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THE RELATIONSHIP BETWEEN G-STROPHANTHIN-BINDING CAPACITY AND ATPASE ACTIVITY IN PLASMA-MEMBRANE FRAGMENTS FROM OX BRAIN

OTTO HANSEN

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

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SUMMARY

1. Binding of labelled g-strophanthin to plasma-membrane fragments from ox brain has been studied using a filtration technique. ATPase-containing particles were separated from medium after incubation with 5 mM Mg^{2+}, 2 mM EDTA, 10 mM Na^+, 3 mM ATP, 60 mM Tris, pH 7.4 (37°), and varying [^{3}H]g-strophanthin concentrations.

2. g-Strophanthin binding was studied as a function of time and concentration The variation of the equilibrium level of bound g-strophanthin (EG) with the concentration of unbound g-strophanthin (G) fits the following simple model for the reaction

$$E + G \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EG$$

where E represents the available part of the enzyme system, *i.e.* $E = EG_{max} - EG$.

3. Addition of unlabelled g-strophanthin, K^+ or EDTA when a steady-state level of g-strophanthin binding was established decreased the binding towards a new equilibrium indicating reversibility of the binding process.

4. Based on the model presented, the binding capacity (EG_{max}) was calculated for a series of enzyme preparations with varying activity. A linear relationship between g-strophanthin-binding capacity and ATPase activity was obtained indicating a constant catalytic centre activity per g-strophanthin binding site.

INTRODUCTION

Since the observation of SCHATZMANN[1] that cardiac glycosides inhibit active cation transport it has been well documented that these steroids are specific inhibitors of the membrane enzyme system which seems to be involved in the active transport of Na^+ and K^+. Studies of the inhibitory effect of g-strophanthin and other cardiac glycosides on the ($Na^+ + K^+$)-activated ATPase system have recently been supplemented by studies on the binding of labelled g-strophanthin to plasma-membrane fragments[2–4]. Determination of binding of g-strophanthin to the particulate preparations has in most cases involved a time-consuming centrifugation step for the separation of the particles from incubation medium.

By using an ultrafiltration technique which allows a rapid separation of enzyme

and solution it is possible to study the time course of the reaction. When the parameter measured is the content of free g-strophanthin in the filtrate, and the difference between added and free glycoside is taken to represent the amount of g-strophanthin bound to the membranes, the method allows an analysis of the kinetics of the binding process. The elucidation of the kinetics was based on the equilibrium situation as determined from the time course. Since the kinetics of enzyme–strophanthin interaction turned out to be compatible with a simple model, an accurate and well-defined value for maximum binding capacity of g-strophanthin was calculated. Finally the relationship between binding capacity and ATPase activity was examined for enzyme preparations with different specific activities.

The present work arose out of a study on the cationic effect on the inhibition of the enzymatic hydrolysis of ATP by g-strophanthin[5].

MATERIALS AND METHODS

Enzyme preparation and chemicals

Microsome fractions from ox brain were prepared as described by SKOU[6]. The established procedure involves homogenization and differential centrifugation in a histidine–sucrose buffer which contains 0.1 % sodium deoxycholate. The centrifugation steps are at 12000 rev./min, approx. 17000 × *g* (sediment B_{12}, usually discarded), 16000 rev./min, approx. 31000 × *g* (sediment B_{16}), and at 35000 rev./min, approx. 100000 × *g* (sediment B_{35}). The pellets are resuspended and stored in histidine–sucrose, pH 6.8, at −18 to −20°.

