Br. J. Pharmac. (1977), **61**, 423–428

EVIDENCE FOR TWO DIFFERENT Na^+-DEPENDENT [^{3}H]-OUABAIN BINDING SITES OF A Na^+-K^+-ATPASE OF GUINEA-PIG HEARTS

U. FRICKE & W. KLAUS

Pharmakologisches Institut der Universität zu Köln, Gleueler Str. 24, D-5000 Köln 41, Germany

1 The influence of various Na^+ concentrations on [^{3}H]-ouabain binding was studied in experiments on a microsomal Na^+-K^+-adenosine triphosphatase (ATPase) from guinea-pig hearts.

2 The ATP-independent cardiac glycoside binding was not influenced by increasing Na^+ concentrations. However, a good correlation was found between the ATP-dependent [^{3}H]-ouabain binding and Na^+ concentration.

3 A more detailed analysis of these results according to Hofstee (1952) revealed two distinct processes involved in this interaction: one ouabain binding process was activated at rather low Na^+ concentrations, ($K_{0.5}$ = 4.5 mM); this type of [^{3}H]-ouabain binding was strongly correlated to the Na^+ concentration necessary for half maximum phosphorylation ($K_{0.5}$ = 1 mM). The other ouabain binding process was predominant at high Na^+ concentrations ($K_{0.5}$ = 69 mM).

4 On the basis of the commonly accepted ATPase reaction cycle a model for the interaction of cardiac glycosides with the Na^+-K^+-ATPase is proposed, assuming two different binding sites for cardiac glycosides (E_2–P and E_1–P) and involving a translocation of these drugs from an outer to an inner compartment of the cell membrane.

Introduction

In experiments on isolated heart muscle preparations the extracellular Na^+ concentration regulates both the inotropic and toxic response of the myocardium to cardiac glycosides: lowering the Na^+ concentration in the perfusion medium results in a delayed onset of the inotropic action (Talbot, 1968), and in a reduced positive inotropic effect (Reiter, 1963; Caprio & Farah, 1967). Furthermore, it is possible to reduce cardiac glycoside-induced toxicity by decreasing the external Na^+ concentration (Toda & West, 1966). On the other hand, a certain dissociation between the therapeutic and the toxic actions of cardiac glycosides was observed with varying extracellular Na^+ concentrations as at low extracellular Na^+ concentrations the toxicity appeared 'earlier', i.e. at a lower inotropic level, than at high extracellular Na^+ concentrations (Klaus & Fricke, 1974). The same dependence on extracellular Na^+ concentration could be demonstrated in studies on the myocardial uptake of cardiac glycosides. Again, a decrease in the external Na^+ concentration led to a decreased uptake of cardiac glycosides in guinea-pig isolated hearts (Dutta & Marks, 1969) or in dogs *in vivo* (Harrison & Wakim, 1969). Similar to this Na^+ dependence of the positive inotropic effect, the toxic action and the myocardial uptake of cardiac glycosides, is the digitalis-induced inhibition of Na^+-K^+-ATPase. This is more pronounced at high Na^+ concentrations (Repke & Portius, 1963; Lindenmayer, 1970). The latter observation is in good agreement with results obtained by Matsui & Schwartz (1968) who showed that the binding of [^{3}H]-digoxin to a Na^+-K^+-dependent adenosine triphosphatase (ATPase) from cardiac tissue was stimulated by Na^+ in the presence of ATP and Mg^{2+}.

The purpose of the present study was to analyse this interaction between Na^+ and cardiac glycosides in a more detailed way using a microsomal Na^+-K^+-ATPase from guinea-pig hearts. In addition, a model relating the results to the mode of action of cardiac glycosides is proposed.

