FACTORS AND AGENTS THAT INFLUENCE CARDIAC GLYCOSIDE-Na^+, K^+-ATPase INTERACTION*

T. Akera, T. M. Brody, R. H-M. So,
T. Tobin, and S. I. Baskin

Department of Pharmacology
Michigan State University
East Lansing, Michigan 48824

The optimal conditions for the binding of cardiac glycosides, such as ouabain, to Na^+, K^+-ATPase *in vitro* are the simultaneous presence of ATP, Na^+ and Mg^{++} or the presence of inorganic phosphate (Pi) and Mg^{++} (Reference 1). It has been postulated that *in vitro* binding of ouabain in the presence of ATP, Na^+ and Mg^{++} reflects the binding of cardiac glycosides to this enzyme system in intact animals,[2] and it is this interaction that ultimately results in the production of the positive inotropic and/or arrhythmic effects.[3] Thus, studies on the factors and agents that influence the cardiac glycoside-Na^+, K^+-ATPase interaction *in vitro* appear important for an understanding of the factors and agents that modify the magnitude of response to cardiac glycosides in patients when the plasma concentrations of cardiac glycosides are maintained at a fixed level. Moreover, such studies may shed light on the mechanism of the Na^+, K^+-ATPase reaction itself.

Monovalent Cations

Na^+, K^+-ATPase has been shown to be an allosteric enzyme and its configurations are determined by monovalent cation and phosphate ligands.[4-11] Therefore, it is reasonable to assume that these ligands affect the ouabain-enzyme interactions. The binding of cardiac glycosides to Na^+, K^+-ATPase *in vitro* in the presence of ATP, Na^+ and Mg^{++} is markedly inhibited by K^+ (Reference 1). Since K^+ has been shown to reverse cardiac glycoside-induced arrhythmias,[12, 13] it was once assumed that K^+ reduces the level of cardiac glycoside bound to the Na^+, K^+-ATPase, presumably by reducing the rate of binding, increasing the rate of dissociation, or both. Supporting this hypothesis, a number of investigators has reported that K^+ antagonized the ouabain-inhibition of the Na^+, K^+-ATPase activity *in vitro*, although the evidence does not quite fit simple competitive inhibition for ouabain with respect to KCl (see Reference 14).

The steady-state levels of the enzyme-bond ouabain at a given drug concentration in the medium are determined by two independent variables, i.e., the rate of binding and the rate of release. These two parameters can be studied separately.

FIGURE 1A shows the results of an experiment in which the rate of [^{3}H] ouabain binding was monitored. Na^+, K^+-ATPase preparations obtained from rat brain microsomal fractions following deoxycholic acid and NaI treatments were incubated at 37°C with 0.01 μM [^{3}H] ouabain (specific activity; 13.2 Ci/mmol) in the presence of 20 mM NaCl, 5 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5). Aliquots were taken at the indicated times and the bound

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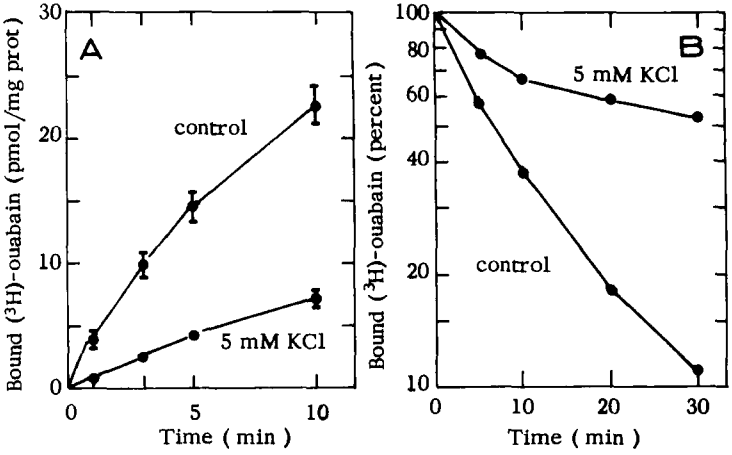

FIGURE 1. The effect of K^+ on [^{3}H]ouabain binding and release. A: Rat brain Na^+, K^+-ATPase preparations were incubated with 0.01 μM [^{3}H]ouabain in the presence of ATP, Na^+ and Mg^{++} at 37°C. B: The [^{3}H]ouabain-enzyme complex prepared with ATP, Na^+ and Mg^{++} (but without K^+) and washed free from ATP, cations, and free [^{3}H]ouabain was suspended in 10 mM Tris-HCl buffer (pH 7.5) and the dissociation was monitored at 37°C. N = 4. Vertical lines: S.E.M.

[^{3}H]ouabain was assayed by liquid scintillation counting after a separation from unbound [^{3}H]ouabain using a Millipore filter system. Nonspecific binding, i.e., the binding in the absence of ATP, was subtracted to calculate the ATP-dependent binding. At this low ouabain concentration, it was possible to monitor the initial rate of [^{3}H]ouabain binding. K^+ markedly reduced the rate of the [^{3}H]ouabain binding. It has been postulated that this effect of K^+ is due to the reduction in the K^+-sensitive form of phosphoenzyme to which ouabain binds preferentially,[7] despite the fact that K^+-induced reduction in phosphoenzyme concentration is disproportionally greater than that in the ouabain binding rate.[15]

FIGURE 1B shows the results of experiments in which the rate of release of [^{3}H]ouabain from Na^+, K^+-ATPase was monitored. The [^{3}H]ouabain-enzyme complex prepared as above in the absence of K^+, was centrifuged at 100,000 X *g* for 30 minutes and the pellets were resuspended in 10 mM Tris-HCl buffer (pH 7.5). This procedure, which was performed at 0°C, effectively removed unbound [^{3}H]ouabain, ATP, Na^+ and Mg^{++}, whereas [^{3}H]ouabain-enzyme complex was retained. The bound [^{3}H]ouabain did not dissociate significantly from the enzyme at this low temperature. The decay of the ouabain-enzyme complex was monitored at 37°C, assaying aliquots of the suspension for bound [^{3}H]ouabain remaining undissociated at the indicated times. The dissociation reaction followed first-order kinetics and the half-life of the ouabain-enzyme complex under these conditions was 6.4 minutes. When 5 mM KCl was added to the suspension of [^{3}H]ouabain-enzyme complex, the rate of the release of [^{3}H]ouabain was markedly reduced (FIGURE 1B). The magnitude of the K^+ effect on dissociation was such that it offset the K^+-induced reduction in ouabain binding rate on the steady state levels. This is consistent with our previous data that K^+ failed to influence the reduction of cardiac Na^+,