Total ATPase activity was determined in 3 mM Mg^{2+}, 3 mM ATP (Tris salt), 120 mM Na^+, 30 mM K^+ and 60 mM Tris–HCl at pH 7.4, 37°; for the Mg^{2+}-activated ATPase activity Na^+ and K^+ were omitted. P_i release was measured by the method of FISKE AND SUBBAROW[7]. Preparations with varying ratios between (Mg^{2+} + Na^+ + K^+)- and Mg^{2+}-activity were obtained (1) as described by SKOU[6], (2) by omission of deoxycholate activation and (3) by heat denaturation of deoxycholate -activated enzyme preparations. In procedure (2) after an initial centrifugation of the homogenate at 7000 rev./min, approx. 6000 × *g* for 30 min (pellet discarded) the supernatant was centrifuged at 20000 rev./min, approx. 48000 × *g* for 30 min and the pellet resuspended in histidine–sucrose (microsomal fraction 18/8). Afterwards part of it was activated with deoxycholate and subjected to differential centrifugation to yield higher levels of specific activity in the following way. One part of the activated microsomal batch was centrifuged at 100000 × *g* for 60 min, and the pellet again resuspended in the same volume of histidine–sucrose (B_{40} 18/8). Another part was centrifuged at 17000 × *g* (B_{12} 28/8), 31000 × *g* and 100000 × *g* (B_{40} 28/8). Part of the protein solubilized after deoxycholate treatment and did not sediment during centrifugation, but the final supernatant did not reveal ATPase activity.

Partial heat denaturation of an enzyme preparation (B_{16} 1/4) was performed by incubating aliquots at 45° for 3 and 10 h. Protein was determined by a modification of the method of LOWRY *et al.*[8] using bovine albumin as standard.

Uniformly labelled g-strophanthin was obtained from New England Nuclear Corp. ([^{3}H]ouabain (G), lot number 184-125, 3.7 C/mmole) and diluted to the desired specific activities (0.5–50 C/mole) with unlabelled g-strophanthin (Merck). All chemicals used were analytical reagent grade.

Binding studies

With the following incubation medium, 5 mM Mg^{2+}, 2 mM EDTA, 10 mM Na^+, 3 mM ATP, and 60 mM Tris, pH 7.4, and at 37°, preliminary experiments seemed to show optimum conditions for g-strophanthin binding, *i.e.* a plateau level of binding was reached during maximally 30–45 min of incubation. The enzyme protein concentration was 0.1–0.3 mg/ml, in one case 0.6 mg/ml. At various intervals, 0.8–1.0-ml samples were withdrawn from the incubation medium and rapidly filtered through Sartorius membrane filters (pore size 0.6 μm) by suction. 500 μl of the filtrate were added to 10 ml of BRAY's scintillation mixture[9] and the radioactivity measured in a Packard Tri-Carb liquid scintillation counter with equipment for the estimation of efficiency. From the radioactivity in the filtrate and the total activity in the same volume of incubation medium added directly to the BRAY fluid[9] without filtration, percentage and absolute amount of bound g-strophanthin was calculated. Preliminary experiments had shown that no enzyme activity and a negligible amount of protein was filtered through the membrane filter.

RESULTS

In studies of the influence of the ionic milieu on g-strophanthin inhibition of the enzymatic hydrolysis of ATP and on g-strophanthin binding[5] it was found that Mg^{2+} was obligatory for g-strophanthin binding and that ATP or P_i promoted binding. Na^+ also influenced binding; its principal effect, when added to a medium containing Mg^{2+} and ATP or Mg^{2+} and P_i, seemed to be an acceleration or deceleration, respectively, of the rate of g-strophanthin binding. Hence the analysis of the kinetics of g-strophanthin binding and the elucidation of the stoichiometric relationship between binding capacity and ATPase activity was attempted as described in MATERIALS AND METHODS.

Incubation of enzyme with various concentrations of g-strophanthin leads to a decrease in the g-strophanthin concentration in the filtrate as compared with what was added to the incubation medium. The difference between the g-strophanthin concenration in the incubation medium and the filtrate is taken to represent the amount of g-strophanthin bound to the membranes. In Fig. 1A the percentage of added g-strophanthin which is bound to the membrane fragments from one enzyme preparation is plotted for g-strophanthin concentrations in the range from $1 \cdot 10^{-6}$–$1 \cdot 10^{-7}$ M. It is seen that the binding is a slow process, but after maximally 30–45 min a plateau level of binding is reached. The percentage of added g-strophanthin which is bound to the membranes increases when the g-strophanthin concentration decreases. From the equilibrium level in each experiment the absolute amount of bound g-strophanthin and the equilibrium concentration of free g-strophanthin can be calculated (mean of at least three samples). From Fig. 1B it is seen that the absolute amount of bound g-strophanthin increases with the g-strophanthin concentration in the medium until a certain saturation level is reached. These results suggest that under conditions where the ionic milieu is kept constant and the g-strophanthin concentration varies the reaction goes to an equilibrium which may be described by the simple equation:

$$E + G \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EG \qquad (1)$$

in which E represents the available part of the enzyme unless it has already bound

g-strophanthin, *i.e.* $E = EG_{max} - EG$, G free g-strophanthin (at equilibrium G_f), and EG the enzyme–strophanthin complex with the maximum value EG_{max}.

