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Binding of Digoxigenin to Sodium- and Potassium-Dependent Adenosine Triphosphatase

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SUMMARY

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[12α-^{3}H]Digoxigenin was prepared by the reduction of 12-dehydrodigoxigenin with NaB^3H_4. Binding to the sodium- and potassium-dependent adenosine triphosphatase was studied at room temperature. Digoxigenin bound to the enzyme with high affinity. This binding was eliminated by prior ouabain treatment and was dependent on the presence of ligands. As in cardiac glycoside binding, mixtures of Mg^{2+} and P_i or of Na^+, Mg^{2+}, and ATP were both effective as ligands. Scatchard plots of binding were linear and showed that the dissociation constant did not change at different concentrations of each ligand except in the very low concentration range, but that the number of binding sites on the enzyme was reduced on descreasing the ligand concentration. The present results show that the binding of digoxigenin to the (Na^+ + K^+)-ATPase does not follow the usual equation representing a reversible reaction. At saturating concentrations of ligands, i.e., 2 mM Mg^{2+} and 2 mM P_i, or 50 mM Na^+, 2 mM Mg^{2+}, and 2 mM ATP, the number of binding sites was close to the number of ouabain binding sites for each set of ligands. The dissociation constant was 0.041 μM in the Mg^{2+}-P_i system and 0.078 μM in the Na^+-Mg^{2+}-ATP system. These dissociation constants are lower than the I_{50} value of digoxigenin (0.4 μM) under the conditions used for assay.

INTRODUCTION

The sodium- and potassium-dependent adenosine triphosphatase, which is considered to be integral to the active transport of Na^+ and K^+, is specifically inhibited by cardiotonic steroids. This inhibition is an excellent model of drug-receptor interaction at the molecular level. Experiments using the radioactive cardiac glycosides ouabain and digoxin (1-4) have established that a cardiac glycoside-enzyme complex is formed in the presence of certain ligands. The two most effective systems are the Mg^{2+}-P_i and Na^+-Mg^{2+}-ATP systems (2). The equilibrium or steady-state condition of binding between (Na^+ + K^+)-ATPase and cardiac glycoside (5, 6) requires a long time, and at low temperatures the binding of cardiac glycosides is nearly irreversible. This contrast with the binding of cardiac aglycones, which is reversible. It is suggested that the cardiac glycoside binds to (Na^+ + K^+)-ATPase at a steroid site and a sugar site (7).

The dissociation constant, association rate constant, and dissociation rate constant of the ouabain-enzyme complex and ligand effects on these constants have been

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studied by several laboratories, using tritium-labeled ouabain (5, 6, 8–12). We have also measured the association and dissociation rate constants of various cardiac glycosides at various temperatures by a different method, and not only have confirmed the binding between the sugar moiety of cardiac glycosides and $(Na^+ + K^+)$-ATPase but also have shown that certain functional groups in the sugar moiety bind to a specific site(s) of the enzyme (13–15).

On the other hand, it has been known for many years, as described by Fieser and Fieser (16), that the toxicity of cardiac glycosides is associated with the steroid portion, although the aglycone is less active than its glycoside, and that the binding site of the cardiac aglycone involves a 17β-unsaturated lactone and a 14β-hydroxyl group (17). Our studies described above suggest that binding of the steroid moiety to the enzyme is the first step in cardiac glycoside binding and that binding of the glycoside moiety follows. Since the cardiac monoglycoside binding is nearly irreversible but the aglycone binding is reversible, it appears that binding of the glycoside moiety shifts the equilibrium of aglycone binding in the direction of less dissociation. Such a binding mechanism raised the question whether the ligands necessary for cardiac glycoside binding are needed for binding of the steroid moiety or for binding of the glycoside moiety. Studies of interaction between cardiac steroids and $(Na^+ + K^+)$-ATPase have involved only inhibition of the ATPase activity, and ligand effects on this interaction, which might answer the above question, have not yet been reported.

The purpose of the present study is to measure the direct binding of [12α-^{3}H]digoxigenin to a $(Na^+ + K^+)$-ATPase preparation and to examine the effects of ligands necessary for this steroid binding.

MATERIALS AND METHODS

The enzyme preparation consisted of microsomes prepared from fresh or frozen beef brain (Pel-Freez Biologicals) by the method of Uesugi *et al.* (18). They were treated further with NaI by the method of Nakao *et al.* (19) according to the modification of Hegyvary and Post (20), and washed by suspension with 0.5 mM $Tris_3$-EDTA and subsequent centrifugation at 48,000 × *g* for 30 min. The washing procedure was repeated several times, until the NaI-treated microsomes did not sediment completely on centrifugation. The whole enzyme preparation was then sedimented by centrifugation (about 100,000 × *g* for 30 min) and stored as a suspension in 0.5 mM $Tris_3$-EDTA at −70°. No significant differences in the binding of digoxigenin were observed with several different batches of the enzyme prepared from fresh or frozen brain.