Methods

Guinea-pig heart Na^+-K^+-ATPase preparation

Na^+-K^+-ATPase from guinea-pig hearts was freshly prepared before use by a slight modification of the procedure of Fricke & Klaus (1974). The hearts of 6 or 7 guinea-pigs (400–500 g) were minced in a micromincer (Braun, Germany) and homogenized (Automatic cell homogenizer, Colora, Germany, 20 strokes/min) in an isotonic sucrose solution (180 mM

sucrose, 100 mM imidazole hydrochloride, 1 mM disodium edetate (Na_2-EDTA), pH 7.4) to give a 15% suspension (g/g). After sedimentation of the resulting homogenate for 15 min at 10 000 *g* (Beckman Spinco L2 65B), the supernatant was passed through a Millipore filter (Type SCWP, 8 µm) and then applied to a Sepharose 4B column (30 cm in height, 5 cm diameter; Pharmacia, Sweden) equilibrated with a KCl-buffer-solution (500 mM KCl, 5 mM imidazole hydrochloride, 1 mM Na_2-EDTA, pH 7.4). Ascending elution of the enzyme was carried out at a flow of 0.8 ml/min maintained by means of a peristaltic pump (MP 4 Ismatec, Switzerland). The eluate was dialyzed on-line against distilled water using two sequential hollow-fibre membrane cartridges (Type H1DX50, Amicon, Netherlands), the flow rate of the dialysate being 10 ml/minute. The enzyme was stored in an imidazole buffer medium (100 mM imidazole hydrochloride, 1 mM Na_2-EDTA, pH 7.4). The contamination with K^+ was checked by flame-photometry and was found to be between 0.01 to 0.04 mM. All steps of the enzyme preparation were carried out at 4°C. The total ATPase activity (5 mM $MgCl_2$, 100 mM NaCl, 5 mM KCl) was 1.61 ± 0.12 units/mg protein ($n = 7$), the Mg^{2+}-dependent ATPase (5 mM $MgCl_2$) was 0.87 ± 0.09 units/mg protein ($n = 7$). One enzyme unit represents the amount of enzyme hydrolyzing 1 µmol ATP per min at 37°C.

[^{3}H]-ouabain binding studies

The binding studies were performed in polyethylene tubes (Sarstedt, Germany) at 37°C. The incubation medium contained the enzyme preparation (about 0.3 mg of protein per 4.0 ml), 5 mM $MgCl_2$, various concentrations of NaCl (0–140 mM), 1 mM Na_2-EDTA and 100 mM imidazole-hydrochloride (pH 7.4) in the presence or absence of 2 mM Na_2-ATP (Serva, Germany). Choline chloride was substituted for NaCl to maintain osmolarity. After a preincubation time of 10 min the reaction was started by the addition of [^{3}H]-ouabain (New England Nuclear Corporation; specific activity: 12 Ci/mmol at various concentrations (5×10^{-9} M to 1×10^{-6} M, final concentration). Unless indicated otherwise the reaction was stopped after 30 min by immersion in an ice bath (5 minutes). Then the mixture was centrifuged at 40,000 *g* for 30 min at 0°C (Beckman Spinco L2 65B). The resulting sediment and an aliquot of the original incubation medium were each mixed with 10 ml of Tritosol scintillation cocktail (Fricke, 1975) and counted in a liquid scintillation counter (Mark II, Nuclear Chicago, U.S.A.). Correction for quenching was performed by the external standard channel ratio method. ATP-dependent [^{3}H]-ouabain binding ('specific binding') was calculated by subtracting the phosphate-ligand independent [^{3}H]-ouabain binding from the [^{3}H]-ouabain binding in the presence of ATP. The [^{3}H]-ouabain binding is expressed as pmol ouabain per mg protein.

Phosphorylation studies

The phosphorylation of the Na^+-K^+-ATPase preparation was carried out in polycarbonate tubes (Beckman, U.S.A.) at 37°C slightly modified according to Neufeld & Levy (1970). The incubation medium contained the enzyme preparation (about 0.3 mg of protein per 2.3 ml), 5 mM $MgCl_2$, various concentrations of NaCl (0 to 140 mM), 1 mM Na_2-EDTA and 100 mM imidazole hydrochloride (pH 7.4). Substitution for NaCl was again made with choline chloride. After a preincubation time of 15 min the reaction was started (with vigorous shaking) by the addition of 100 µM (final concentration) [^{32}P]-ATP (New England Nuclear Corporation, U.S.A.; specific activity: 41.6 Ci/mmol), stopped after 10 s by the addition of 20 ml of an ice-cold trichloroacetic acid solution (400 mM) containing 0.6 mM Na_2-ATP and 0.6 mM K_2HPO_4. The mixture was then quickly centrifuged at 25,000 *g* for 10 min (Beckman Spinco L2 65B). The pellet was resuspended in 20 ml of an ice-cold trichloroacetic acid solution (400 mM) and recentrifuged and washing was repeated. The final sediment was solubilized for 15 min at 100°C in 1.5 ml of a solution containing 200 mM NaOH and 200 mM Na_2CO_3. Aliquots of the initial incubation medium and the solubilized labelled enzyme were each mixed with 10 ml of Tritosol (see above) and counted in a liquid scintillation counter (Mark II, Nuclear Chicago, U.S.A.). Correction for quenching was carried out by the external standard channel ratio method. The terminal phosphate incorporation was calculated on the basis of pmol ^{32}P per mg protein after individual correction for the protein recovery. The mean protein recovery was 63%.