Relation between saturation level of g-strophanthin binding and ATPase activity

The observation that an increase in the g-strophanthin concentration leads to a final saturation level of bound g-strophanthin as shown in Fig. 1 offers the possibility of investigating how the experimentally determined maximal amount of g-strophanthin bound to a membrane preparation varies with the specific ATPase activity of the preparation. Results of such experiments are shown in Fig. 2. The different specific

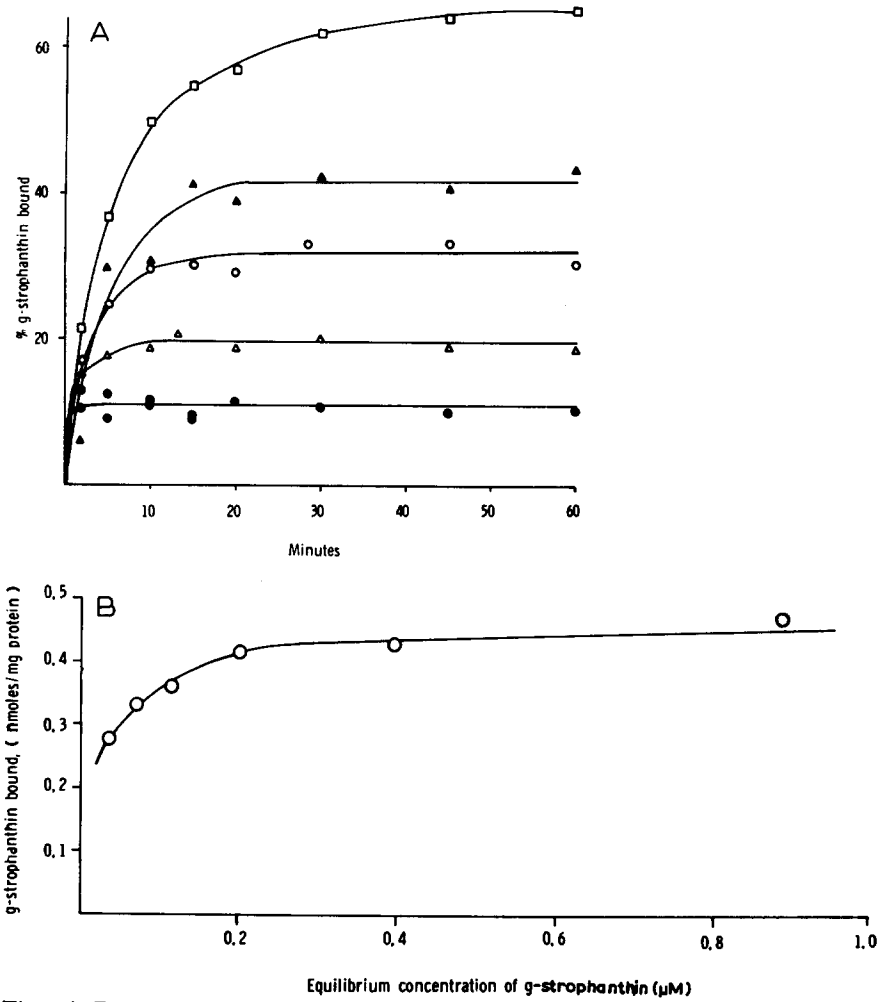


Fig. 1.A. Percentage of added g-strophanthin bound to a preparation of microsomes from ox brain as a function of time and with different initial g-strophanthin concentrations. (□—□, 0.1 μM; ▲—▲, 0.2 μM; ○—○, 0.3 μM; △—△, 0.5 μM; ●—●, 1 μM). Incubation medium, 5 mM Mg^{2+}, 2 mM EDTA, 10 mM Na^+, 3 mM ATP, and 60 mM Tris–HCl, pH 7.4 at 37°. Enzyme protein, 0.238 mg/ml. B. Absolute amount of bound g-strophanthin in nmoles per mg protein calculated from the equilibrium data in A and expressed as a function of the equilibrium concentration of free g-strophanthin.

activities were obtained as mentioned under MATERIALS AND METHODS using procedures (1), (2), and (3).

