MOLECULAR PHARMACOLOGY, 14, 624-632

Influence of pH on the Interaction of Cardiotonic Steroids with Sodium- and Potassium-Dependent Adenosine Triphosphatase

ATSUNOBU YODA AND SHIZUKO YODA

Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin 53706

(Received October 24, 1977)

(Accepted February 24, 1978)

SUMMARY

YODA, ATSUNOBU & YODA, SHIZUKO (1978) Influence of pH on the interaction of cardiotonic steroids with sodium- and potassium-dependent adenosine triphosphatase. *Mol. Pharmacol.*, **14**, 624-632.

pH influences the equilibrium binding and the association and dissociation rate constants (k_a and k_d) of ouabain and digoxigenin for ($Na^+ + K^+$)-ATPase. When the cardiotonic steroid-enzyme complexes are formed in the presence of Mg^{2+} and P_i (type II complex) between pH 6.5 and 8.5, the k_a values of ouabain and digoxigenin and the apparent number of sites according to Scatchard plots of digoxigenin binding are reduced as the pH is increased. These changes correspond to changes in enzyme phosphorylation by Mg^{2+} and P_i. The k_d values of type II complexes with ouabain and digoxigenin are constant below pH 8.0, but they decrease significantly to a new plateau at about pH 8.0 and above. This change is slow and reversible, and it is produced only by changing the pH of the dissociation medium. In the cardiotonic steroid-enzyme complex formed in the presence of Na^+, Mg^{2+}, and ATP (type I complex), the k_a values of ouabain, the apparent number of sites for digoxigenin binding, and enzyme phosphorylation by ATP are not influenced by pH. On the other hand, the k_d of the type I ouabain-enzyme complex decreases with increasing pH; the k_d at pH 8.0 is one-fifth the value at pH 6.5. The k_d of the type I ouabain-enzyme complex is not dependent on pH during complex formation but is dependent on pH during dissociation. These effects of pH on the k_a values of both cardiac glycoside and steroid support the reaction mechanism previously proposed: that cardiac steroids or glycosides bind to the phosphorylated form of ($Na^+ + K^+$)-ATPase and that binding of the steroid portion results in activation of the sugar-binding site, with consequent binding of the glycoside portion.

INTRODUCTION

Cardiotonic steroids inhibit sodium and potassium transport and ($Na^+ + K^+$)-ATPase (EC 3.6.1.3), which is an integral part of this transport process. It is well known 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 the Na^+-Mg^{2+}-ATP systems (1-4). Our kinetic rate studies on cardiac glycoside-($Na^+ + K^+$)-ATPase complexes (5-7) indicated that cardiac glycosides were bound to the enzyme at two sites, one specific for the steroid moiety and the other for the sugar. The binding of the steroid moiety results in activation of the sugar-binding site, with consequent binding of the glycoside portion. The steroid moiety of the cardiac glycoside is the active inhibitory component. The role of the sugar moiety in binding to the enzyme is to increase the stability of the cardiac glycoside-($Na^+ + K^+$)-ATPase complex. The effect of pH on

This work was supported by Grant HL 16549 from the National Heart and Lung Institute.

0026-895X/78/0144-0624$02.00/0

ouabain binding has been briefly examined in the Mg^{2+} (8), Na^+-Mg^{2+}-ATP (8), and Mg^{2+}-P_i (9) systems, and it was found that ouabain binding was insensitive to pH in the Na^+-Mg^{2+}-ATP and Mg^{2+}-P_i systems compared with enzymatic ATP hydrolysis.

Recently we have studied the binding of [^{3}H]digoxigenin to $(Na^+ + K^+)$-ATPase at equilibrium (10), and the k_a and k_d values of several cardiac aglycones for the enzyme in the Mg^{2+}-P_i system (11). In these studies the effects of the sugar moiety of cardiac glycoside were eliminated. From these results, the complexity of cardiac steroid binding to the enzyme was shown, and a new reaction scheme was proposed. In this study we have examined the effects of pH on the interactions between $(Na^+ + K^+)$-ATPase and ouabain as a cardiac glycoside, or digoxigenin as a cardiac aglycone. For convenience, we refer to the cardotonic steroid-$(Na^+ + K^+)$-ATPase complex formed in the presence of Na^+, Mg^{2+}, and ATP as the type I complex, and to that formed in the presence of Mg^{2+} and P_i, as the type II complex.