Tritium-labeled sodium borohydride and uniformly labeled [^{3}H]ouabain were obtained from New England Nuclear Corporation. Unlabeled ouabain, digoxigenin, and Tris-ATP were purchased from Aldrich Chemical Company, Boehringer/Mannheim, and Sigma Chemical Company, respectively.

Enzyme activity was measured by the lactate dehydrogenase–pyruvate kinase linked spectrophotometric method as reported previously (13), and protein was determined by the method of Lowry *et al.* (21). Specific activity is expressed as micromoles of P_i liberated per milligram of protein per hour at 25°.

All radioactivity was measured with a Packard Tri-Carb liquid scintillation spectrometer, using 5 ml of scintillation medium (5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 liter of toluene and 500 ml of Triton X-100) (22).

Synthesis of [12α-^{3}H]-digoxigenin. Reduction of the carbonyl to the hydroxyl group by sodium borohydride was used to prepare the tritium-labeled compound [12α-^{3}H]digoxigenin (Fig. 1). 12-Dehydrodigoxigenin was prepared from digoxin by the method of Yamada (23) and purified by silicic acid column chromatography; 220°/274–275° [reported, 210°/275° (24), 269–272° (23)]. The product was homogeneous on silica gel G thin-layer chromatography. The presence of hydroxyl, ester, carbonyl, and butenolide groups was shown by the infrared spectrum (KBr pellet).

For the preparation of [12α-^{3}H]digoxi-

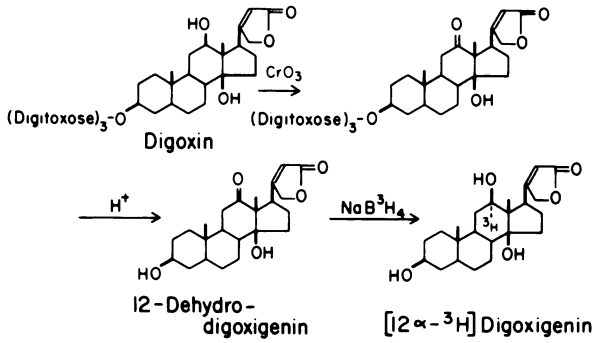


FIG. 1. *Synthesis scheme for [12α-^{3}H]digoxigenin*

genin from 12-dehydrodigoxigenin, the method of Van Watburg *et al.* (24), used for the preparation of [12α-^{3}H]digoxin, was modified as follows. An open ampoule containing 2.2 mg of solid NaB^3H_4 (859 mCi) was placed in a tube with a side arm containing a stopcock and closed with a rubber stopper (sleeve type). This tube was evacuated to about 300 mm Hg and kept under reduced pressure before applying a nitrogen stream. All additions were made through the rubber stopper, using a syringe. A solution of 12-dehydrodigoxigenin in 75% aqueous dioxane (120 mg in 2.0 ml) was added to the ampoule. When all the NaB^3H_4 had been solubilized by gentle swirling, the tube was allowed to stand overnight at room temperature, then acidified with 0.3 ml of glacial acetic acid. The side arm was connected to a tritium gas trap which contained a suspension of platinum oxide in water (under constant stirring), and the outlet was opened under an efficient fume hood. The solvent was evaporated with a gentle nitrogen stream blown into the ampoule through the rubber stopper by a hypodermic needle. After separation of a white solid, the nitrogen stream was continued for 3 hr. This aqueous suspension of radioactive digoxigenin was solubilized with dioxane, and the evaporation was repeated. The solid material in the ampoule was then treated with chloroform, transferred to a test tube, washed successively with 0.1 N HCL, 0.1 N NaOH, and water, and then dried with anhydrous sodium sulfate. This chloroform solution was applied to a silicic acid column (2 × 20 cm), and two tritium-containing fractions were eluted with 20% acetone in chloroform.