It is seen that the maximal binding of g-strophanthin increases proportionally with the specific activity of the preparation, and that this is the case both for the preparations which have not been fully activated and for those in which the activity has been decreased by heat denaturation after optimal activation.

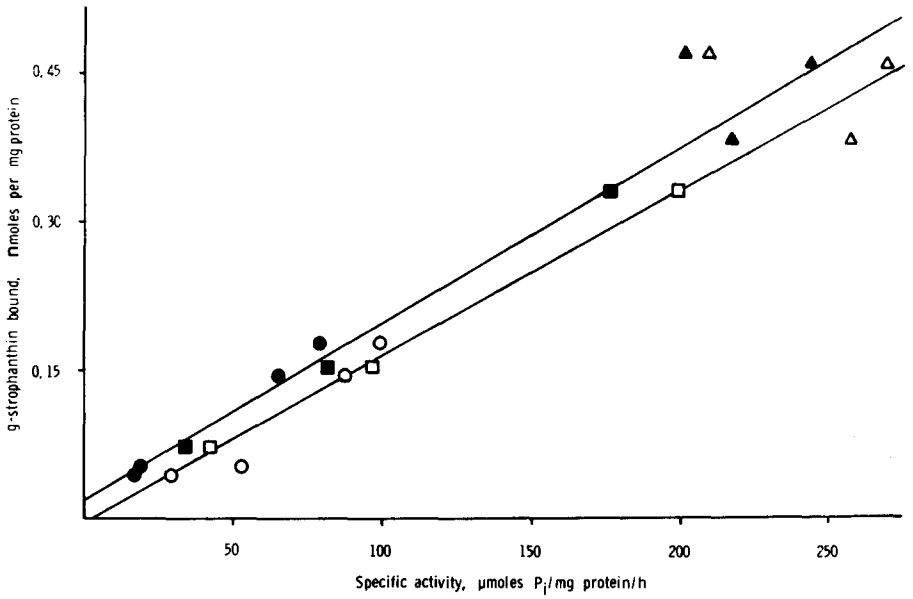


Fig. 2. Experimentally determined maximum amount of bound g-strophanthin per mg protein *versus* the specific total (open symbols) or ($Na^+ + K^+$)-activated ATPase activity (filled symbols). △—▲, binding to enzyme preparations optimally activated with deoxycholate; □—■, binding to enzyme preparation optimally activated and afterwards heat-denatured to two lower steps of activity by incubation at 45° for 3 h or 10 h; ○—●, binding to microsomal fraction before deoxycholate activation and to the same preparation after activation and differential centrifugation to increasing steps of specific activity.

Two objections may be raised against this method for determination of maximum binding. One is that at g-strophanthin concentrations that give nearly maximum binding the percentage of g-strophanthin removed from the solutions may be relatively low. The method measures the difference between added and free g-strophanthin, *i.e.* near saturation a small difference between two large figures, and this may introduce a considerable error in the determinations. The other is that the experimentally determined maximum binding may be lower than the real EG_{max}.

If, however, the binding of g-strophanthin follows the simple Eqn. 1, it should be possible by a kinetic analysis to determine EG_{max}, and by this to overcome the problems related to the direct measurements of the maximal binding.

Kinetics of g-strophanthin binding

If the reaction at equilibrium can be described by Eqn. 1, then plots of G_f/EG *versus* G_f should give straight lines according to the equation

$$\frac{G_f}{EG} = \frac{1}{EG_{max}}\left(\frac{k_{-1}}{k_1} + G_f\right) \qquad (2)$$

which represents an elaboration of the formula used for the more common Lineweaver–Burk plot. Equilibrium concentrations are used instead of velocities and the equation is multiplied by the factor G_f.