K^+-ATPase activity observed with the intravenous infusion of ouabain in anesthetized dogs.[16] Since K^+ reduced the rates of both binding and release of [^{3}H] ouabain, it was postulated that K^+ reduced the accessibility of ouabain binding sites.[17]

Certain agents are capable of modifying the K^+ effect on ouabain binding and dissociation. Among these is the metabolic inhibitor, phloridzin. The effect of phloridzin on the release of [^{3}H] ouabain from Na^+, K^+-ATPase is shown in TABLE 1. Robinson demonstrated that phloridzin increases the apparent affinity of Na^+, K^+-ATPase for K^+ and decreases it for Na^+, and concluded that these actions of phloridzin are due to a heterotropic allosteric modification of the enzyme.[18] Therefore, the effects of K^+ on the ouabain-enzyme complex were studied with or without phloridzin in order to compare with those on the native enzyme. The [^{3}H] ouabain-enzyme complex was prepared as described above, and the dissociation rate was monitored at 37°C in the presence of 10 mM Tris-HCl buffer (pH 7.5). The amount of bound [^{3}H] ouabain remaining undissociated after a 10-minute incubation period is shown in TABLE 1. Potassium stabilized the ouabain-enzyme complex in a dose-dependent manner. Phloridzin failed to affect the dissociation rate either in the absence of KCl or in the presence of saturating concentrations of KCl. However, the stabilizing effect of 0.2 mM KCl, which produced approximately half-maximal stabilization of the complex, was significantly enhanced by 3 mM phloridzin ($P < 0.01$ by paired *t* test). Phloridzin also potentiates the K^+ effect on binding.

The effects of other cations on the ouabain-enzyme complex are shown in TABLE 2. The full activation of Na^+, K^+-ATPase activity requires the simultaneous presence of Na^+ and K^+.[19] Whereas Na^+ cannot be substituted by other cations, K^+ may be replaced by NH_4^+, Rb^+, Cs^+ or Tl^+.[19-22] These cations also stabilized the ouabain-enzyme complex prepared with ATP, Na^+, and Mg^{++} (TABLE 2). The amount of bound [^{3}H] ouabain remaining after a 30-minute incubation at 37°C was significantly increased by NH_4^+, Rb^+, K^+, Cs^+ and Tl^+, whereas the same concentration of Na^+ and Li^+ were markedly less effective. Although Na^+ stabilized the complex at higher concentrations it was obvious that the ouabain-enzyme complex discriminated Na^+ and Li^+ from other cations in a manner similar to that by which the native phosphoenzyme discriminates these cations.

Thus, the effects of K^+ on the ouabain-enzyme complex prepared with ATP, Na^+ and Mg^{++} appeared to be related to the effect of K^+ on the native enzyme. Since the ouabain-enzyme complex is likely to be dephosphorylated prior to the

TABLE 1

Phloridzin Enhancement of K^+ Effect on the Ouabain-Na^+, K^+-ATPase Complex*

	KCl Concentrations		
	0 (%)†	0.2 mM (%)	5 mM (%)
Control	16.8 ± 1.4	37.4 ± 1.8	73.3 ± 1.7
Phloridzin (3 mM)	17.9 ± 1.8	44.0 ± 3.9‡	74.0 ± 1.2

*The [^{3}H] ouabain-enzyme complex was prepared with ATP, Na^+ and Mg^{++}. Free [^{3}H] ouabain, ATP, Na^+ and Mg^{++} were removed by centrifugation.

†Percentage of bound [^{3}H] ouabain remaining after a 10-minute incubation at 37°C. Mean ± S.E.M. of five experiments with different enzyme preparations.

‡Significantly different from corresponding control values ($P < 0.01$ by paired *t* test).

TABLE 2
Effects of Monovalent Cations on the Ouabain-Na^+, K^+-ATPase Complex*

Cations	Bound [^{3}H]ouabain (%)†
None	9.6 ± 0.3
NH_4^+ 1 mM	58.6 ± 2.9
Rb^+ 1 mM	51.4 ± 2.9
K^+ 1 mM	50.8 ± 6.8
Cs^+ 1 mM	48.0 ± 2.9
Tl^+ 1 mM	40.6 ± 2.3
Na^+ 1 mM	17.2 ± 0.4
Li^+ 1 mM	16.7 ± 0.6

*The [^{3}H]ouabain-enzyme complex was prepared with ATP, Na^+ and Mg^{++}. Free [^{3}H]ouabain, ATP, Na^+ and Mg^{++} were removed by centrifugation.
†Percentage of bound [^{3}H]ouabain remaining after a 30-minute incubation at 37°C. Mean ± S.E.M. of three experiments with different enzyme preparations.

dissociation reaction, the effect of K^+ would appear to result from a K^+-induced conformational change in the complex.

Phosphate Ligands

The ouabain-enzyme complex formed with various phosphate ligands dissociated at different rates in the absence of K^+ (TABLE 3). In these experiments, [^{3}H]ouabain-enzyme complexes were prepared by incubating the enzyme preparation and 0.1 μM [^{3}H]ouabain with 10 mM NaCl, 5 mM $MgCl_2$, and 50 mM Tris-HCl buffer (pH 7.5) in the presence of 5 mM ATP, *p*-nitrophenylphosphate, acetylphosphate, or carbamylphosphate, or with 1 mM Tris-phosphate, 1 mM $MgCl_2$ and 10 mM Tris-HCl buffer (pH 7.5). All these phosphate ligands were capable of supporting ouabain binding. After a 10-minute reaction period

TABLE 3
Dissociation of Ouabain-Na^+, K^+-ATPase Complexes Formed with Various Phosphate Ligands*