The radiochemical purity of each fraction was examined by chromatography in instant thin-layer chromatography medium ("ITLC," type SA, Gelman Instrument Company, Ann Arbor). Small aliquots of each fraction were chromatographed with unlabeled digoxigenin and 12-dehydrodigoxigenin in acetone–benzene (3:7, v/v). The radioactivity was located by counting 0.5-cm segments, and the steroid spots were visualized using 3% ceric sulfate in 2 N H_2SO_4. These experiments showed that the second fraction from the silicic acid column contained about 95% tritiated digoxigenin (R_F 0.31), but the first fraction, only 30%; the latter fraction also contained an unknown radioactive material (R_F 0.36) which was probably the digoxigenin isomer 12-*epi*-digoxigenin. The total concentration of digoxigenin was determined by inhibition of $(Na^+ + K^+)$-ATPase under the standard assay conditions. The first fraction contained 8.0 μmoles of digoxigenin and 72.5 mCi of radioactivity, and the second fraction, 33.3 μmoles of digoxigenin and 135 mCi. The chemical yield of the second fraction was 14.5%, and the yield based on radioactivity was 15.7%. This second fraction was kept as a benzene–ethanol (1:1, v/v) solution at −20° and used in this study.

Digoxigenin binding assay. In the standard assay procedure, 0.8–1.2 mg/ml of enzyme preparation was treated with various concentrations of [12α-^{3}H]digoxigenin (0.01–0.7 μM) in the presence of various ligands and 50 mM imidazole HCl

buffer (pH 7.3) for 35 min at room temperature (22–24°) in thick-walled polycarbonate centrifuge tubes. Two 0.1-ml aliquots were taken for assay of total digoxigenin, and the suspension was centrifuged at 40,000 rpm (Spinco type 40 rotor; 23 ± 1°) for 20 min. In addition, 0.1 ml of the supernatant was used for assay of unbound digoxigenin. Bound digoxigenin was calculated by subtracting unbound from total digoxigenin. Unless otherwise indicated, all values for digoxigenin binding represent the concentration of digoxigenin under each experimental condition. Nonspecific binding (i.e., binding of ouabain-treated enzyme) was not subtracted because it was insignificant, as described below.

Ouabain binding was assayed by the method used for digoxigenin binding, except that a 60-min incubation period was used instead of 35 min, and the amount of bound ouabain was calculated from the difference between total ouabain and that in the supernatant. This value, for 0.1 mg of protein, showed saturation of binding around 0.2 μM ouabain. Nonspecific binding of ouabain, measured in the absence of ligands, was negligible and about the same as the counting error.

RESULTS

In order to examine the time for the reaction to reach equilibrium, 1 mg/ml of the enzyme preparation was incubated at room temperature under eight sets of experimental conditions: with 0.05 μM or 0.5 μM digoxigenin and with one of two different saturated ligand mixtures, 2 mM Mg^{2+} and 2 mM P_i or 50 mM Na^+, 2 mM Mg^{2+}, and 2 mM ATP, or with one of two low-ligand mixtures, 0.15 mM Mg^{2+} and 0.15 mM P_i or 2 mM Na^+, 0.1 mM Mg^{2+}, and 0.3 mM ATP. After incubation for various periods from 15 to 60 min at room temperature, the enzyme-digoxigenin mixtures were centrifuged. The concentration of digoxigenin in the supernatant did not change with incubation time under each set of experimental conditions. Therefore, in all experiments in this study, after 30–35 min of incubation the samples were centrifuged for 20 min at 40,000 rpm. All incubations and centrifugations were carried out at room temperature (22–24°).

As a preliminary study, the binding of digoxigenin was examined over a wide concentration range, using a high enzyme concentration (3.9 mg of protein per milliliter). In the control experiment, the enzyme was treated initially with 25 μM unlabeled ouabain for 60 min in the presence of the same ligands and added digoxigenin. In the presence of Mg^{2+} and P_i, nonspecific binding (binding of the ouabain-treated enzyme) increased linearly with digoxigenin (Fig. 2), but binding of the untreated enzyme increased markedly with low concentrations of digoxigenin (less than 1 μM) and increased gradually with higher concentrations of the drug. The difference in binding between untreated enzyme and ouabain-treated enzyme appears biphasic, but in the present study it was not obvious that the low-affinity binding represented binding to the (Na^+ + K^+)-ATPase because the purity of the enzyme preparation was low. Similarly, high-affinity binding was observed in the Na^+-Mg^{2+}-ATP system, but the presence of low-affinity binding was not as clear because of wide data scatter.

In this study only the high-affinity binding of digoxigenin at low concentrations was examined, because it appears more likely to resemble binding *in vivo*. When the concentration of digoxigenin was less than 0.6 μM, binding to the ouabain-treated enzyme in the presence of ligands and binding to the untreated enzyme in the absence of ligands were less than 8% of binding to the untreated enzyme in the presence of ligands (Fig. 3). These experiments were done in the Na^+-Mg^{2+}-ATP system; similar results were obtained in the Mg^{2+}-P_i system. In the following binding experiments, the binding of 0.03 μM, 0.1 μM, or 0.6 μM digoxigenin to the ouabain-treated enzyme was measured as a blank. Each blank value was less than 8% of the binding to the untreated enzyme at each concentration.