From the slopes of the lines and from the intercept with the ordinate, EG_{max} and the apparent dissociation constant, $K_s = k_{-1}/k_1$, can be calculated. Eqn. 2 is valid for molar concentrations (moles/l). In the experiments the amount of bound g-strophanthin was related to the protein concentration, *i.e.* expressed as specific values, EG/mg protein. Accordingly, EG of Eqn. 2 was divided by mg protein/l to obtain EG/mg protein.

In Figs. 3–5, G_f/EG is plotted as a function of G_f for the entire series of preparations used in the experiments shown in Fig. 2. For each of the preparations EG and G_f were determined at a number of different g-strophanthin concentrations as shown in Fig. 1. The regression line was drawn from the method of least squares.

For all the preparations the figures show that the points which relate G_f/EG to G_f fall on a straight line. This suggests that the binding of g-strophanthin measured under the experimental conditions used can be described by the simple model given in Eqn. 1. The slopes of the lines may therefore represent $(EG_{max})^{-1}$.

The values for EG_{max} are given in Table I. The table also shows the measured values for the maximum amount of g-strophanthin bound to the enzyme preparations. From the intercept and the slope of the lines, the apparent dissociation constant, K_s, has been calculated (Table I, column 6).

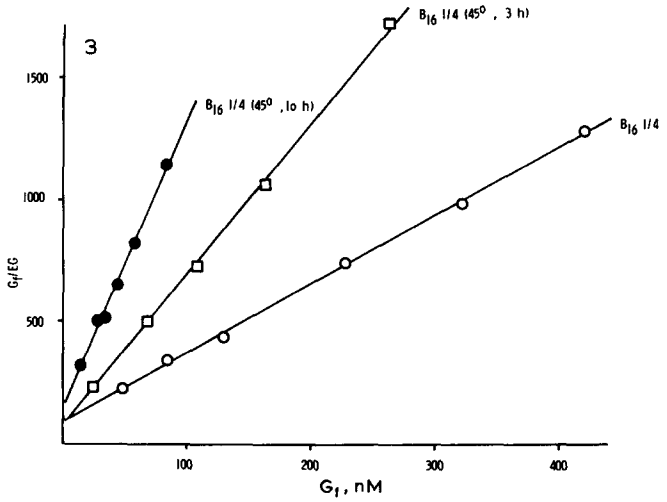
TABLE I

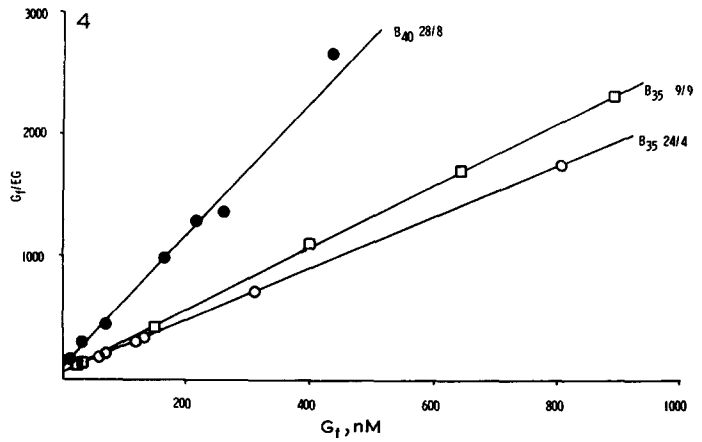
MAXIMUM g-STROPHANTHIN BINDING EXPERIMENTALLY DETERMINED AND CALCULATED BINDING CAPACITY, CORRESPONDING CALCULATED MOLECULAR ACTIVITIES AND APPARENT DISSOCIATION CONSTANT, K_s, FOR ENZYME PREPARATIONS WITH VARYING SPECIFIC ACTIVITIES