MATERIALS AND METHODS

Two different $(Na^+ + K^+)$-ATPase preparations were used in this study. Both were prepared from frozen beef brain (Pel-Freez Biologicals). One was NaI-treated microsomes prepared by the method of Nakao *et al.* (12) according to the modification of Hegyvary and Post (13), the same method that has been used in previous studies. The other enzyme preparation was the NaSCN-treated microsomes described by Klodos *et al.* (14). The specific enzymatic activity of the latter preparation was 60–70 μmoles of P_i per hour per milligram of protein at 30°, and that of NaI-treated microsomes was about 50–60 μmoles/hr/mg of protein at 30°. No significant differences were observed in the k_a, k_d, and binding constants of ouabain and digoxigenin. Digoxigenin and ouabain were purchased from Boehringer/Mannheim and Sigma, respectively. [^{3}H]Ouabain was obtained from New England Nuclear, and [12α-^{3}H]digoxigenin was prepared by the reduction of 12-dehydrodigoxigenin with NaB^3H_4 as reported previously (10). [γ-^{32}P]ATP was prepared by the method of Glynn and Chappel (15) and used as the Tris salt.

Binding of ouabain or digoxigenin was determined as reported previously (10). After incubation of the enzyme with [^{3}H]-ouabain or [^{3}H]digoxigenin in the presence of 100 mM Na^+, 4 mM Mg^{2+}, and 4 mM ATP (for the type I complex) or 4 mM Mg^{2+} and 4 mM P_i (for the type II complex) at 22–24°, the medium was separated from the enzyme by centrifugation at 145,000 × *g* for 20 min at 22°, using a Beckman L2-65 centrifuge equipped with a high-temperature kit, and the bound ouabain or digoxigenin was calculated from the difference between the radioactivity of the enzyme suspension before centrifugation and that of the supernatant after centrifugation. From the results obtained with 12 different concentrations of added digoxigenin (0.02–0.5 μM), a Scatchard plot was constructed, and the apparent number of sites and the apparent binding constant were determined.

The k_a and k_d of ouabain were determined as reported previously (5, 6). These methods are based on the fact that the enzyme-cardiac glycoside complex remains relatively stable during the assay of enzymatic activity after 10–20-fold dilution. The reaction mixture containing the enzyme and ouabain was diluted after suitable time intervals in order to stop the association reaction, and the ATPase activity was assayed by the linked pyruvate kinase-lactate dehydrogenase spectrophotometric method. The rate of increase of ATPase inhibition is a measure of k_a. After the enzyme inhibited by incubation with ouabain was diluted to minimize the association reaction, the dissociation reaction predominated, and the rate of reappearance of $(Na^+ + K^+)$-ATPase activity permitted us to measure the k_d. Since the *n*-fold dilution decreased the association rate to $1/n^2$, the association of ouabain during the ATPase assay after 20-fold dilution was negligible. Dissociation of the ouabain complex during that assay is an important source of error in the k_a value. As the k_d of ouabain was less than 0.03 min^{-1} and the assay time was between 0.5 and 2 min, this error in the k_a or k_d value is not large (less than 10%). On the other hand, for k_d determination, a rather long incubation (20–70 min) is

needed for dissociation, and the inhibition during this period should be minimized to decrease the error in k_d. For this reason, the concentration of ouabain during the preliminary incubation was kept as low as possible to obtain more than 60% inhibition. In this study, the inhibition after dilution (during the dissociation period) was kept at less than 15%.

Since the $(Na^+ + K^+)$-ATPase–cardiac steroid complex is not stable after dilution (16), the methods used in the case of ouabain are not suitable for k_a or k_d measurements of digoxigenin, so that the previously reported rapid assay method for the active enzyme was used for this purpose (11). The assay of EP,[1] which is affected by the amount of bound digoxigenin, is too rapid to be affected by the change in the enzyme-digoxigenin complex during the assay.

The rate of increase of $(Na^+ + K^+)$-ATPase inhibition in the mixture containing the enzyme and digoxigenin can be measured by this assay without stopping the interaction between the enzyme and digoxigenin, and permits us to obtain the k_a value of digoxigenin. If the enzyme, equilibrated with digoxigenin, is diluted, the rate of reappearance of $(Na^+ + K^+)$-ATPase is also determined by this assay, and from that the k_d value of digoxigenin is calculated. In these determinations of k_a and k_d of digoxigenin, the assay of EP was performed as described in the previous report, and the use of the semirapid mixing apparatus described by Kanazawa *et al.* (17) improved the accuracy of the results.

Radioactivity was measured with a Packard Tri-Carb liquid scintillation counter, using 5 ml of scintillation medium (5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenoxyazolyl)]benzene in 1 liter of toluene and 500 ml of Triton X-100). Protein was measured according to Lowry *et al.* (18), using bovine serum albumin as a standard.