Miscellaneous

Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951) using Labtrol (Merz & Dade, Germany) as a standard. All data were analysed by standard statistical methods (mean value and s.e. mean, Student's *t*-test, regression analysis). The given half times ($T_{\frac{1}{2}}$) and the exponential curve-fitting were computed using the BMDO7B (Biomedical computer programs, Univ. California, Los Angeles, U.S.A., non-linear least square method according to Hartley, 1961, CMS-version, programmed by P. Gille, Inst. f. Biometrie, Med. Hochschule, Hannover, Germany) on an IBM 360/67. All reagents unless stated otherwise were of reagent grade and were obtained from Merck AG (Germany).

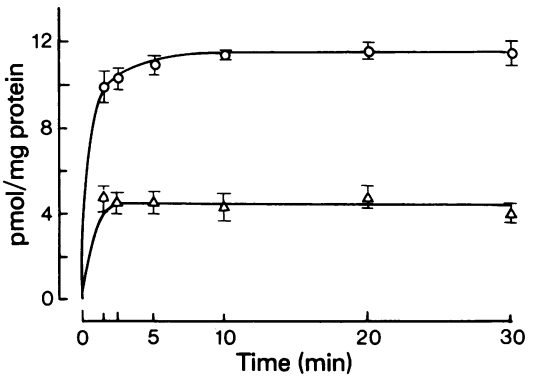

Figure 1 Time course of [^{3}H]-ouabain binding to a microsomal Na^+-K^+-ATPase from guinea-pig hearts in the presence (○) and absence (△) of ATP. Na^+ concentration: 146 mM; [^{3}H]-ouabain concentration: 5×10^{-7} M. For details see Methods section. The means of 3–4 single experiments are given. Vertical lines show s.e. means.

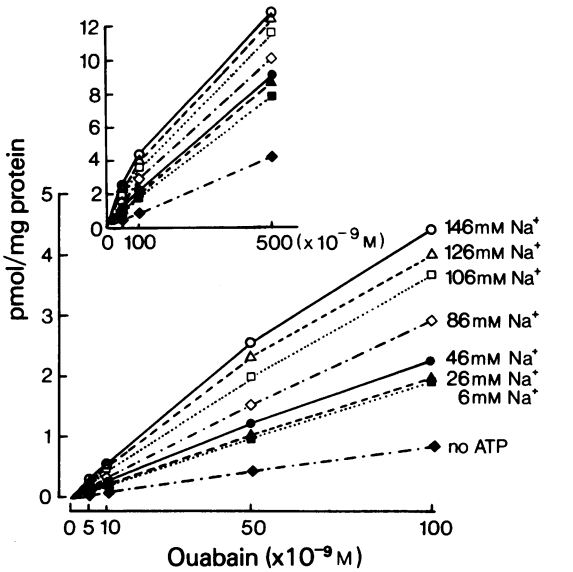

Figure 2 Na^+-dependence of [^{3}H]-ouabain binding to a microsomal Na^+-K^+-ATPase of guinea-pig hearts at different [^{3}H]-ouabain concentrations (abscissa scale, in M) in the presence and absence of ATP. For details see Methods section. The means of 3–4 single experiments are shown.