Digoxigenin binding was measured at various concentrations of each ligand. For each set of parameters, the equilibrium values of bound and free digoxigenin were determined with 10–12 different concentrations of added digoxigenin (0.01 μM ~ 0.6 μM) for each ligand condition. A Scatchard

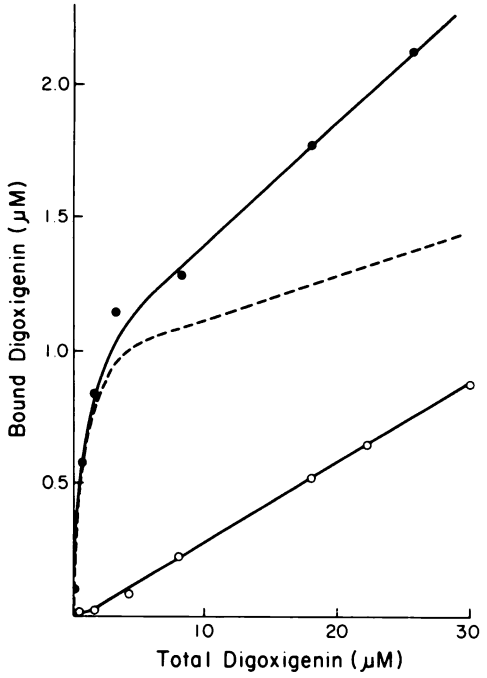


FIG. 2. *Binding of digoxigenin and (Na^+ + K^+)-ATPase.*

The enzyme preparation (3.9 mg/ml; specific activity 30.7) was treated with or without 25 μM ouabain in the presence of 2 mM Mg^{2+}, 2 mM P_i, and 50 mM imidazole HCl buffer (pH 7.3) for 60 min, and various concentrations of digoxigenin were added. After 35 min of incubation, the suspension was centrifuged. Bound digoxigenin was calculated from the difference between the total digoxigenin in the suspension and the free digoxigenin in the supernatant. ●——●, digoxigenin bound to untreated enzyme; ○——○, digoxigenin bound to ouabain-treated enzyme; - - -, difference between these two values, namely, specifically bound digoxigenin.

plot was then constructed from the equilibrium binding results. As all the plots obtained were linear, the ordinate intercept corresponds to the apparent total number of binding sites, and the slope corresponds to the apparent dissociation constant.

Figure 4 shows the effect of variation of the P_i concentration with a constant Mg^{2+} concentration (2 mM). The effect of variations in the Mg^{2+} concentration with P_i concentration held constant at 2 mM, is shown in Fig. 5. In Fig. 6, simultaneous variation of the Mg^{2+} and P_i concentrations caused a change in binding which was similar to that seen with variation of Mg^{2+} or P_i alone. Thus, in the Mg^{2+}-P_i system, the apparent number of sites changed with the concentration of ligands, but the apparent dissociation constant did not change excpet at very low concentrations of ligands. At saturating ligand concentrations (more than 2 mM Mg^{2+} or P_i) the dissociation constant was 0.041 μM. This value is an order of magnitude lower than the I_{50} value of digoxigenin (0.4 μM) obtained under assay conditions. Furthermore, the apparent total number of binding sites was a little less than the number of ouabain binding sites.

In the Na^+-Mg^{2+}-ATP system, the effect of ATP on digoxigenin binding was similar to the effects of Mg^{2+} and P_i in the Mg^{2+}-P_i system (Fig. 7). The dissociation constant of digoxigenin was not changed except at low concentrations of ATP (less than 0.1 mM), and the apparent number of binding sites decreased as the concentration of ATP decreased. The Mg^{2+} effect was similar to that of ATP, except that the dissociation constant did not change at the lowest concentration of Mg^{2+} used (Fig. 8).