RESULTS

pH effects on binding of ouabain. The interaction of ouabain and $(Na^+ + K^+)$-ATPase is nearly irreversible and takes a long time to reach equilibrium. In the case of the type I complex, the time course of ouabain binding at pH 6.5 was not different from that at pH 8.0 (Fig. 1). For the type II complex, the change of pH did not affect the amount of ouabain bound at equilibrium, even though at higher pH binding took a longer time to reach equilibrium.

pH effects on k_a and k_d of ouabain. Increases in pH markedly decreased the k_a of ouabain in the type II complex, but not in the type I complex (Fig. 2). Although the k_d of the type I ouabain-enzyme complex was reduced with increasing pH, and the value at pH 8.5 was about one-fifth that at pH 6.5, the k_d of the type II complex

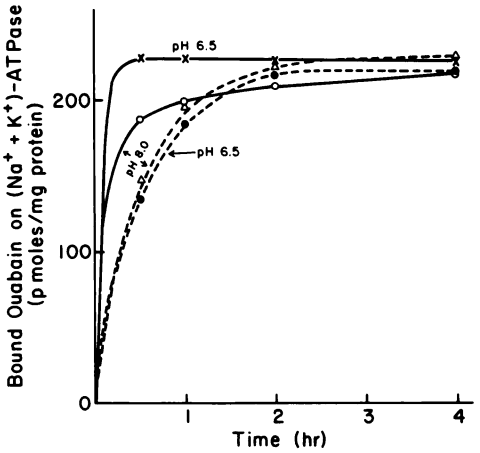

FIG. 1. *Time course of [^{3}H]ouabain binding at 22°* For formation of the type I complex, NaSCN-treated microsomes (0.31 mg/ml) were treated with 0.1 μM [^{3}H]ouabain in the presence of 100 mM Na^+, 4 mM Mg^{2+}, 4 mM ATP, and 50 mM imidazole HCl buffer (pH 6.5) (●– – –●) or 50 mM Tris-HCl buffer (pH 8.0) (△– – –△). After various periods of incubation, as indicated, the suspension was centrifuged at 145,000 × *g* for 20 min at 22°. Bound ouabain was calculated from the difference between the radioactivity in the suspension (total ouabain) and that in the supernatant (unbound ouabain). For formation of the type II complex, the same enzyme preparation (1.44 mg/ml) was treated with 0.4 μM [^{3}H]ouabain in the presence of 4 mM Mg^{2+}, 4 mM P_i, and 50 mM imidazole HCl buffer (pH 6.5) (×——×) or 50 mM Tris-HCl buffer (pH 8.0) (○——○). Other experimental conditions were the same as those for formation of the type I complex. The values are averages of three sets of experiments; in each experiment, measurements of bound ouabain were duplicated and the average of experimental error in each point was ±7 pmoles/mg of protein.

[1] The abbreviations used are: EP, $(Na^+ + K^+)$-ATPase phosphorylated by ATP in the presence of Na^+ and Mg^{2+}; TCA, trichloroacetic acid.

changed significantly only between pH 7.5 and 7.75 (Fig. 3). As reported earlier (19–21), the presence of potassium ion in the dilution medium reduced the k_d value of the type I ouabain-complex at every pH value examined.

In order to examine the reversibility of changes in k_d with pH, the pH of the dilution medium was varied during inhibition by ouabain. The k_d value of the type I complex seemed to depend on the pH of the dilution medium (Table 1). When the

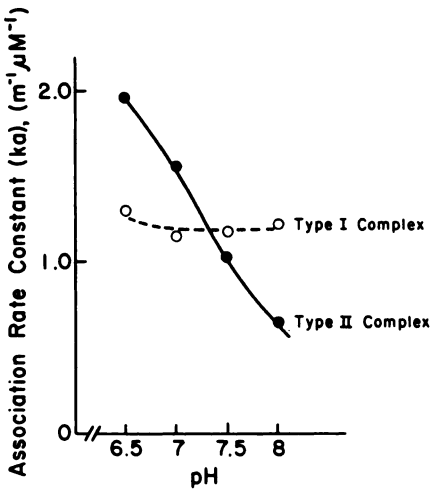

FIG. 2. *Effect of pH on association rate constants (k_a) of ouabain at 30°*

For the type I complex (O- - -O), the reaction mixture (0.25 ml), containing enzyme (NaSCN-treated microsomes) and 0.2 μM ouabain, was incubated at 30° with 100 mM Na^+, 4 mM Mg^{2+}, 4 mM ATP, and 50 mM imidazole buffer for a suitable interval, and enzyme inhibition was determined after dilution with 5 ml of 10 mM imidazole buffer (pH 7.35) containing 1 mM EDTA. Eight to nine measurements at different intervals were carried out in each experiment. A time course curve for ouabain inhibition was constructed. From its slope, the k_a value was calculated by a second-order equation. The averages of duplicate experiments are shown. The total average of k_a values of type I complexes obtained was 1.22 $min^{-1}\ \mu M^{-1}$ ($\sigma = 0.05$, $n = 8$). For the type II complex (●——●), 4 mM Mg^{2+} and 4 mM P_i were used as the promoting ligands instead of Na^+, Mg^{2+}, and ATP. The concentration of ouabain varied from 0.12 to 0.5 μM, depending on the pH value. Three sets of experiments, using different concentrations of ouabain, were performed. The experimental error at each point was less than ±0.12 $min^{-1}\ \mu M^{-1}$. Other experimental conditions were the same as those for the type I complex.