Phosphate Ligands	Bound [^{3}H]ouabain	
	No KCl (%)†	KCl 5 mM (%)†
ATP	23.6 ± 1.0	53.2 ± 2.5
p-Nitrophenylphosphate	30.1 ± 2.3	43.1 ± 4.6
Acetylphosphate	33.9 ± 1.9	48.6 ± 6.1
Carbamylphosphate	40.6 ± 2.0	50.1 ± 4.4
Inorganic phosphate	50.9 ± 3.4	51.6 ± 4.7

*The binding of [^{3}H]ouabain was stopped by the dilution and the addition of excess cold ouabain.
†Percent of bound [^{3}H]ouabain remaining after a 30-minute incubation at 37°C. Mean ± S.E.M. of five experiments with different enzyme preparations.
‡Since 1 mM NaCl was present during the dissociation reaction, the control (no KCl) dissociation rate was slower than those shown in TABLES 1 and 2.

at 37°C, the binding of the labeled ouabain was stopped by adding cold ouabain and diluting the mixture 10-fold (final concentration of cold ouabain, 0.1 mM). Since this procedure effectively stopped further binding of the labeled ouabain, it was possible to monitor the dissociation of the [^{3}H]ouabain-enzyme complex. The percentage of bound [^{3}H]ouabain remaining after a 30-minute incubation at 37°C was smallest with the ouabain-enzyme complex prepared with ATP (unstable complex) and largest with the complex prepared with Pi (stable complex). Those complexes prepared with *p*-nitrophenylphosphate, acetylphosphate, and carbamylphosphate had intermediate rates of dissociation. K^+ stabilized the unstable complexes to the levels of the most stable complex, i.e., the ouabain-enzyme complex prepared with Mg^{++} and Pi, but failed to further stabilize the latter complex. The rate of [^{3}H]ouabain binding in the presence of Mg^{++} and Pi was also minimally affected by K^+. It appeared that the ouabain-enzyme complexes prepared with *p*-nitrophenylphosphate, acetylphosphate, and carbamylphosphate are mixtures of at least two distinct forms, namely, a K^+-sensitive unstable form and a K^+-insensitive stable form.

Temperature

The Na^+, K^+-ATPase reaction has been shown to have a critical temperature which lies between 18° and 20°C (see Reference 23). Charnock, Cook, and Opit have postulated that the phosphoenzyme loses its ability to assume a K^+-sensitive conformation below this temperature.[23] The temperature dependency of the K^+-effect on the ouabain-enzyme complex is shown in TABLE 4. In these experi-

TABLE 4

Dissociation of [^{3}H] Ouabain-Na^+, K^+-ATPase Complex at Various Temperatures*

	12°C (%)†	17°C (%)	22°C (%)	27°C (%)	37°C (%)
No KCl	80.9 ± 1.3	76.2 ± 2.9	58.5 ± 4.6	45.0 ± 4.1	11.0 ± 1.1
KCl 5 mM	83.8 ± 0.9	82.1 ± 1.8	76.2 ± 3.6	74.8 ± 3.6	48.2 ± 7.0
	(7)‡	(7)	(7)	(7)	(4)

*The [^{3}H]ouabain-enzyme complex was prepared at 37°C in the presence of ATP, Na^+ and Mg^{++}. Binding of [^{3}H]ouabain was stopped by the dilution and the addition of cold ouabain.

†Percentage of bound [^{3}H]ouabain remaining after a 30-minute incubation. Mean ± S.E.M.

‡Numbers of experiments with different enzyme preparations.

ments, the binding of [^{3}H]ouabain was stopped by the addition of cold ouabain as in the experiments shown in TABLE 3. The rates of dissociation of the [^{3}H]ouabain-enzyme complex in the absence of KCl were highly temperature-sensitive, whereas those in the presence of 5 mM KCl were relatively temperature-insensitive. Below 17°C, i.e., below the reported critical temperature for the Na^+, K^+-ATPase reaction, the dissociation rates of the ouabain-enzyme complex were minimally affected by K^+. These data indicate that the same critical temperature exists for ATP hydrolysis and K^+-induced stabilization of the ouabain-enzyme complex and may support the hypothesis proposed by Charnock and his coworkers.[23]

TABLE 5
Effects of Deoxycholic Acid on [^{3}H]ouabain Binding and Dissociation

KCl	Deoxycholic Acid	Binding* 1 min.	Binding* 3 min.	Binding* 10 min.	Dissociation† 10 min.	Dissociation† 30 min.
		(pmol/mg protein)			(%)‡	
–	–	104.0 ± 0.5§	153.7 ± 18.3	177.5 ± 27.3	34.1 ± 0.6	4.9 ± 1.3
–	0.3 mM	25.2 ± 3.0	53.6 ± 10.9	77.5 ± 15.9	36.4 ± 3.1	3.0 ± 1.8
5 mM	–	17.4 ± 2.7	29.6 ± 2.6	50.0 ± 4.4	41.1 ± 1.4	30.0 ± 1.3
5 mM	0.3 mM	24.9 ± 1.9	44.4 ± 2.7	59.2 ± 3.5	23.5 ± 1.6	9.4 ± 2.1

*Binding in the presence of ATP, Na^+ and Mg^{++}.
†The [^{3}H]ouabain-enzyme complex was prepared with ATP, Na^+ and Mg^{++} in the presence or absence of KCl and deoxycholic acid. The binding of [^{3}H]ouabain was stopped by the addition of cold ouabain.
‡Percentage of bound [^{3}H]ouabain remained after incubation at 37°C.
§Values are mean ± S.E.M. of five experiments with different enzyme preparations.

Deoxycholic Acid

Deoxycholic acid affected the rates of both [^{3}H]ouabain binding and release in the presence of K^+ (TABLE 5). These enzymes were exposed to 0.37 mM deoxycholic acid during the process of preparation. This treatment at 0°–5°C increased the enzyme activity. At 37°C, however, 0.3 mM deoxycholic acid produced 35.2 ± 2.4% decrease in enzyme activity with a concomitant decrease in [^{3}H]ouabain binding. In the presence of KCl, however, deoxycholic aicd increased the initial rate of [^{3}H]ouabain binding (TABLE 5). When the [^{3}H]ouabain enzyme complex was prepared with ATP, Na^+ and Mg^{++} and the rate of [^{3}H]ouabain dissociation was monitored at 37°C in the absence of KCl, 0.3 mM deoxycholic acid failed to influence the dissociation rate. In the presence of 5 mM KCl, however, 0.3 mM deoxycholic acid increased the release of [^{3}H]ouabain from the enzyme. These data are in agreement with the observations of Taniguchi and Iida[24] and may suggest that the K^+-induced conformation that reduces the accessibility of the ouabain binding sites involves a lipoprotein.