When the Na^+ concentration was higher than 20 mM, the apparent number of di-

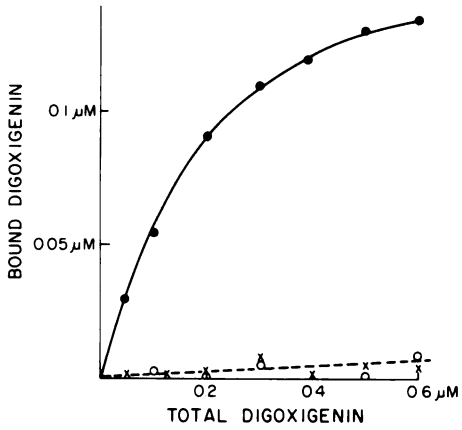


FIG. 3. *High-affinity binding of digoxigenin*

The enzyme preparation (0.94 mg/ml; specific activity 34.7) was treated with various concentrations of digoxigenin in the presence of 50 mM Na^+, 2 mM Mg^{2+}, 2 mM ATP, and 50 mM imidazole HCl buffer (pH 7.3). Other experimental conditions were the same as in Fig. 2. ●——●, digoxigenin bound to untreated enzyme; ×- - -×, digoxigenin bound to untreated enzyme in the absence of Na^+, Mg^{2+}, and ATP; ○——○, digoxigenin bound to ouabain-treated enzyme in the presence of Na^+, Mg^{2+}, and ATP.

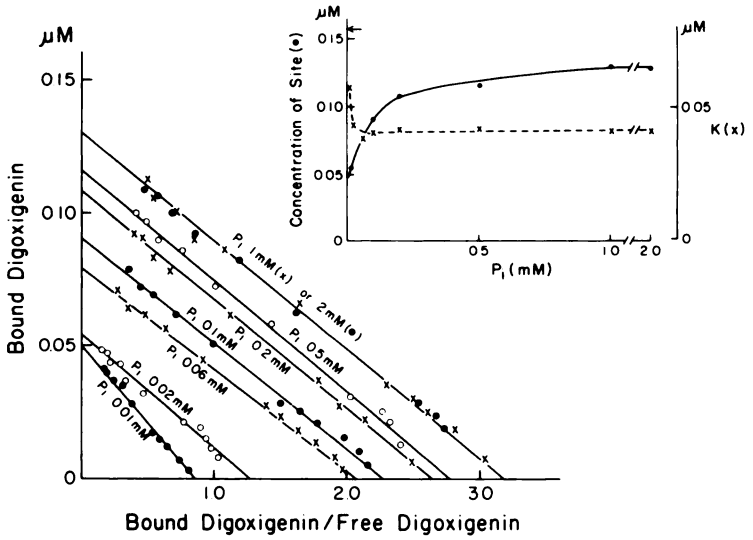


FIG. 4. *Scatchard plot of digoxigenin binding in Mg^{2+}-P_i system with P_i concentration varied*

The incubation medium contained 2 mM Mg^{2+}, 0.01-2.0 mM P_i, 50 mM imidazole HCl (pH 7.3), 0.015-0.60 μM digoxigenin, and enzyme, 1.05 mg/ml (specific activity, 30.7). The inset shows the micromolar concentration of total binding sites (●——●) and the apparend dissociation constant K (×- - -×) plotted against P_i concentration. The arrow indicates the maximum concentration of ouabain binding sites (0.16 μM) under these experimental conditions.

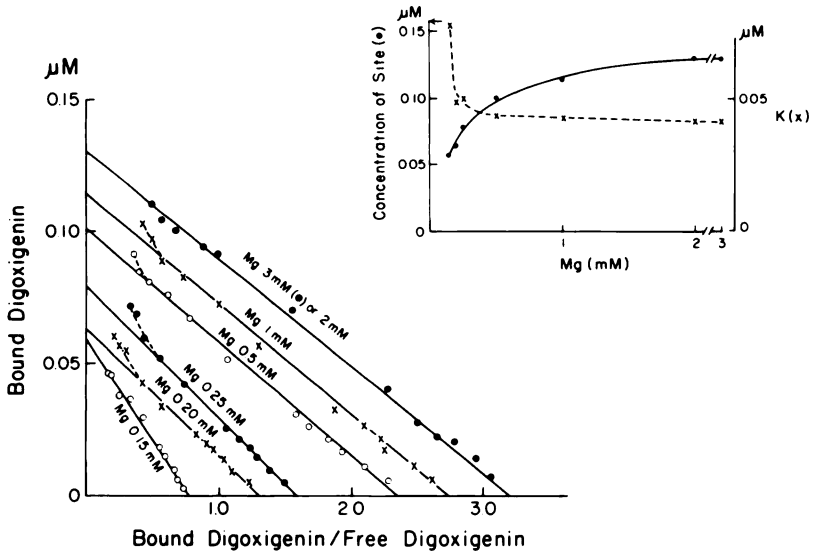


FIG. 5. *Scatchard plot of digoxigenin binding in Mg^{2+}-P_i system with Mg^{2+} concentration varied*

Conditions were the same as in Fig. 4, except that the P_i concentration was 2 mM and the Mg^{2+} concentration was varied from 0.15 to 3 mM.

goxigenin binding sites decreased with increasing Na^+ concentration, but the dissociation constant did not change (Fig. 9). In the lower Na^+ concentration range (less than 15 mM), however, the apparent number of binding sites was constant and the apparent dissociation constant increased as the Na^+ concentration decreased.