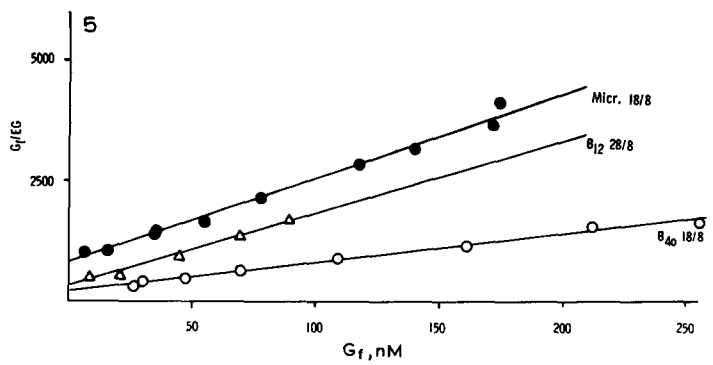
For further explanation, see text.

Enzyme preparation	*Specific activity (μmoles P_i/mg protein per h)*		*Max. g-strophanthin bound (nmoles/mg protein)*		*Calculated apparent dissociation constant, K_s (nM)*	*Molecular activity related to (Na^+-K^+)-activity (P_i released/min per site)*	
	Total	*(Na^++K^+)-activated*	*Measured*	*Calculated = EG_{max}*		*Calculated from max. binding measured*	*Calculated from binding capacity, EG_{max}*
Microsome fraction 18/8	30	17	0.044	0.0571	44.89	6454	4962
B_{12} 28/8	53	19	0.053	0.0660	19.24	6032	4798
B_{16}* 1/4	43	34	0.073	0.0853	12.39	7763	6643
B_{40} 18/8	88	66	0.144	0.1706	36.80	7639	6448
B_{40} 28/8	100	80	0.177	0.1761	10.82	7533	7571
B_{16}** 1/4	97	82	0.153	0.1619	10.96	8932	8441
B_{16} 1/4	200	177	0.330	0.3580	32.12	8939	8240
B_{35} 19/3	211	203	0.470	0.4816	34.61	7199	7025
B_{35} 9/9	259	218	0.383	0.3931	22.45	9487	9243
B_{35} 24/4	272	246	0.461	0.4769	26.78	8894	8597
Mean	—	—	—	—	25.11	7887	7197
± S.D.	—	—	—	—	± 11.91	±1150	±1510

* B_{16} 1/4, heat-denatured at 45° for 10 h.
** B_{16} 1/4, heat-denatured at 45° for 3 h.







Figs. 3, 4, and 5. Plots of G_f/EG (= free g-strophanthin concentration in nM/bound g-strophanthin in nmoles/mg protein) *versus* G_f (= the free g-strophanthin concentration in nM) at equilibrium for nine enzyme preparations. For further explanation, see text.

It is seen from the table that there is a good correlation between the calculated and experimentally determined EG_{max}, and that EG_{max} increases proportionally with the specific ATPase activities of the preparations. This is also illustrated in Fig. 6, which shows the EG_{max} calculated as a function of the specific enzyme activities.

Linear regression analysis gives high correlation coefficients, both when binding is related to $(Na^+ + K^+)$-activated and to $(Mg^{2+} + Na^+ + K^+)$-activated ATPase activity. One point (B_{35} 19/3) was omitted in this analysis as there were reasons to believe that the specific activity had been underestimated for this preparation. When the g-strophanthin binding is related to $(Na^+ + K^+)$-activated ATPase activity the curve in Fig. 6 is described by the equation $y = 0.00177x + 0.0319$ (ordinate intercept $\neq 0$;