Mechanical Shaking

Kim, Bailey, and Dresel[25] have reported that a 5-minute shaking with a Vortex mixer causes a release of [^{3}H]ouabain which was "loosely" bound to microsomal fractions isolated from the [^{3}H]ouabain-perfused guinea pig hearts. These authors have demonstrated a positive correlation between such loosely bound drug and the positive inotropic action. Moreover, Na^+, K^+-ATPase preparations are frequently shaken during the processes of isolation and assay. Therefore, the effects of mechanical shaking of Na^+, K^+-ATPase suspensions were studied using partially purified rat brain enzyme preparations and guinea pig heart homogenates. Mechanical shaking was performed by placing a test tube containing a 2.5 ml aliquot of the enzyme suspension on a Vortex mechanical mixer (A. H. Thomas Scientific Company, Philadelphia, Pa.). The enzyme suspension (0.2–0.5 mg of protein per milliliter) contained 0.32 M sucrose, 1 mM EDTA, and 5 mM histidine (pH 7.0) and was shaken at 50 Hz. The diameter of the circular movement of the test tube was approximately 1.5 cm. Shaking of the ouabain-enzyme complex, which was pre-formed by incubating

rat brain enzyme or guinea pig heart homogenates in the presence of [^{3}H] ouabain, ATP, Na^+ and Mg^{++}, caused a significant release of bound ouabain. The bound ouabain released by the shaking was primarily that formed by ATP-dependent binding. [^{3}H] ouabain bound to the enzyme in the absence of ATP was only minimally affected by the shaking (TABLE 6). Further release of [^{3}H] ouabain from rat brain enzyme at 0°C after the termination of the shaking was minimal. In control experiments in which the ouabain-enzyme complex was not subjected to mechanical shaking, but was stored at 0°, the release of [^{3}H] ouabain from rat brain enzyme was also very slow (TABLE 6). The addition of 5 mM KCl to the mixture failed to prevent the release of [^{3}H] ouabain by mechanical shaking, although K^+ reduces the rate of spontaneous dissociation of the ouabain-enzyme complex at 37°C. Thus, mechanical shaking of enzyme preparations released specifically prebound ouabain regardless of the presence or absence of KCl.

Mechanical shaking of the enzyme suspension also affected the enzyme activity. The Na^+, K^+-ATPase and K^+-NPPase (K^+-stimulated *p*-nitrophenylphosphatase) activities of the enzyme preparations used in these studies were approximately 240 μmol of Pi released from ATP and 60 μmoles of *p*-nitrophenol produced from *p*-nitrophenylphosphate per milligram protein in 60 minutes, respectively. Mechanical shaking prior to the enzyme assay resulted in a loss of these enzyme activities (FIGURE 2). The magnitude of the inactivation was dependent on the duration of the shaking. The loss of K^+-NPPase activity, a partial reaction of Na^+, K^+-ATPase,[11] was significantly less than that of Na^+, K^+-ATPase activity after a 5-minute shaking period ($P < 0.05$). Attempts to protect Na^+, K^+-ATPase from inactivation by adding such agents as 5 mM Tris-ATP, 5 mM $MgCl_2$, 100 mM NaCl, 3 mM EDTA, 10 μM dithiothreitol, phosphatidyl serine (0.75 or 1.5 mg/ml), and various combinations of these reagents to the enzyme suspension

TABLE 6

The Effect of Mechanical Shaking on the [^{3}H] Ouabain-Na^+, K^+-ATPase Complex

	Rat Brain Enzyme		Guinea Pig Heart Homogenate	
	Control	Treated	Control	Treated
Number of experiments	4	4	5	5
Bound [^{3}H] ouabain	(pmol/mg protein)		(pmol/mg protein)	
Specific*	101.0 ± 4.5 (96.2 ± 5.0)‡	15.4 ± 3.1† (14.1 ± 3.8)†‡	1.90 ± 0.24	0.52 ± 0.11†
Nonspecific*	2.04 ± 0.35§	1.92 ± 0.12§‖	0.50 ± 0.07	0.39 ± 0.06‖

NOTE: The [^{3}H] ouabain-enzyme complex was prepared by incubating the enzyme preparation (0.02 mg of rat brain enzyme protein or 1 mg of guinea pig heart homogenate protein per ml) with [^{3}H] ouabain (0.1 μM for rat brain enzyme or 0.02 μM for guinea pig heart homogenate) in the presence of 20 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 50 mM Tris-HCl buffer (pH 7.5) at 37° for 10 min. At the end of the incubation period, the mixture was rapidly cooled in ice and subjected to mechanical shaking at 0° for 5 minutes (treated) or stored at 0° for a comparable period (control). Bound [^{3}H] ouabain was then assayed immediately. Values are mean ± S.E.M.

*Specific: ATP-dependent portion; nonspecific: ATP-independent portion.

†Significantly different from control ($P < 0.01$).

‡Numbers in parenthesis indicate the values obtained 40 minutes after the treatment. Control and treated preparations were stored at 0° during this time.

§Data from 0 and 40 minutes were pooled. Mean ± S.E.M. of eight experiments.

‖Values from control and treated preparations were statistically not different.

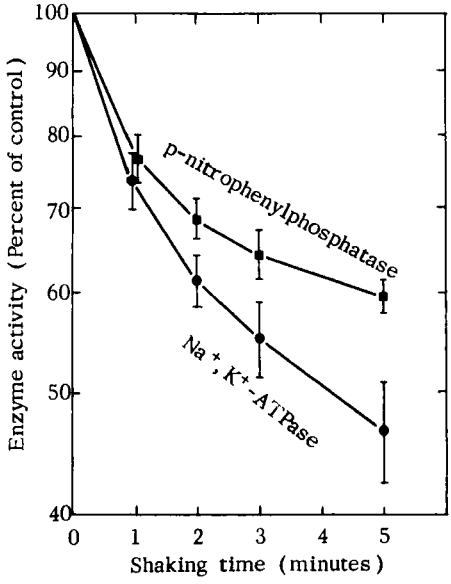

FIGURE 2. Inactivation of Na^+, K^+-ATPase and K^+-*p*-nitrophenylphosphatase activities by shaking of the enzyme suspension. Rat brain enzyme preparation was shaken with Vortex mixer for indicated time at 27°C. N = 5. Vertical lines: S.E.M.

were unsuccessful. Exclusion of sucrose, EDTA, or histidine from the medium had little influence on the loss of the enzyme activity due to shaking.