At saturating concentrations of ligands (more than 50 mM Na^+, 2 mM Mg^{2+}, or 2 mM ATP), the apparent dissociation constant was 0.078 μM. This value is about

twice that in the Mg^{2+}-P_i system but is still lower than the I_{50} value of digoxigenin under assay conditions. Under these conditions the apparent total number of binding sites was a little less than the number of ouabain binding sites.

DISCUSSION

In this study bound digoxigenin was calculated from the difference between total and free digoxigenin instead of by direct measurement of the precipitated digoxigenin, in order to avoid the error caused by

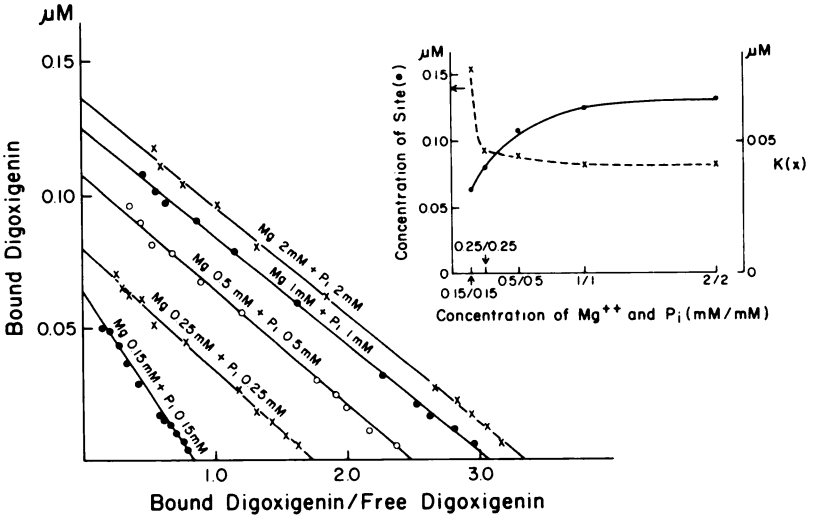


FIG. 6. *Scatchard plot of digoxigenin binding in Mg^{2+}-P_i system with both ligand concentrations varied*

Conditions were the same as in Fig. 4, except that the Mg^{2+} and P_i concentrations were both varied simultaneously from 0.15 to 2 mM and the enzyme concentration was 1.2 mg/ml (specific activity, 28.2). The maximum concentration of ouabain binding sites was 0.14 μM.

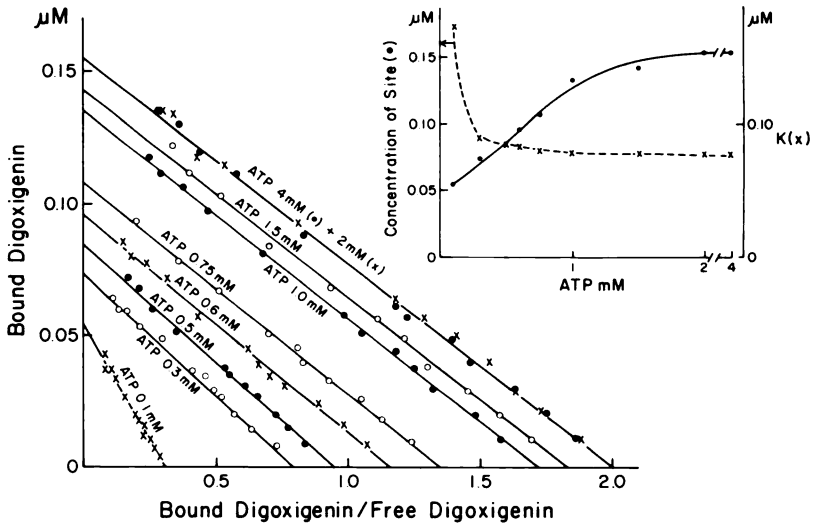


FIG. 7. *Scatchard plot of digoxigenin binding in Na^+-Mg^{2+}-ATP system with ATP concentration varied*

The incubation medium contained 50 mM Na^+, 2 mM Mg^{2+}, 0.1–4.0 mM ATP, 50 mM imidazole HCl (pH 7.3), 0.015–0.60 μM digoxigenin, and enzyme, 0.94 mg/ml (specific activity, 34.7). The inset shows the micromolar concentration of total binding sites (●——●) and the apparent dissociation constant K (×- - -×) plotted against ATP concentration. The arrow indicates the maximum concentration of ouabain binding sites (0.16 μM) under these experimental conditions.