Results

Time-dependence of [^{3}H]-ouabain binding

The binding of [^{3}H]-ouabain to the Na^+-K^+-ATPase preparation showed very short half times ($T_{\frac{1}{2}}$). Depending on the external Na^+ concentration the mean half times varied between 159 s (6 mM Na^+) and 54 s (146 mM Na^+). There was no significant difference in the time course of [^{3}H]-ouabain binding between the experiments in the presence and in the absence of ATP. Maximum [^{3}H]-ouabain binding was attained within about 5 min under all conditions studied (Figure 1).

Na^+-dependence of [^{3}H]-ouabain binding

The binding of [^{3}H]-ouabain to the Na^+-K^+-ATPase preparation was studied within a concentration range of the cardiac glycoside of 5×10^{-9} M to 1×10^{-6} M using an incubation period of 30 min (Figure 2). In the presence of Mg^{2+} and Na^+ the [^{3}H]-ouabain binding was significantly dependent on ATP. [^{3}H]-ouabain binding in the range of 6 mM to 146 mM Na^+ and in the presence of ATP and Mg^{2+} was significantly activated by increasing the Na^+ concentration. However, in the absence of ATP there was no dependence of the [^{3}H]-ouabain binding on the Na^+ concentration in the incubation medium. The [^{3}H]-ouabain binding in the presence of ATP nearly equalled the ATP-independent binding at high concentrations of the cardiac glycoside (1×10^{-6} M; not shown).

When the data were analysed according to Hofstee (1952), which unlike other plots (e.g. Scatchard-plot), favours the detection of side reactions, two different [^{3}H]-ouabain binding processes depending on the Na^+-concentration in the incubation medium could be distinguished (Figure 3); one [^{3}H]-ouabain binding site predominant at low Na^+ concentrations with a Na^+ concentration for half maximum binding ($K_{0.5}$) of 4.5 mM ('high affinity binding site'), and a second [^{3}H]-ouabain binding site prevailing at high Na^+ concentrations ($K_{0.5} = 69$ mM; 'low affinity binding site'). The calculated maximum binding capacities of the 'low' and the 'high' affinity site decreased with decreasing [^{3}H]-ouabain concentrations in the incubation medium, parallelled by an increase of the Na^+ concentration necessary for half maximum [^{3}H]-ouabain binding. A plot of the calculated maximum binding capacities ('low' and 'high' affinity binding process) obtained at the different [^{3}H]-ouabain concentrations studied in the presence of various Na^+ concentrations against the respective ouabain concentration is shown in Figure 4.

Phosphorylation of the Na^+-K^+-ATPase

The influence of increasing the Na^+-concentration from 2 mM to 142 mM on the phosphorylation of the guinea-pig heart Na^+-K^+-ATPase preparation by [^{32}P]-ATP is shown in Figure 5a. A maximum phosphorylation in the range of 150 pmol ^{32}P/mg

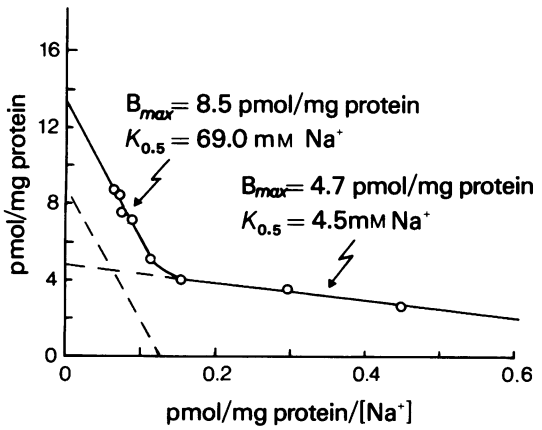

Figure 3 Hofstee-plot of the influence of increasing Na^+ concentrations on the ATP-dependent [^{3}H]-ouabain binding to a microsomal Na^+-K^+-ATPase of guinea-pig hearts. [^{3}H]-ouabain concentration: 5×10^{-7} M. Data according to Figure 2.