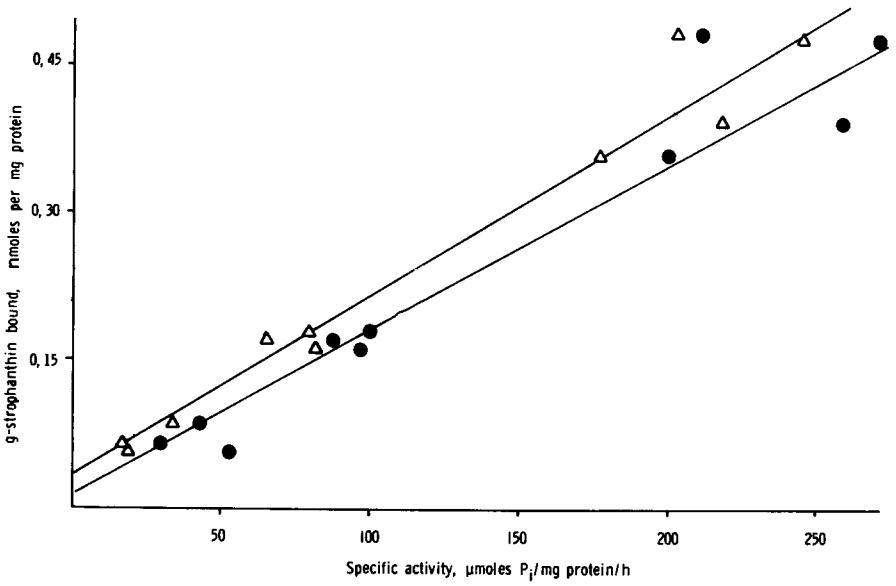


Fig. 6. Calculated maximum amount of bound g-strophanthin per mg protein (EG_{max}) as a function of the specific total (●—●) or $(Na^+ + K^+)$-activated (△—△) ATPase activity. The EG_{max} was calculated from the slope of the regression line drawn from the method of least squares on the binding data from the single enzyme preparation (Figs. 3–5).

TABLE II

CALCULATION OF MOLECULAR ACTIVITY BASED ON THE SLOPE OF LINEAR REGRESSION ANALYSIS ON ATPASE ACTIVITIES *versus* g-STROPHANTHIN-BINDING CAPACITIES

	Molecular activity (P_i *released per site per min*, ± *SE*)	
	*Based on measured max. binding of g-strophanthin**	*Based on calculated max. binding capacity of g-strophanthin***
Related to total activity	10 200 ± 500	10 100 ± 600
Related to $(Na^+ + K^+)$-activity	9 500 ± 300	9 400 + 300

* Calculated from the slope of the lines in Fig. 2.
** Calculated from the slope of the lines in Fig. 6.

$P = 0.05$), where y represents bound g-strophanthin in nmoles/mg protein and x the specific ($Na^+ + K^+$)-activity in μmoles P_i/mg protein per h.

Concerning the variation in the calculated dissociation constants as seen from Table I, see DISCUSSION.

If it is assumed that each g-strophanthin-binding site represents one hydrolysis site, it is possible to calculate the molecular activity or the catalytic centre activity. This is done in Table I from the measured and calculated maximum binding capacity of each preparation and in Table II from the slopes of the linear regression analysis on measured and calculated maximum binding capacities of all enzyme preparations. Molecular activity is related to both total and ($Na^+ + K^+$)-ATPase activity in Table II. By using the slope the assumption was made that the ordinate intercept represented unspecific binding.

The model presented here, based on equilibrium situations, requires a forward and a backward reaction. Having established an equilibrium level of bound g-strophanthin, the reversibility could be shown directly by addition of unlabelled g-strophanthin. If the ionic conditions were changed by addition of K^+ or EDTA (which complexes part of the Mg^{2+}; see Fig. 7) the binding level was similarly lowered towards a new equilibrium.

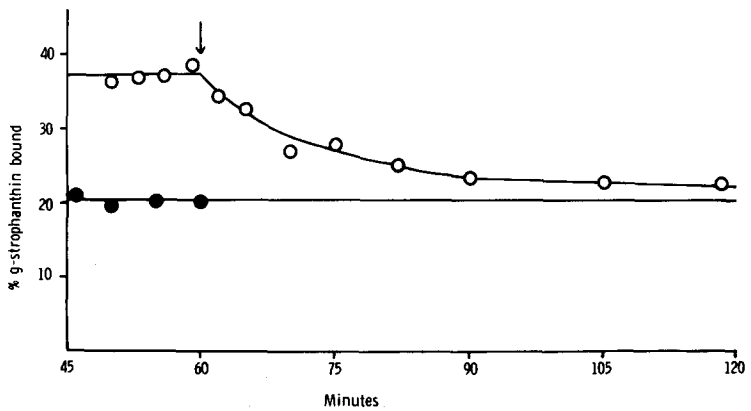


Fig. 7. Incubation of enzyme with $5 \cdot 10^{-8}$ M g-strophanthin in the same incubation medium as in Fig. 1A. Enzyme protein, 0.122 mg/ml; ($Mg^{2+} + Na^+ + K^+$)-activity, 188; ($Na^+ + K^+$)-activity, 141 μmoles P_i/mg protein per h. After 1 h, when an equilibrium level was established, addition of EDTA (arrow) to a final concentration of 12 mM (○—○). Incubation of enzyme as above except with an initial concentration of 12 mM EDTA (●—●).