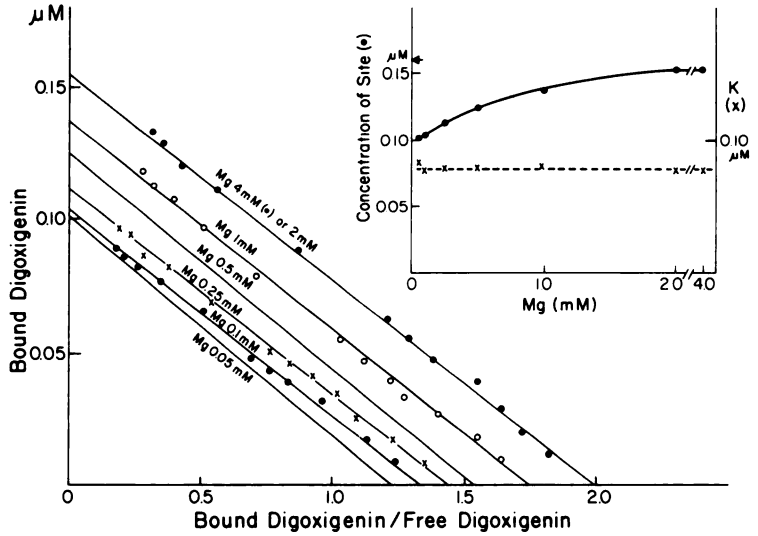


FIG. 8. *Scatchard plot of digoxigenin binding in Na^+-Mg^{2+}-ATP system with Mg^{2+} concentration varied*
Conditions were the same as in Fig. 7, except that the Mg^{2+} concentration was varied from 0.05 to 4.0 mM and the Na^+ and ATP concentrations were 50 mM and 2 mM, respectively. The values at 0.05 and 0.5 mM Mg^{2+} were obtained with a different batch of enzyme (1.0 mg/ml; specific activity, 29.0), and corrected lines were inserted.

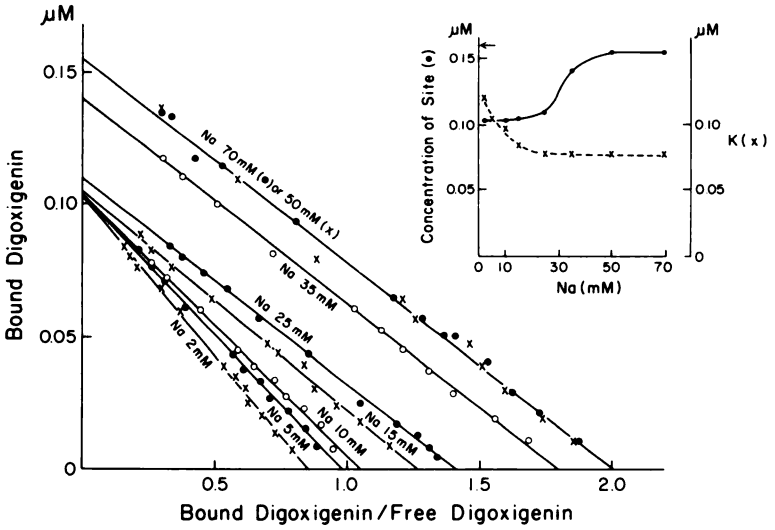


FIG. 9. *Scatchard plot of digoxigenin binding in Na^+-Mg^{2+}-ATP system with Na^+ concentration varied*
Conditions were the same as in Fig. 7, except that the Na^+ concentration was varied from 2 to 70 mM and the Mg^{2+} and ATP concentrations were 2 mM each.

the presence of digoxigenin in the adherent supernatant. Since it is difficult by this method to obtain an accurate value for binding at high concentrations of digoxigenin, or for weak binding, this study is limited to high-affinity binding. Therefore ligand systems other than Mg^{2+}-P_i and Na^+-Mg^{2+}-ATP were not examined.

In all the Scatchard plots shown in Figs. 4–9, good linearity was obtained, indicating that all high-affinity sites in each experimental set were identical and noninteracting. In the present study digoxigenin binding was almost completely eliminated by prior treatment of the enzyme with ouabain, and the maximum number of digoxi-

genin sites agreed well with the number of ouabain sites. Previously, competition for the association of ouabain by the cardiac aglycones digitoxigenin (14) and ouabagenin (25) has been observed. Therefore it is likely that the binding site for digoxigenin, or cardiac aglycone, is the same as the steroid-specific site for ouabain, or cardiac glycoside.