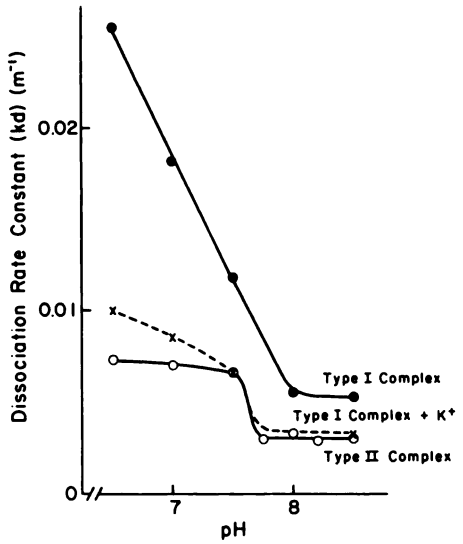

FIG. 3. *Effect of pH on dissociation rate constants (k_d) of ouabain at 30°*

The type I complex was formed by a 10-min incubation of NaI-treated microsomes and 0.2 μM ouabain in the presence of 50 mM Na^+, 2 mM Mg^{2+}, 1 mM ATP, and 50 mM imidazole HCl buffer (under pH 8.0) or 50 mM Tris-HCl buffer (for pH 8.0 and above) and then diluted 20-fold with 10 mM imidazole or Tris buffer containing 1 mM EDTA; its pH was the same as that for complex formation. Enzyme inhibition was determined at seven or eight intervals. From the slope of this time course curve of enzyme inhibition, the k_d value of ouabain at each pH value was calculated. When the dilution medium contained 2 mM KCl, the change in k_d with pH is shown by the dashed line (×- - -×). In the case of the type II complex, 4 mM Mg^{2+} and 4 mM P_i were used as the promoting ligands instead of Na^+, Mg^{2+}, and ATP, and the concentration of ouabain and the incubation time were selected in the ranges of 0.1–2.0 μM ouabain and 5–10 min so that the inhibition would be 60–85%. Other experimental conditions were the same as those for the type I complex. The averages of experimental error were examined at pH 6.5 and 8.5 for both the type I and II complexes and are shown in Table 1.

type II complex of ouabain with enzyme was formed at pH 6.5 and dissociated at pH 8.2, the time course of dissociation was not linear. The initial rate of dissociation was higher than that of complex formed at pH 8.5, but it was gradually reduced to a constant final value near the k_d value of the pH 8.5 complex. The experiment in which the type II ouabain complex was formed at pH 8.5 and diluted at pH 6.5 was difficult, because a high concentration of ouabain (2

TABLE 1

Reversibility of pH effect on k_d values of ouabain-(Na^+ + K^+)-ATPase complex

Each 0.25-ml suspension of type I and II complexes was formed as described for Fig. 3, except that 20 mM imidazole or Tris buffer was used instead of 50 mM buffer; these were diluted with 5 ml of 20 mM buffer containing 1 mM EDTA. pH values were measured 10–20 min after dilution. In the last experiment, the type II complex formed at pH 6.5 (0.25 ml) as described above was incubated for 30 min with 0.05 ml of 500 mM Tris buffer (pH 8.5) at 30° and then diluted with 5 ml of 20 mM imidazole buffer (pH 6.5) containing 1 mM EDTA. The final pH was 6.7. Other experimental conditions were the same as in Fig. 3. When the pH of the dilution medium for the type II complex was different from the pH during complex formation, the stability curve after dilution was biphasic, and the initial and final rate constants of the curve are given.

Complex type	pH during complex formation	pH after dilution	k_d at 30°
			$min^{-1} \pm SD$
I	6.5	6.5	0.030 ± 0.003 ($n = 4$)
	8.5	8.5	0.0059 ± 0.0005 ($n = 4$)
	6.5	8.3	0.0055 ± 0.0010 ($n = 4$)
	8.5	6.5	0.025 ± 0.004[a] ($n = 3$)
II	6.5	6.5	0.0068 ± 0.0005 ($n = 6$)
	8.5	8.5	0.0040 ± 0.0007 ($n = 5$)
	6.5	8.2–8.3	0.0080[b] ± 0.0007[a] ($n = 3$)
			0.0034[c] ± 0.0003[a] ($n = 3$)
	6.5 (8.5[d])	6.7	0.0068[b] ± 0.0013 ($n = 4$)
			0.0038[c] ± 0.0005 ($n = 4$)

[a] Standard error.
[b] Initial.
[c] Final.
[d] pH after alkali treatment.