DISCUSSION

It has been shown by several authors that the binding of cardiac glycosides to the ATPase system from membrane fragments depends on the ionic milieu of the incubation medium. In the present experiments the solution in which binding of g-strophanthin has been performed contains Mg^{2+}, ATP and Na^+ in concentrations which according to SKOU *et al.*[5] should give favourable conditions for reaction with g-strophanthin. Even with such conditions the rate of the enzyme–strophanthin reaction is remarkably slow. The observation that the bound amount reaches an equilibrium level depending on the concentration of g-strophanthin at equilibrium makes it possible to

devise a reaction scheme. Based on the time course of the binding curve, BAKER AND WILLIS[10] have suggested a similar model for intact cells. Since the equilibrium level obtained depends on the ionic milieu, the model probably represents an overall reaction scheme. An attempt at describing the possible kinetics of g-strophanthin binding, allowing for ionic requirements, demands a modification of the summarizing scheme. Consequently, the calculated dissociation constant may be an apparent constant covering more complex reactions. It also means that variations in the apparent dissociation constant do not disprove the summarizing scheme based on equilibrium levels. The lower dissociation constant after partial heat denaturation of the enzyme system may have some meaning. Moreover, it has been shown that K^+ greatly influences (increases) the apparent constant (O. HANSEN, unpublished observation, and ref. 10), and variation in small impurities of contamination with K^+ of the enzyme preparations easily explains the variable dissociation constant as shown in Table I.

Addition of unlabelled g-strophanthin reverses the process and thus supports the equilibrium model, which requires a forward and a backward reaction. Addition of K^+ or EDTA changes the ionic conditions and the reversibility is then presumably due to a lowering of that part of the enzyme system which is now available for g-strophanthin binding. That part of the enzyme which is in equilibrium with the enzyme–strophanthin complex is diminished; the complex itself is probably not affected directly.

The relationship between g-strophanthin-binding capacity and ATPase activity is evident, and binding does not seem to be related to latent ATPase activity until treatment with detergents has exposed this. The results thus do not support the finding of ELLORY AND SMITH[11] that the effect of detergents on intestinal ATPase from fish is an acceleration of the turnover number per g-strophanthin-binding site. The question whether binding should be related to total activity or to $(Na^+ + K^+)$-activity only, is unsolved and is connected with the question whether these enzymes are quite different or related. g-Strophanthin inhibits only the $(Na^+ + K^+)$-activated ATPase, but still the Mg^{2+}-activated part could be due to the same enzyme which might be partly changed or "go wrong" when there is no Na^+ and K^+ in the medium[12].

The concentration of the enzyme system expressed as g-strophanthin equivalents is difficult to compare with the results of others as no specific activities are reported. However, the findings are in agreement with the results of binding experiments on human erythrocyte ghosts[13]. Based on this, ELLORY AND KEYNES calculated a catalytic centre activity of 10500 min^{-1}. Also the catalytic centre activities calculated by ALBERS *et al.*[2] from g-strophanthin binding, and by BADER *et al.*[14] from ^{32}P incorporation experiments for mammalian tissues are of the same order of magnitude as found here, indicating a one-to-one correlation between g-strophanthin binding and phosphorylation. As we made no correction for unspecific binding in Table I, this will especially affect the calculated molecular activity of preparations with low specific activities. Assuming that the ordinate intercept represents unspecific binding when binding capacity is plotted *versus* ATPase activity (Fig. 7), correction for unspecific binding is possible and makes the molecular activity of the preparations with lower specific activity coincide with the molecular activity of the preparations with higher activity. For preparations of high specific activity the calculated molecular activity approaches the value calculated from the slope of the linear regression analysis on the entire series of preparations.

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