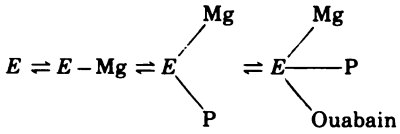
It was also observed here that certain ligands promote high-affinity binding of the cardiac aglycone digoxigenin to the enzyme. Both the Mg^{2+}-P_i and Na^+-Mg^{2+}-ATP systems are very effective in promoting this binding. However, the apparent dissociation constants of digoxigenin under these saturating conditions are different. In the Mg^{2+}-P_i system digoxigenin has more affinity for the enzyme than in the Na^+-Mg^{2+}-ATP system, although the total number of binding sites is almost the same. The apparent dissociation constants are lower than the I_{50} value obtained by measuring inhibition of the enzyme, 0.40 μM (13). This difference suggests that the affinity of digoxigenin may be reduced by K^+ in the same way as the affinity of ouabain is reduced by K^+, as suggested by Wallick *et al.* (25).

The salient and unexpected finding in this study is that none of the ligands examined changed the dissociation constant of digoxigenin binding, but all promoted binding by increasing the number of binding sites on the enzyme, except at low concentrations of Na^+ in the Na^+-Mg^{2+}-ATP system. If the binding follows the usual reversible equation, $E + M \rightleftharpoons EM$, $EM + I \rightleftharpoons EMI$, where E is the enzyme or receptor, M a modifier, and I the inhibitor or drug, the apparent total number of binding sites obtained at equilibrium by Scatchard plots does not vary with the concentration of the modifier whereas the dissociation constant does. Although it has been shown that cardiac aglycone binding is reversed by dilution (7, 14, 25), the findings for digoxigenin binding obtained here are opposite to those expected from the usual reversible equation.

From the present binding results, it is apparent that the activation step is regulated exclusively by concentrations of the modifier mixtures Na^+-Mg^{2+}-ATP and Mg^{2+}-P_i. Only this activated form can bind digoxigenin. Since these modifiers can phosphorylate the enzyme, activation of the enzyme may include phosphorylation and a conformational change. Moreover, some reaction step(s) in the association process may be irreversible, and the enzyme-digoxigenin complex may dissociate via another reaction sequence. The present results do not clarify the reaction sequence.

In the case of ouabain binding, the phosphorylated form[1] of $(Na^+ + K^+)$-ATPase has been suggested as the direct reactant with ouabain (1, 3, 6, 8, 26–28), although evidence has been presented that the nonphosphorylated enzyme can bind cardiac glycosides under appropriate conditions (2, 29). Recently Post *et al.* (30) have presented additional support for the former idea. They have shown that the phosphorylated enzyme, whether it is formed by the Na^+-Mg^{2+}-ATP system or by the Mg^{2+}-P_i system, is stabilized by ouabain (30). As described under INTRODUCTION, steroid binding is thought to be the first step in cardiac glycoside binding. It is plausible that these phosphorylated forms[1] of the enzyme are the active forms which bind the cardiac steroid.

Hansen and Skou (6) claimed that ouabain binding could be defined by a simple reversible equation. In particular, in the Mg^{2+}-P_i system, the number of ouabain binding sites did not change with ligand concentration, unlike the dissociation constant, and they proposed the following reaction scheme (6):



The discrepancy between ouabain binding and digoxigenin binding described

[1] The phosphorylated site of $(Na^+ + K^+)$-ATPase is apparently different from the binding site of cardiac aglycone or glycoside, because phosphorylation occurs on the inside of erythrocyte membranes but binding of ouabain takes place on the outside. The conformation of the steroid binding site may usually be induced to the active form by phosphorylation, but it is also possible that the conformation of the binding site may be activated in another way.

here may be explained by our previously reported results (13). We suggested that binding of cardiac glycosides proceeds by the following reaction sequence: step 1, binding of the steroid moiety to the steroid-specific site on the $(Na^+ + K^+)$-ATPase; step 2, rearrangement of the sugar site from an unreactive to a reactive form; step 3, binding of the sugar moiety to its specific site. These steps would be reversed during dissociation. Since the rate-determining step in the Mg^{2+}-P_i system is suggested to be the rearrangement step of the sugar-specific site (step 2), as shown previously (13), dissociation of the steroid moiety from the steroid site should be masked by dissociation of the sugar moiety, and consequently the whole binding reaction of cardiac glycosides appears to follow the single reversible equation.

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