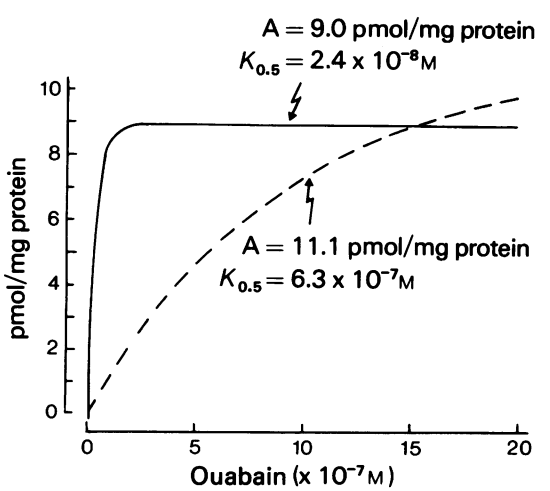

Figure 4 Plot of the calculated maximum binding capacities at various Na^+ concentrations versus the respective ouabain-concentrations in the incubation medium. The maximum binding capacities are obtained from the data presented in Figure 2 analysed according to Hofstee (1952). The solid line represents the maximum [^{3}H]-ouabain binding in the presence of high Na^+ ('low affinity Na^+-site'), the dashed line represents the respective data obtained at low Na^+-concentrations ('high affinity Na^+-site'). The curve-fit was obtained assuming a single exponential function ($y = a(1 - e^{-ct})$). For details see Methods section.

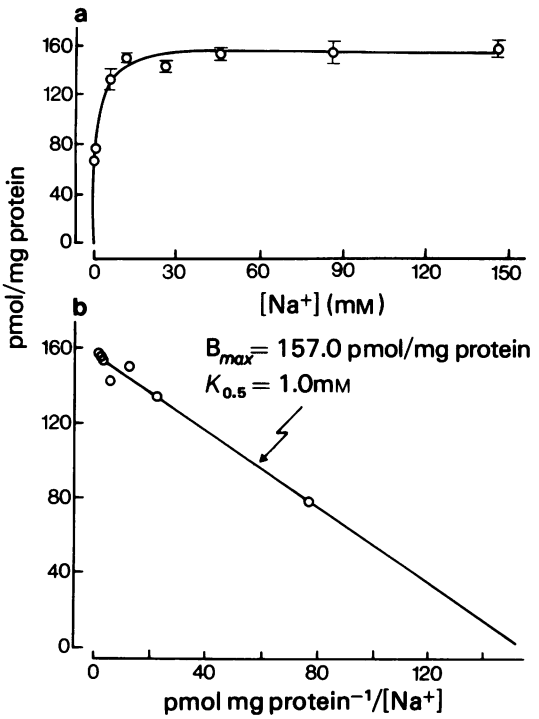

Figure 5 (a) Na^+-dependence of the phosphorylation of a microsomal Na^+-K^+-ATPase of guinea-pig hearts. For details see Methods section. The means of 4 single experiments are given. Vertical lines show s.e. means. (b) Hofstee-plot of the data presented in Figure 5a.

protein could be obtained at 22 mM Na^+. An analysis of these data according to Hoffstee (1952) revealed one phosphorylation reaction with a calculated maximum binding capacity of 157 pmol ^{32}P/mg protein and a Na^+ concentration of 1.0 mM for half maximum phosphorylation ($K_{0.5}$) of the enzyme preparation (Figure 5b).

Discussion

Na^+ plays an important role in the modulation of the cardiac glycoside action in isolated heart muscle preparations, in the myocardial uptake of these drugs and in the inhibitory action of digitalis on the Na^+-K^+-ATPase activity (see Introduction).

A more detailed analysis of the influence of Na^+ on the binding of ouabain to a microsomal Na^+-K^+-ATPase based on the results of the present study revealed two distinct processes involved in this interaction (Figure 3). One binding site is activated at rather low Na^+ concentrations with a calculated Na^+

concentration for half maximum ouabain binding between 8.1 mM and 4.5 mM (decreasing with increasing cardiac glycoside concentrations in the incubation medium). These $K_{0.5}$ values for Na^+ are in good agreement with the Na^+ concentration necessary for half maximum phosphorylation ($K_{0.5} = 1.0$ mM; Figure 5a, b). Therefore, it is tempting to assume on the basis of the commonly accepted ATPase reaction cycle (Post, Kume, Tobin, Orcutt & Sen, 1969) and the results obtained recently by Hegyvary (1976) that the binding of ouabain to the phosphorylated intermediate of the enzyme at low Na^+ concentrations reflects the interaction with the (E_1–P)-conformation. The other ouabain binding process predominant at high Na^+ concentrations with a Na^+ concentration for half maximum ouabain binding between 479 mM and 69 mM (decreasing with increasing cardiac glycoside concentrations in the incubation medium) is the result of a different activating influence of the Na^+ ions and seems to be more related to the (E_2–P)-conformation (see also Figure 6).