The Na^+, K^+-ATPase reaction consists of at least two distinct steps, namely, a Na^+, Mg^{++}-dependent phosphorylation reaction and K^+-stimulated dephosphorylation reaction.[4,26] The latter reaction appears to be related to the K^+-NPPase activity.[11] The former step, i.e., that Na^+, Mg^{++}-dependent phosphorylation reaction, was studied at 0°C by monitoring the phosphorylation of the enzyme from $\gamma[^{32}P]$ATP.[27] Prior mechanical shaking of the enzyme for 5 minutes at room temperature reduced the maximal levels of phosphorylation of the enzyme with $\gamma[^{32}P]$ATP (FIGURE 3). The rate of dephosphorylation at 0°C in the absence of K^+ appeared to be faster with the shaken enzyme than with controls (FIGURE 3). At 15 seconds, the K^+-sensitive portion of phosphoenzyme was lost almost completely with the shaken enzyme whereas with the nontreated enzyme approximately 30% of such phosphoenzyme remained. However, due to the difficulty of estimating the levels of ^{32}P bound nonspecifically to the enzyme preparation, quantitative conclusions are not possible.

An alternative and indirect method to study the phosphorylation reaction of Na^+, K^+-ATPase is to monitor the rate of [^{3}H] ouabain binding to the enzyme in the presence of Na^+, Mg^{++} and ATP.[27] Under these conditions, phosphoenzyme would be formed, and ouabain would bind to this phosphoenzyme at a rate proportional to the concentration of the phosphoenzyme.[27-29] Although this method is indirect, it has advantages in that the reaction may be monitored at 37°C and a more accurate assay may be performed than by the direct estimation of phosphoenzyme formation. Prior mechanical shaking of the enzyme suspension

reduced the rate of ATP-dependent [^{3}H]ouabain binding (FIGURE 4). The reduction of the level of [^{3}H]ouabain binding at 5 minutes by prior mechanical shaking was 58.6 ± 4.6% (mean ± S.E.M. of four experiments). Na^+, K^+-ATPase activity in the same preparations was inhibited 72.4 ± 3.0% (n = 4).

Upon the termination of binding of [^{3}H]ouabain, the [^{3}H]ouabain-enzyme complex dissociated rapidly (FIGURE 5). The addition of 5 mM KCl to the mixture after the binding reaction delayed the dissociation. The K^+-induced delay in the release of ouabain has been postulated to be related to the K^+-induced conformational change in the enzyme protein and thus to the function of the transport ATPase.[17] Prior shaking of the enzyme preparation failed to alter the rates of dissociation of subsequently formed ouabain-enzyme complex either in the presence or absence of KCl. The ouabain-enzyme complex formed after shaking responded to KCl in a manner similar to the control ouabain-enzyme complex (FIGURE 5). Thus, it appears that the K^+-sensitivity of the ouabain-enzyme complex formed from shaken enzyme preparations is similar to that of control preparations.

Thus, mechanical shaking of Na^+, K^+-ATPase preparations resulted in a loss of catalytic activity. The nature of the inactivation is not clear since all attempts to protect the enzyme by adding various reagents were unsuccessful. It should be noted, however, that partial reactions associated with ATP hydrolysis, such as K^+-NPPase activity and the rate of the [^{3}H]ouabain binding reaction, which requires phosphorylation of the enzyme, were less affected by shaking than the overall ATPase reaction.

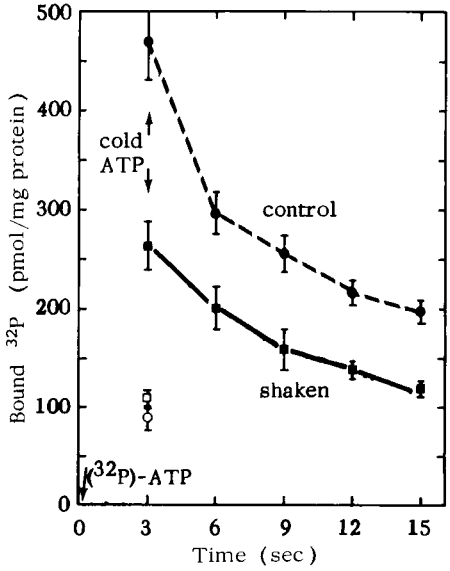

FIGURE 3. Effects of mechanical shaking on the formation of phosphoenzyme from γ[^{32}P]ATP and the decay of the phosphoenzyme. Rat brain enzyme preparation was shaken for 5 minutes. Phosphorylation was assayed at 0°C in the presence of 16 mM NaCl, 1 mM $MgCl_2$ and 50 mM Tris-HCl buffer (pH 7.5). γ[^{32}P]ATP concentration was 0.05 mM. Excess cold ATP was added at 3 seconds. Open symbols: 15 mM KCl present. N = 6. Vertical lines: S.E.M.

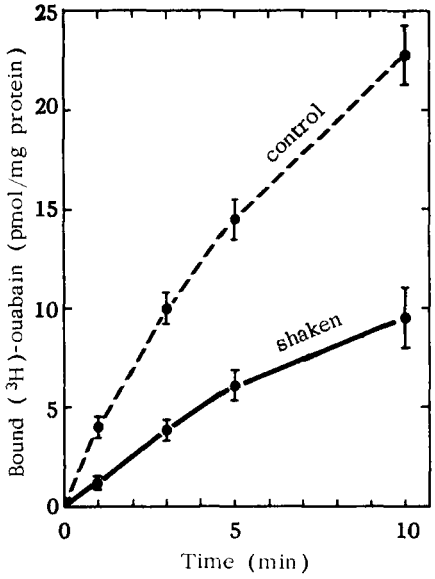

FIGURE 4. The effect of prior shaking of enzyme suspension on ATP-dependent [^{3}H]ouabain binding. Rat brain enzyme preparation was shaken for 5 minutes prior to [^{3}H]ouabain binding reaction. The shaking reduced Na^+, K^+-ATPase activity 72.5 ± 3.0 percent. N = 4. Vertical lines: S.E.M.