μM) was needed for inhibition at pH 8.5, and as little as 0.1 μM ouabain (the concentration after dilution) would inhibit the enzyme significantly at pH 6.5 during the dissociation, as suggested by Fig. 2. Therefore the inhibited enzyme at pH 6.5 was treated at pH 8.5 for 30 min to convert it to the pH 8.5 complex and then diluted with pH 6.5 solution. The pH after dilution was 6.7. The result was that the initial dissociation rate constant was 0.0078–0.0049 min^{-1} and the final constant was 0.0038 min^{-1}. This was the same as the k_d at pH 8.5. The increase in k_d produced by the change from pH 8.5 to pH 6.7 seemed very slow.

pH effects of binding of digoxigenin at equilibrium. The binding of digoxigenin was examined by Scatchard analysis. For the type I complex, the change from pH 6.0 to 9.0 did not significantly influence either the apparent binding constant or the apparent number of binding sites (Fig. 4). For the type II complex, the apparent number of sites was reduced markedly with increasing pH. The apparent number of digoxigenin binding sites at pH 6.5 was about 1.7 times the number at pH 8.0, but the change in apparent binding constant between pH 6.5 and 8.0 was negligible.

At both pH 6.5 and 8.0, dilution of ligands (Mg^{2+} or P_i) decreased the apparent number of binding sites but did not change the apparent binding constant seen at pH 7.35 (10) (data not shown).

pH effects on the k_a and k_d values of digoxigenin. Because of the lack of a suitable method for the type I complex, the k_a and k_d of digoxigenin could be examined only in the type II complex. The k_a of digoxigenin changed in a manner similar to that of ouabain (Fig. 5), and the ratio of ouabain to digoxigenin k_a values did not vary with pH. At pH 8.0, the time course of dissociation not linear (Fig. 6). The k_d of the digoxigenin-enzyme complex changed significantly at pH 8.0 (Fig. 7), as did that of ouabain (Fig. 3).

pH effect on EP values and inhibition of EP formation with Mg^{2+} and P_i. Since it seems that cardiac aglycones (11) and ouabain (22) can bind to the phosphorylated form of (Na^+ + K^+)-ATPase, the effect of

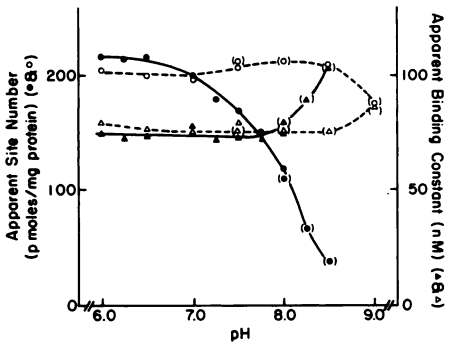

FIG. 4. *Effect of pH on digoxigenin binding*

For the type I complex, the incubation medium, containing 100 mM Na^+, 4 mM Mg^{2+}, 4 mM ATP, 50 mM imidazole HCl, 0.020–0.50 μM digoxigenin, and NaI-treated microsomes (0.64 mg/ml), was incubated at 22° for 35 min, when it had reached equilibrium, and then centrifuged at 145,000 × *g* for 20 min at 22°. From the values for bound and free digoxigenin obtained at 12 different concentrations of digoxigenin, a Scatchard plot was constructed by selecting a straight line into which more than 10 points would fit within ±5% error, and the apparent total number of sites and apparent binding constant were plotted against pH. The points in parentheses represent the results when 50 mM Tris-HCl was used as the buffer instead of imidazole HCl. For the type II complex, the suspension contained 4 mM Mg^{2+} and 4 mM P_i as the ligand. The enzyme concentration was 0.86 mg/ml. Other experimental conditions were the same as those for the type I complex. ○- - -○, apparent number of sites in the type I complex; △- - -△, apparent binding constant for the type I complex; ●——●, apparent number of sites in the type II complex; ▲——▲, apparent binding constant for the type II complex.

pH on quantity of the phosphorylated form was examined. The *E*P value did not change significantly between pH 6.0 and 8.0 (Fig. 8), like the k_a of the type I ouabain complex. Since prior phosphorylation of the enzyme by Mg^{2+} and P_i reduced the *E*P formed by Na^+, Mg^{2+} and ATP, the reduced level of *E*P may correspond to the amount of phosphoprotein formed by Mg^{2+} and P_i (11, 22). This inhibition decreased with increasing pH, and, especially in the case of *E*P formation with 1 mM Mg^{2+} and 1 mM P_i, the decrease paralleled the decrease in k_a values of ouabain and digoxigenin in the Mg^{2+}-P_i system.

Since the inhibition of *E*P formation by Mg^{2+} and P_i at pH 8.0 did not increase when the P_i concentration was raised from 2 to 8 mM in the presence of 4 mM Mg^{2+}, the effect of pH probably does not result from a decrease in phosphate monoanion, $H_2PO_4^-$, as was seen for the k_a of the type II ouabain complex (data not shown).