If the calculated maximum binding capacities of the binding process with either low affinity or high affinity for Na^+ obtained at different ouabain concentrations in the incubation medium are plotted against the actual cardiac glycoside concentration in the incubation medium, two different 'dissociation constants' (indicating the ouabain concentration necessary for half maximum ouabain binding at various Na^+ concentrations) can be obtained (Figure 4). As the result of a 'positive cooperativity' of Na^+ and ouabain the maximum binding capacity of both processes is nearly identical.

A similar differentiation of the ouabain binding sites by Na^+ as described above was also observed by Inagaki, Lindenmayer & Schwartz (1974) in studies on a Na^+-K^+-ATPase from the outer medulla of canine kidney. However, these authors suggested the ouabain binding site had only the (E_2–P)-conformation. In their opinion the two different Na^+ concentration ranges were supposed to be necessary for the formation (low Na^+) and for the stabilization (high Na^+) of the ouabain enzyme complex.

On the basis of the ATPase reaction sequence proposed by Post *et al.* (1969) and the above interpretations of our results, a model for the cardiac glycoside ATPase interaction was developed which involves translocation of ouabain from an outer to an inner compartment of the cell membrane (Figure 6): (E_1) representing the non-phosphorylated enzyme facing inward is phosphorylated by ATP resulting in the high energy intermediate (E_1–P). A conformational change of the enzyme leads then to (E_2–P) facing outwards. The following dephosphorylation of this complex results in the non-phosphorylated intermediate of the enzyme (E_2) which returns by another conformational change to the inward facing enzyme (E_1). The two conformational changes of the enzyme (E_1–P → E_2–P and E_2 → E_1 are connected with the active transmembranous transport of Na^+ and K^+.

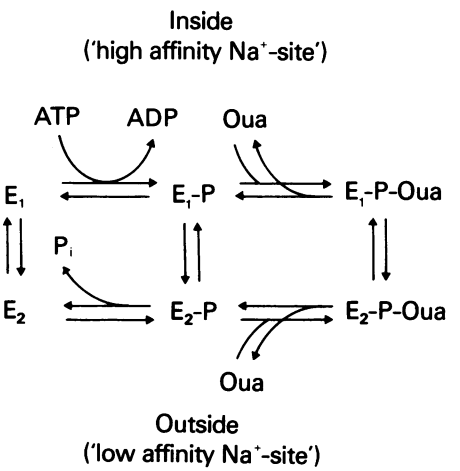

Figure 6 Proposed interaction scheme of cardiac glycosides (Oua) and Na^+-K^+-ATPase. For details see text.

The favoured binding site for cardiac glycosides within this system is the (E_2–P)-conformation. In our opinion this reaction is activated by rather high Na^+ concentrations ('low affinity Na^+-site') and results in a Na^+-dependent transport of cardiac glycosides from an outer to an inner compartment of the cell membrane (E_2–P · Oua → E_1–P · Oua). On the other hand, cardiac glycosides may bind the inward facing complex (E_1–P) at rather low Na^+ concentrations ('high affinity Na^+-site'), if relevant intracellular drug concentrations are present. Therefore the latter reaction step may represent an outward transport of cardiac glycosides.

This proposed carrier-mediated translocation system for cardiac glycosides operative in the heart muscle cell membrane is comparable (on the basis of the Na^+-dependence and the saturability) with other specific active transport mechanisms for cardiac glycosides observed in the small intestine (see Lauterbach, 1975) and the liver (Kupferberg & Shanker, 1968).

The skilful technical assistance of Miss Christa Busche and Miss Eva Kruse is greatly appreciated. The study was supported by a grant from the Deutsche Forschungsgemeinschaft (Fr 407/3). Some of these results were presented to the 8th International Meeting of the International Study Group for Research in Cardiac Metabolism, Tokyo, May 1976.