The present results indicate that Na^+, K^+-ATPase should not be subjected to vigorous shaking during its preparation and assay. Such a procedure not only results in a loss of activity but also releases cardiac glycosides prebound to the enzyme. The shaking of the ouabain-enzyme complex primarily released the ATP-dependent portion of the bound ouabain. This type of binding is saturable, results in enzyme inhibition, and is considered to be the specific binding to the Na^+, K^+-ATPase protein.[1, 14, 15, 30] The ATP-independent portion of the bound ouabain, which is nonsaturable and hence considered to represent nonspecific binding to the enzyme preparation,[15] was only minimally influenced by mechanical shaking. Thus, mechanical shaking may provide a means of distinguishing that component of the cardiac glycosides specifically bound to Na^+, K^+-ATPase from other fractions of cardiac glycosides present in tissue preparations. The release was not complete under the present experimental conditions, however, since a significant portion (15–27%) of the specifically bound ouabain still remained after a 5-minute shaking.

The data strongly suggest that the "loosely bound ouabain" described by Kim and coworkers[25] represents that fraction of the drug bound specifically to Na^+, K^+-ATPase.

Cardiac Glycosides

The stability of a cardiac glycoside-Na^+, K^+-ATPase complex is dependent on the particular cardiac glycoside.[31] Compared with the complex formed with ouabain, those formed with digoxin and digitoxin were significantly more stable in the absence of KCl (FIGURE 6). K^+ stabilized these complexes, and those

complexes formed with digoxin or digitoxin were more stable than that formed with ouabain in the presence of K^+.

Source of Enzyme

Marked species differences in sensitivity to the inotropic and toxic effects of cardiac glycosides have been known for a long time. It has been shown that these differences *in vivo* may be accounted for by the differences in sensitivity of cardiac Na^+, K^+-ATPase.[32-36] From the half-time of the dissociation reaction of the ouabain-enzyme complexes, first-order dissocation rate constants were obtained (TABLE 7). If one can assume that the association rate constants are similar for different enzyme preparations, it is possible to calculate the Ki values for the *in vitro* inhibition of Na^+, K^+-ATPase by ouabain.[35] Such calculations, based upon the assumption that various enzyme preparations have the association rates similar to those for guinea pig kidney enzyme, are shown in TABLE 7. The calculated K_i's are in good agreement with the observed K_i's. Thus, it would appear that differences in sensitivity of Na^+, K^+-ATPase to ouabain mainly depends upon the differences in dissociation rates, whereas association rates are rather similar.

Na^+, K^+-ATPase preparations from rat brain formed a rather stable complex with ouabain. The half-time for the dissociation in the presence of KCl was greater than 30 minutes (FIGURE 1). The ouabain-enzyme complex prepared

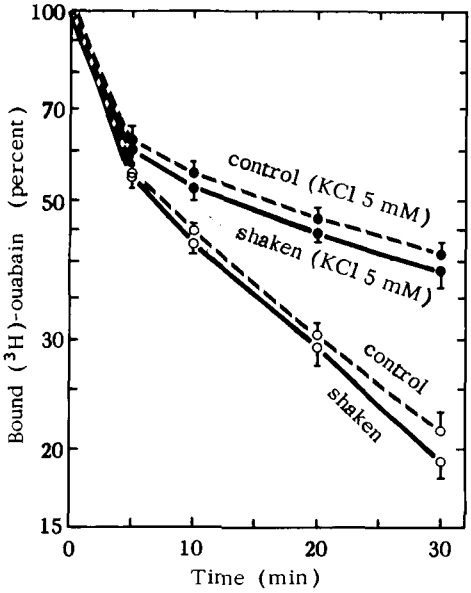

FIGURE 5. Failure of prior shaking of enzyme preparation to alter rates of dissociation of subsequently formed ouabain-enzyme complex. After the binding reaction, 0.1 mM cold ouabain was added to stop [^{3}H]ouabain binding at time zero. Data are expressed as percentage of bound [^{3}H]ouabain in each preparation immediately before the addition of cold ouabain (time zero). These values are 22.6 ± 1.5 (control) and 9.6 ± 1.6 pmol mg protein (previously shaken enzyme), respectively. N = 5. Vertical lines: S.E.M.

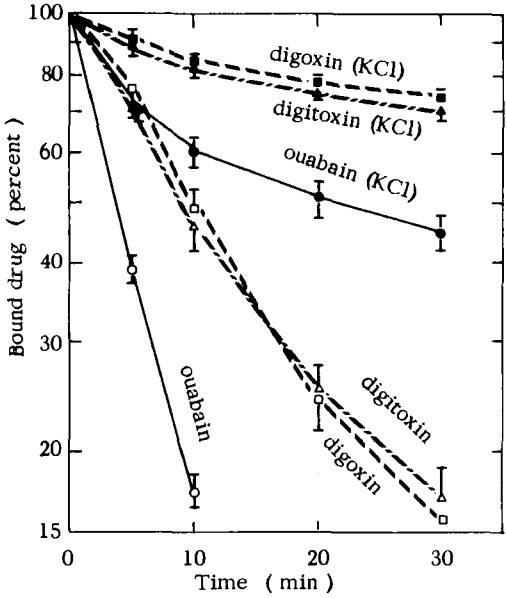

FIGURE 6. Dissociation of ouabain, digoxin, and digitoxin from Na^+, K^+-ATPase. The cardiac glycoside-enzyme complexes were formed with ATP, Na^+ and Mg^{++}, washed to remove ATP, cations and free glycosides, and allowed to dissociated at 37°C. N = 4. Vertical lines: S.E.M.

with rat cardiac enzyme, on the contrary, had an extremely rapid dissociation rate and high K_i value (TABLE 7). Experimental determination of dissociation rates, however, was not possible with rat cardiac enzyme. The number in parenthesis in TABLE 7, i.e., half-time of 3 seconds, indicates the half-time of the dissociation reaction calculated from the observed K_i value. Thus, the stability of the ouabain-enzyme complex is greatly influenced by the source of the enzyme.