DISCUSSION

Two brief reports on the effect of pH on ouabain binding, by Whittam and Chipper-

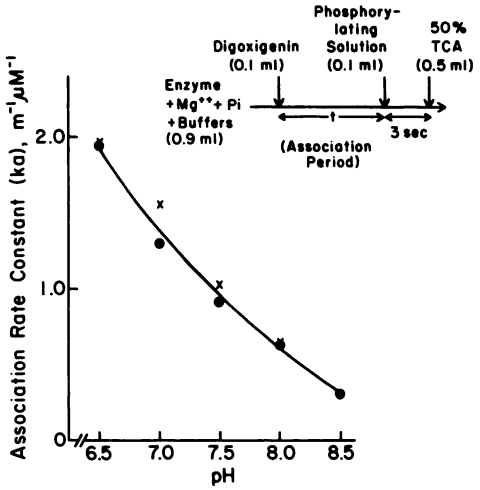

FIG. 5. *Effect of pH on association rate constants (k_a) of type II complex of digoxigenin at 15°*

As the flow sheet in the inset shows, the association reaction of 0.5 μM digoxigenin with the enzyme (NaSCN-treated microsomes, 50–80 μg/ml) was carried out in the presence of 4 mM Mg^{2+}, 4 mM P_i, and 50 mM imidazole HCl buffer, using a semirapid mixing apparatus. At pH 8.5, 50 mM Tris-HCl buffer was used instead of imidazole HCl, and the association was started by the addition of Mg^{2+} to the mixture of digoxigenin, P_i, buffer, and enzyme in order to avoid the precipitation of magnesium phosphate. *E*P formation was performed by the addition of 0.1 ml of phosphorylating solution containing 1 M Na^+, 50 mM Mg^{2+}, and 0.2–0.3 mM [γ-^{32}P]ATP. After 3 sec of phosphorylation, the reaction was quenched by the addition of 40% TCA. An aliquot of the TCA suspension was filtered with a Millipore filter (pore size, 0.45 μm), the precipitate was washed with ice-cold 5% TCA containing 1 mM P_i and 0.1 mM unlabeled ATP, and its radioactivity was measured as described previously (11). From the data obtained at six intervals, a time course curve for digoxigenin inhibition was constructed. The k_a values of digoxigenin (●) were calculated as described for Fig. 2 and are averages of three sets of experiments, with an experimental error at each point of less than ±0.05 min^{-1} μM^{-1}. For comparison, the k_a values of the type II ouabain complex at 30° (×) shown in Fig. 2 are replotted here.

field (8) and Erdmann and Schoner (9), and one report on the rate of dissociation of the ouabain-enzyme complex, by Akera *et al.* (23), have appeared. Insufficient details were present in the latter study to permit comparison of these results with those presented here. The present studies on the influence of pH on the k_a and k_d of the ouabain-enzyme complex and on the binding of cardiac aglycones are the first data reported on these facets of the cardiotonic steroid-$(Na^+ + K^+)$-ATPase complex.

Changes in pH strikingly affect the k_a values of type II complexes, but not type I complexes. Therefore, although the velocity of ouabain inhibition in the Na^+-Mg^{2+}-ATP system is greater than in the Mg^{2+}-P_i system at pH 7.5, this order is reversed at pH 7.0 (Fig. 2).

The type I complex of digoxigenin was not influenced at equilibrium by changes in pH. In the type II complex, the apparent number of sites was profoundly decreased

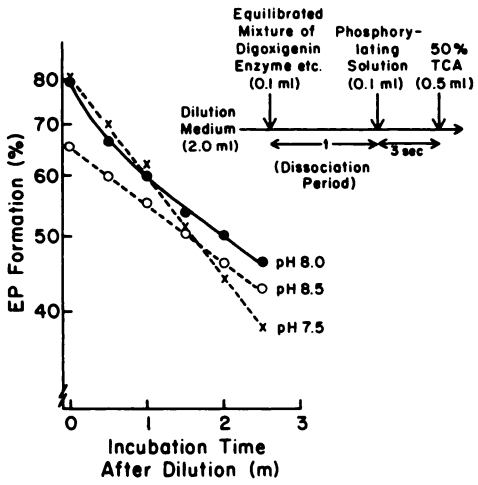

FIG. 6. *Stability of type II digoxigenin-enzyme complex at 23° after dilution*

NaI-treated microsomes (60–75 μg/ml) were incubated with a mixture of 2 mM Mg^{2+}, 2 mM P_i, 0.6–1.2 μM digoxigenin, and 50 mM imidazole, adjusted to the pH shown, for 30–60 min in order to reach equilibrium. As the flow sheet in the inset shows, the equilibrated mixture (0.1 ml) was diluted with 20 mM imidazole HCl buffer (the same pH as the equilibrated mixture) containing 1 mM EDTA and was phosphorylated at each interval. For experiments at pH 8.5, 50 mM Tris-HCl buffer and 6 μM digoxigenin were used. The procedures for phosphorylation and the *E*P assay were the same as described for Fig. 5.