References

CAPRIO, A. & FARAH, A. (1967). The effect of the ionic milieu on the response of rabbit cardiac muscle to ouabain. *J. Pharmac. exp. Ther.*, **155**, 403–414.

DUTTA, S. & MARKS, B.H. (1969). Factors that regulate ouabain-H3 accumulation by the isolated guinea-pig heart. *J. Pharmac. exp. Ther.*, **170**, 318–325.

FRICKE, U. (1975). Tritosol: A new scintillation cocktail based on Triton X-100. *Analyt. Biochem.*, **63**, 555–558.

FRICKE, U. & KLAUS, W. (1974). A simple preparation technique for a microsomal Na^+-K^+-activated ATPase from cardiac tissues of different species. *Prep. Biochem.*, **4**, 13–29.

HARRISON, C.E. & WAKIM, K.G. (1969). Inhibiton of binding of tritiated digoxin to myocardium by sodium depletion in dogs. *Circulation Res.*, **24**, 263–268.

HARTLEY, H.O. (1961). Modified Gauss-Newton method for fitting of non linear regression functions. *Technometrics*, **3**, 269–280.

HEGYVARY, C. (1976). Ouabain binding and phosphorylation of ($Na^+ + K^+$) ATPase treated with N-ethylmaleimide or oligomycin. *Biochim. Biophys. Acta*, **422**, 365–380.

HOFSTEE, B.H.J. (1952). On the evaluation of the constants V_m and K_m in enzyme reactions. *Science*, **116**, 329–331.

INAGAKI, C., LINDENMAYER, G.E. & SCHWARTZ, A. (1974). Effects of sodium and potassium on binding of ouabain to the transport adenosine triphosphatase. *J. biol. Chem.*, **249**, 5135–5140.

KLAUS, W. & FRICKE, U. (1974). Interactions of cardiac glycosides and electrolytes at the cellular and subcellular level. *Second Congress of the Hungarian Pharmacological Society*, Budapest, 2–5 October 1974, R5/21.

KUPFERBERG, H.J. & SCHANKER, L.S. (1968). Biliary secretion of ouabain-^{3}H and its uptake by liver slices in the rat. *Am. J. Physiol.*, **214**, 1048–1053.

LAUTERBACH, F. (1975). Resorption und Sekretion von Arzneistoffen durch die Mukosaepithelien des Gastrointestinaltraktes. *Arzneim.-Forsch.*, **25**, 479–488.

LINDENMAYER, G.A. (1970). Biochemical responses of the cardiac cell to various stresses, a molecular mechanism for cardiac glycoside-induced compensation. *Thesis*, Houston, Texas.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.I. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

MATSUI, H. & SCHWARTZ, A. (1968). Mechanism of cardiac glycoside inhibition of the (Na^+-K^+)-dependent ATPase from cardiac tissue. *Biochim. Biophys. Acta*, **151**, 655–663.

NEUFELD, A.H. & LEVY, H.M. (1970). The steady state level of phosphorylated intermediate in relation to the two sodium-dependent adenosine triphosphatases of calf brain microsomes. *J. biol. Chem.*, **245**, 4962–4967.

POST, R.L., KUME, S., TOBIN, T., ORCUTT, B. & SEN, A.K. (1969). Flexibility of an active center in sodium-plus-potassium adenosine triphosphatase. *J. gen. Physiol.*, **54**, 306–326S.

REITER, M. (1963). The relation of calcium and sodium to the inotropic glycoside effect. *Naunyn Schmiedebergs Arch. exp. Path. Pharmak.*, **245**, 487–499.

REPKE, K. & PORTIUS, H.J. (1963). Über die Identität der Ionenpumpen-ATPase in der Zellmembran des Herzmuskels mit einem Digitalis-Rezeptorenenzym. *Experientia*, **19**, 452–458.

TALBOT, M.S. (1968). Sodium dependence of the rate of onset of the ouabain-induced positive inotropic effect on cardiac muscle. *Nature, Lond.*, **219**, 1053–1054.

TODA, N. & WEST, T.C. (1966). Modification by sodium and calcium of the cardiotoxicity induced by ouabain. *J. Pharmac. exp. Ther.*, **154**, 239–249.

(Received March 24, 1977)