The ouabain-enzyme complexes formed with cardiac Na^+, K^+-ATPase preparations obtained from guinea pig and rabbit dissociated more rapidly than those

TABLE 7

Relationship between K_i for Ouabain and the Dissociation Rate of the Ouabain-Enzyme Complex

Enzyme Source	Measured T½ (min)*	K dissociation (min^{-1})	Calculated K_i (μM)	Observed K_i (μM)
Dog brain	87	0.008	0.11	0.06–0.76
Dog heart	45	0.016	0.22	–0.79
Rat brain	45	0.016	0.02	0.18–1.6
Rabbit kidney	10	0.070	1.0	0.50–1.5
Guinea pig kidney	2.5	0.280	–	4.0
Rat heart	(0.05)			180

*[^{3}H] ouabain-enzyme complexes were prepared with Mg^{++} and Pi.

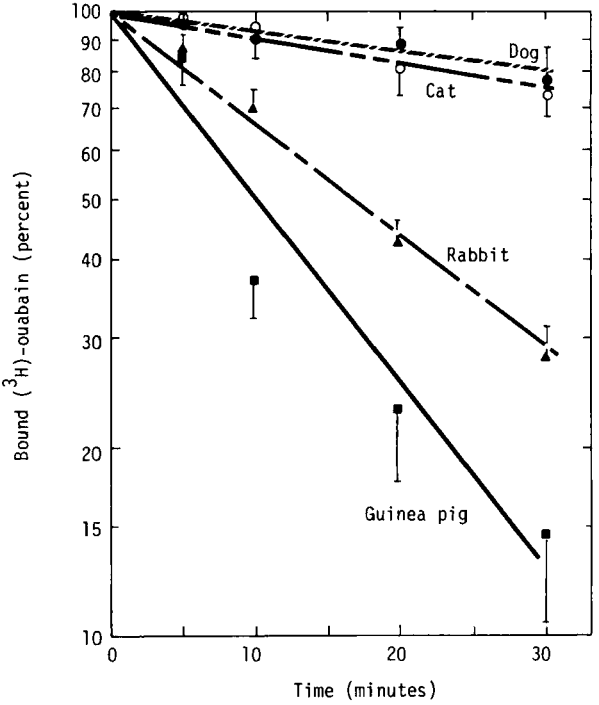

FIGURE 7. Dissociation of [^{3}H]ouabain from cardiac Na^+, K^+-ATPase *in vitro*. The ouabain-enzyme complex was prepared with ATP, Na^+ and Mg^{++}. Dissociation was monitored at 27°C in the presence of 5 mM KCl (see Reference 2). N = 5. Vertical lines: S.E.M. (From Akera *et al.*[2] By permission of the publisher of *Naunyn-Schmiedebergs Archiv fuer Pharmacologie und Experimentelle Pathologie.*)

from kitten and puppy (FIGURE 7). Thus, it is of interest to determine whether the loss of inotropic effects is faster in guinea pig and rabbit and slower in kitten and puppy hearts. When Langendorff preparations of guinea pig, rabbit, kitten, and puppy hearts were perfused with 0.6 μM ouabain for 20 to 30 minutes, 63 ± 13, 72 ± 24, 58 ± 20 and 66 ± 16% increases (mean ± S.E.M. of four experiments) in the right ventricular pressure, a measure of contractile force in Langendorff preparation, were observed, respectively. When the perfusing solution was switched to a ouabain-free solution, the inotropic response disappeared gradually. The loss of the inotropic response followed first-order kinetics, and thus, it was possible to compare the half-time of the loss of inotropic response and that of the dissociation of ouabain from the cardiac Na^+, K^+-ATPase. Such a comparison indicated that these two parameters are indeed closely related (FIGURE 8). Thus, it appeared that the dissociation of ouabain from the cardiac Na^+, K^+-ATPase results in a loss of the inotropic response.

Effects of Other Drugs

Since both inotropic and toxic effects of cardiac glycosides appeared to involve Na^+, K^+-ATPase inhibition, it is reasonable to assume that an agent that facilitates

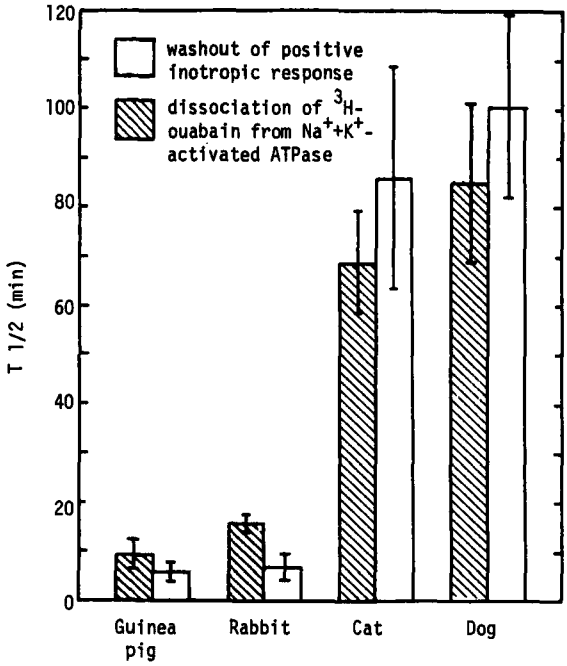

FIGURE 8. Half-times of the inotropic effect of ouabain and the dissociation of [^{3}H] ouabain from cardiac Na^+, K^+-ATPase. Inotropic effect was monitored with Langendorff preparations at 27°C. Half-time of dissociation reaction was calculated from FIGURE 7 (see Reference 2). N = 4 (inotropic data) and 5 (dissociation data). Vertical lines: S.E.M. (From Akera *et al.*[2] By permission of the publisher of *Naunyn-Schmiedebergs Archiv fuer Pharmacologie und Experimentelle Pathologie.*)

the dissociation of cardiac glycosides from the enzyme might be useful in the management of digitalis overdose. Therefore, agents that have been reported to possess antiarrhythmic properties against digitalis-induced arrhythmias were tested *in vitro* for possible enhancement of the dissociation of the [^{3}H] ouabain-Na^+, K^+-ATPase complex. The [^{3}H] ouabain-enzyme complex was prepared with ATP, Na^+ and Mg^{++} and the rate of dissociation was monitored at 37°C in the presence of 5 mM KCl. Diphenylhydantoin (0.1 mM), glucagon (0.05 mM), heparin (5 units/ml), or potassium canrenoate (0.5 mM) failed to alter the dissociation rate significantly. Urea (4 M) also failed to facilitate the dissociation of the [^{3}H] ouabain-enzyme complex, suggesting that the interaction of ouabain with Na^+, K^+-ATPase is not a simple hydrophobic binding. Ca^{++} (2.5 mM) also failed to affect the dissociation rate of the [^{3}H] ouabain-enzyme complex.