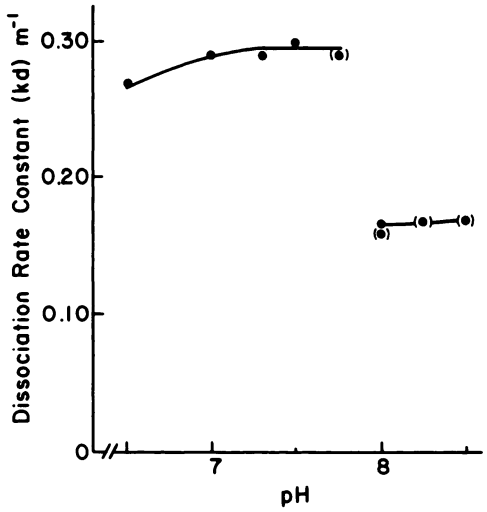

FIG. 7. *Effect of pH on dissociation rate constant (k_d) of type II digoxigenin-enzyme complex at 23°*

Each k_d value was calculated from the slope of the stability curve (average of two sets of experiments) shown in Fig. 6. The points in parentheses represent the results when 50 mM Tris-HCl was used as the buffer instead of imidazole HCl. The total average of k_d values between pH 6.5 and 7.75 was 0.29 min^{-1} (σ = 0.01, n = 10), and at pH 8.25 and 8.50, 0.17 min^{-1} (σ = 0.01, n = 4).

with increasing pH, but the apparent binding constant did not change below pH 8.0. However, the pH effects on ouabain binding at equilibrium are obscure in both types (Fig. 1). This discrepancy between a cardiac aglycone (digoxigenin) and a glycoside (ouabain) can be explained by the high affinity of the cardiac glycoside. If the total concentration of ouabain is 100 nM, more than 9 times greater than the binding constant (less than 10 nM), more than 90% of the enzyme binds with ouabain.[2] Under such conditions, usually used in measuring ouabain binding, the change in bound ouabain will be too small to detect, even when the binding constant is increased 2-fold or decreased by one-half. When the incubation time is not long enough for ouabain binding to reach equilibrium, the results obtained may represent the association rate inaccurately. We have already indicated that such a high affinity of the cardiac glycoside for $(Na^+ + K^+)$-ATPase origi-

[2] This calculation is based on a hypothetical equation: enzyme + ouabain ⇌ enzyme-ouabain complex.

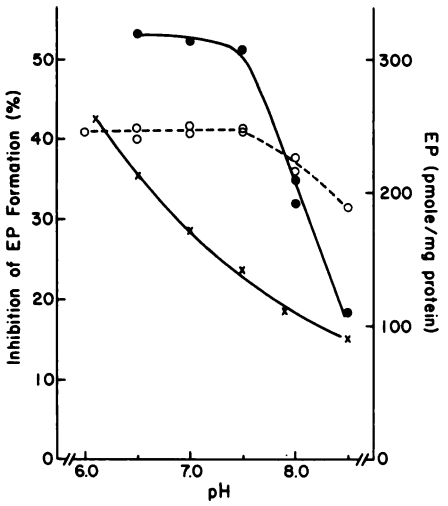

FIG. 8. *Effect of pH on EP formation and its inhibition by Mg^{2+} and P_i at 15°*

For determination of the *E*P value, 1 ml of the reaction mixture, containing NaSCN-treated microsomes (40–50 μg/ml) and 50 mM imidazole buffer and adjusted to the pH shown, was incubated for 3 min and then phosphorylated by 0.1 ml of the phosphorylating solution. When Mg^{2+} and P_i were added to the same enzyme and buffer, the value of *E*P inhibited with Mg^{2+} and P_i was obtained by the same procedure, and the inhibition of *E*P formation by Mg^{2+} and P_i was calculated. O- - -O, *E*P value; ●——●, inhibition of *E*P formation with 4 mM Mg^{2+} and 4 mM P_i; ×——×, inhibition of *E*P formation with 1 mM Mg^{2+} and 1 mM P_i. Each value is the average of the four measurements. The standard deviation for the *E*P value was 15 pmoles/mg of protein, and that of the inhibition of *E*P formation was 3%. Since two batches of enzyme preparation were used in this experiment, the same sets of experiments were repeated at several pH values. These results are shown as separate points, but no significant differences between batches were observed.

nates in the interaction between the first sugar group of the glycoside and the sugar-specific site on the enzyme (5, 7, 11, 24, 25).

In both the Na^+-Mg^{2+}-ATP and Mg^{2+}-P_i systems, (a) the apparent number of sites of digoxigenin binding, (b) the k_a of ouabain or digoxigenin, and (c) the *E*P value (in the Na^+-Mg^{2+}-ATP system) or the inhibition of *E*P formation by Mg^{2+} and P_i varied similarly with changes in pH. These similarities support our previous conclusions about the reaction mechanisms: that the phosphorylated form of $(Na^+ + K^+)$-ATPase is the active form, which binds with the cardiac steroid or the steroid moiety of the cardiac glycoside (11), and that binding of the steroid moiety activates the sugar-binding site of the enzyme, with consequent binding of the glycoside portion (6). The differences between type I and II complexes seem to reflect the differences between two types of phosphorylated enzyme formed by ATP and P_i.

