The binding constants given in Table I for enzyme–Mg and enzyme–P_i interaction are calculated from Eqn VI and from binding data similar to those given in Figs 2 and 3 but within a more narrow range of Mg and P_i concentrations giving a more safe determination of the ordinate intercept and the slope of the straight line. For magnesium and inorganic phosphate, the calculations are based on the total concentrations of the ions added. Only one form of phosphate, *e.g.* HPO_4^{2-}, may be effective in promoting binding. Moreover due to magnesium–phosphate complex formation, the total concentrations of free magnesium and phosphate are somewhat reduced. However, because the association constant is low, only a small fraction of the magnesium and the inorganic phosphate added is complexed and this fraction is nearly independent of the absolute concentrations.

TABLE I

ION–(Na^+–K^+)-ATPase DISSOCIATION CONSTANTS

The binding constants were calculated from ion–enzyme binding models deduced from the ionic interaction with g-strophanthin binding to (Na^++K^+)-activated ATPase from ox brain.

	Dissociation constant (mM)				
	Mg^{2+}	P_i	K^+	Na^+	$Tris^+$
Enzyme < ion	5.75	0.64	0.27	4.45	19.4
Enzyme < ion ion	—	—	0.94		

The dissociation constants for K^+, Na^+ and $Tris^+$ are estimated from similar but more numerous observations than those given in Figs 4, 5, 6, 8, 9 and 10. The constants are calculated from Eqn VIa by means of the method of least squares. The effect of $Tris^+$ may be purely due to an effect of the ionic strength or $Tris^+$ may have a specific effect on g-strophanthin binding. Experiments with the addition of Na_2SO_4 and NaCl+Tris–HCl giving the same concentration of Na^+ and the same ionic strength favoured the first suggestion. However, calculation of enzyme–$Tris^+$ binding constants seems justified because the effects of increasing concentrations of $Tris^+$ are similar to the effects of increasing concentrations of K^+, although the effects of K^+ were studied in a much narrower concentration range practically without variation in the ionic strength. The method of least squares applied to the Na^+- and $Tris^+$–binding values did not give reliable values unless the first binding constant was assumed to be very high, in which case an approximated overall constant for the two step reaction might be calculated. The sodium experiments were carried out at increasing ionic strength, and the experimental sodium curve giving the relationship between the overall dissociation constant for g-strophanthin binding and sodium concentration may thus be the result of the sodium effect and an effect of increasing ionic strength (*e.g.* the $Tris^+$ effect). The calculation of the overall enzyme–Na constant was based on this assumption.

DISCUSSION

Several studies of the factors which affect g-strophanthin binding and inhibition, and which also influence rate of ATP hydrolysis by $(Na^+ + K^+)$-activated ATPase, have appeared recently[2–4,6]. This study represents an attempt to gather several, often conflicting, observations and to make them available for quantitative analysis. The model given for the ionic influence on g-strophanthin binding supported by $(Mg^{2+} + P_i)$ and inhibited by monovalent cations may help to explain some observations and clarify some of the controversy in the literature. Thus, the present results explain why it is claimed that K^+ decrease the rates of binding and inhibition[3,9] but does not affect the extent of binding and inhibition[10], and on the other hand that the effect of K^+ on glycoside interaction with the enzyme is sensitive to drug concentration[11]. The crucial point seems to be that the effect of K^+ depends on the glycoside concentration as well as on the concentration of the ions which facilitate g-strophanthin binding (here $Mg^{2+} + P_i$), and that the effect of K^+ on g-strophanthin binding therefore may be hidden at high concentrations of potassium antagonists. This is demonstrated in the Scatchard plots by the identical ordinate intercept independent of the conditions for binding.

The g-strophanthin binding data seem to be compatible with a one-to-one relationship between g-strophanthin–binding units of the enzyme and Mg^{2+} and P_i, but only with stepwise binding of more than one Na^+ or K^+ per g-strophanthin-binding unit. According to the model used for the calculation of ion–enzyme binding constants, binding of Na^+ and/or K^+ excludes binding of g-strophanthin. However, by increasing the potassium concentration it does not seem possible to overcome totally the g-strophanthin binding, as seen from Fig. 11 where the extent of binding first declines but then reaches and maintains a lower level at high K^+ concentration. From this one would predict that the sigmoid curve in Fig. 8 will eventually reach a maximum and then flatten out; it has not been possible experimentally to show whether the low binding data obtained with high concentration of K^+ still fit the model. Fig. 11 indicates binding of K^+ as well as of g-strophanthin simultaneously, implying a much higher dissociation constant for the overall reaction of g-strophanthin binding when the enzyme is saturated with K^+. This again implies that in one or more of the individual reactions the enzyme–ligand affinity is decreased. Saturation with K^+ means that the apparent dissociation constant must have increased considerably, and hence the low affinity binding (corresponding to reactions IXa and IXb) has been neglected in the calculations of ion–enzyme binding constants. It should be pointed out that the dissociation constants given in Table I are only tentative. For instance, the experimental data with Na^+ and K^+ may just as well be explained by simple displacement of Mg^{2+} from its site instead of the binding of Na^+ and K^+ to their specific sites. Eqns I–IIIa and IIIb do not exclude this possibility.

The direct determination of ion binding is not possible due to the relatively low ion–enzyme affinities and to the lack of a pure enzyme. Based on kinetic analysis, and with the assumption that the K^+-dependent rate constant for dephosphorylation is much higher than the Na^+-dependent rate constant for phosphorylation, Green and Taylor[12] assume that the binding constants for Na^+ and K^+ are of equal magnitude, approximately 20–40 mM. From the influence of K^+ on

ATP binding to ox brain microsomes, Nørby and Jensen[13] calculate a dissociation constant for enzyme–K^+ of 87 μM.

Although the ($Na^+ + K^+$)-activated enzyme used in this study is attached to particulate plasma membrane fragments, no attempts have been made to distinguish in the model between the two sides of the membrane. The enzyme is simply represented by E in the proposed model. Experiments on intact systems have shown that the effect of g-strophanthin is from the outside[14]. The promoting effect of Mg^{2+} and P_i is probably on the inside[15,16]. For maximal ATPase activity of intact systems, K^+ is needed on the outside and Na^+ on the inside[17], and high concentrations of both ions inhibit to an extent inversely related to the concentration of the other ion[12,18].

As discussed above, the sigmoid shape of the curve for the relationship between the concentration of potassium or sodium and the apparent dissociation constant for g-strophanthin binding suggests a reaction with more than one sodium or potassium ion per g-strophanthin-binding unit. It may either mean that the cations react initially with one side of the system followed by a reaction with the other side, or that one side of the system reacts with more than one cation, or both. It is not possible to overcome the g-strophanthin binding by increasing the potassium concentration, contrary to the effect of increasing the concentration of sodium. It shows that they have different effects and it is tempting to suggest that it is due to effects on different sides of the membrane. Sodium may act on the inside and hinder the reaction with g-strophanthin from outside by shifting the equilibrium of the system towards the inside. The non-competitive effect of K^+ on g-strophanthin binding might be visualized by its moving the enzyme towards the outside and merely decreasing the affinity for g-strophanthin. If this is the case, the sigmoid curves should not be explained as a successive effect first on one side of the membrane, and then on the other, but as the result of stepwise equilibria for binding of at least two alkali metal ions on the same side of the membrane per g-strophanthin-binding unit. Stepwise binding can account for what is interpreted as homotropic cooperativity in binding, ligand-induced conformational changes, or allosterism[8,19–21].

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