From the changes in k_a values for type II complexes with digoxigenin or ouabain, it might seem that only phosphate monoanion, $H_2PO_4^-$, is the effective form for the type II complex, i.e., that a decrease in concentration of the monoanion at higher pH causes the k_a of the cardiotonic steroid to decrease. However, in the inhibition of ouabain binding, 1.0 mM P_i is already saturated at pH 7.3 (6), whereas the k_a of ouabain in the presence of 4 mM P_i is nearly half the k_a at pH 6.5 (Fig. 2). We have recently found that 2 mM P_i is saturated at pH 8.5 for type II complex formation with ouabain.[3] Therefore a decrease in the concentration of $H_2PO_4^-$ cannot be the reason for changes in the k_a values for type II complexes; instead, the main reason seems to be an ionic change in the enzyme.

The k_d values of the type II complexes change around pH 8.0, but in other pH ranges they remain constant. Such changes are observed for both digoxogenin and ouabain. These observations suggest that the type II complex has two different subtypes, one (type IIA) present below pH 8.0, and the other (type IIB) above pH 8.0. The coexistence of both subtypes was observed at pH 8.0 in the case of digoxigenin (Fig. 6). The conversion of type IIA to type IIB and the reverse occurred spontaneously only when the pH changed, but both reactions, especially that from type IIB to type IIA, were slow. Another important effect of pH change on k_d is the marked decrease in k_d with increasing pH for the type I ouabain complex. The k_d value of this ouabain complex changes reversibly with the pH of the dilution medium, but the stabilizing effect of K^+ in the dilution medium on the type I ouabain complex is not affected by pH. More studies are needed to explain such k_d changes with pH.

[3] Unpublished observations.

REFERENCES

1. Matsui, H. & Schwartz, A. (1968) *Biochim. Biophys. Acta,* **151,** 655–663.
2. Schwartz, A., Matsui, H. & Laughter, A. H. (1968) *Science,* **160,** 323–325.
3. Sen, A. K., Tobin, T. & Post, R. L. (1969) *J. Biol. Chem.,* **254,** 6596–6604.
4. Albers, R. W., Koval, G. J. & Siegel, G. J. (1970) *Mol. Pharmacol.,* **4,** 324–336.
5. Yoda, A. (1973) *Mol. Pharmacol.,* **9,** 51–60.
6. Yoda, A., Yoda, S. & Sarrif, A. M. (1973) *Mol. Pharmacol.,* **9,** 766–773.
7. Yoda, A. & Yoda, S. (1974) *Mol. Pharmacol.,* **10,** 494–500.
8. Whittam, R. & Chipperfield, A. R. (1973) *Biochim. Biophys. Acta,* **307,** 563–577.
9. Erdmann, E. & Schoner, W. (1973) *Biochim. Biophys. Acta,* **330,** 302–315.
10. Yoda, A. (1976) *Mol. Pharmacol.,* **12,** 399–408.
11. Yoda, A. & Yoda, S. (1977) *Mol. Pharmacol.,* **13,** 352–361.
12. Nakao, T., Tashima, K., Nagano, K. & Nakao, M. (1965) *Biochem. Biophys. Res. Commun.,* **40,** 880–886.
13. Hegyvary, C. & Post, R. L. (1971) *J. Biol. Chem.,* **193,** 265–275.
14. Klodos, I., Ottolenghi, P. & Boldgren, A. (1975) *Anal. Biochem.,* **67,** 397–403.
15. Glynn, I. M. & Chappel, J. B. (1964) *Biochem. J.,* **90,** 147–149.
16. Yoda, A. & Hokin, L. E. (1970) *Biochem. Biophys. Res. Commun.,* **40,** 880–886.
17. Kanazawa, T., Saito, M. & Tonomura, Y. (1970) *J. Biochem. (Tokyo),* **67,** 693–711.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.,* **193,** 265–275.
19. Akera, T. & Brody, T. M. (1971) *J. Pharmacol. Exp. Ther.,* **176,** 545–557.
20. Allen, J. C., Harris, R. A. & Schwartz, A. (1971) *Biochem. Biophys. Res. Commun.,* **42,** 366–370.
21. Yoda, A. & Yoda, S. (1974) *Mol. Pharmacol.,* **10,** 810–819.
22. Post, R. L., Toda, G. & Rogers, R. U. (1975) *J. Biol. Chem.,* **250,** 691–701.
23. Akera, T., Brody, T. M., So, R. H.-M., Tobin, T. & Baskin, S. I. (1974) *Ann. N. Y. Acad. Sci.,* **242,** 617–632.
24. Yoda, S., Sarrif, A. W. & Yoda, A. (1975) *Mol. Pharmacol.,* **11,** 647–652.
25. Yoda, A. & Yoda, S. (1975) *Mol. Pharmacol.,* **11,** 653–662.