The dissociation rates of [^{3}H] ouabain from Na^+, K^+-ATPase, however, were significantly enhanced by low pH (FIGURE 9). Concomitantly, the rate of washout of [^{3}H] ouabain from Langendorff preparations of guinea pig heart after perfusion of [^{3}H] ouabain was significantly enhanced by lowering the pH of the perfusing solution. Thus, a factor or agent that facilitates the dissociation of [^{3}H] ouabain from Na^+, K^+-ATPase would appear to facilitate the washout of ouabain from the cardiac tissue.

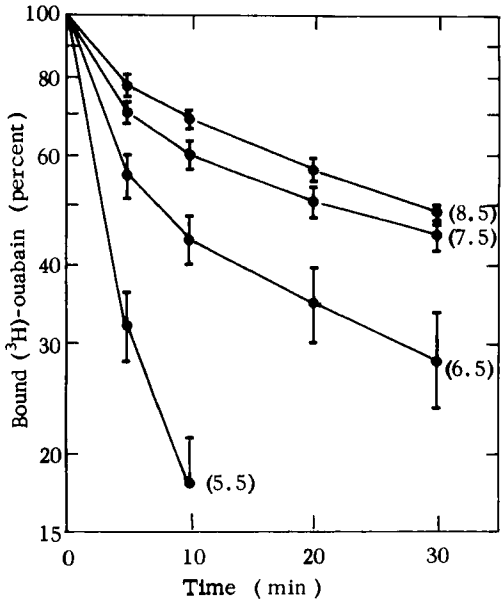

FIGURE 9. Dissociation of [^{3}H]ouabain-Na^+, K^+-ATPase complex at various pH. The ouabain-enzyme complex was prepared with ATP, Na^+ and Mg^{++} and washed free from cations, ATP and ouabain. Dissociation was monitored at 37°C. N = 5. Vertical lines: S.E.M.

Summary

The [^{3}H]ouabain-Na^+, K^+-ATPase complex prepared with ATP, Na^+ and Mg^{++} dissociated rapidly at 37°C, whereas that prepared with Mg^{++} and Pi was relatively stable. Those complexes prepared with *p*-nitrophenylphosphate, acetylphosphate or carbamylphosphate in the presence of Na^+ and Mg^{++} were intermediate. K^+ stabilized labile complexes to the level of the stable complex prepared with Mg^{++} and Pi, but failed to further stabilize the latter complex. NH_4^+, Rb^+, Cs^+ and Tl^+ had similar effects as K^+ but Na^+ and Li^+ were markedly less effective, and Ca^{++} was ineffective. The stabilizing effect of K^+ was reduced at low temperatures, abolished below 17°C, and enhanced by phloridzin. The dissociation rate in the presence of K^+ was relatively temperature-insensitive. Deoxycholic acid also reduced the K^+-effect. The dissociation rates of the cardiac glycoside-enzyme complexes were dependent on the cardiac glycoside and the source of the enzyme. Cardiac enzymes from relatively ouabain-insensitive species had faster ouabain dissociation rates than those from ouabain-sensitive species in the presence of K^+. Half-lives of ouabain-cardiac enzyme complexes were similar to half-times of the washout of inotropic responses to ouabain in Langendorff preparations in each species. Mechanical shaking of the ouabain-enzyme complex produced a marked release of specifically bound ouabain. Similar shaking of the enzyme preparations resulted in a loss of catalytic activity. Such agents as diphenylhydantoin, glucagon, heparin, potassium canrenoate or urea failed to alter the dissociation rate of [^{3}H]ouabain from Na^+, K^+-ATPase in the presence of K^+. Low pH media caused facilitated release of [^{3}H]ouabain

from the enzyme and from guinea pig Langendorff preparations preloaded with [^{3}H]ouabain. Thus, *in vitro* studies of cardiac glycoside-Na^+, K^+-ATPase interactions will prove a means to uncover an agent or condition useful in controlling pharmacologic and toxic responses to this drug.

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Discussion

DR. SCHWARTZ: What is the significance of studying association under one series of conditions and dissociation under another? As I understand it, the materials that support binding are removed after ouabain is bound and then,

in order to study the dissociation, the ouabain-enzyme complexes are put into another environment, an environment with low ionic strength in which potassium inhibits the dissociation. But isn't it true also that other monovalent cations, for example, sodium, choline or large amounts of Tris also inhibit the dissociation? If you carry out your reaction to produce, let us say, what you can call an unstable complex in the presence of magnesium, ATP, and sodium, then stop the reaction by adding the large excess of unlabeled ouabain, and then allow the reaction to continue, is there any effect of potassium on that dissociation?

DR. AKERA: It depends upon the sodium concentration. If the sodium concentration is as high as 200 mM, the addition of potassium after the binding does not slow down the dissociation. The dissociation in the presence of 200 mM sodium is already slow. However, if sodium concentration is lower, the addition of potassium slows down the dissociation rate. In other words, the discrimination is not absolute but there is a clear distinction, between sodium and lithium, and Tris perhaps, on one side, and other monovalent cations on the other. If potassium is present during the binding reaction, the picture is very different. The resulting ouabain-enzyme complex has intermediate dissociation rate between stable and unstable complexes and it is insensitive to potassium. The study of dissociation of the ouabain-enzyme complexes under artificial environment allows us to differentiate and characterize the complexes formed under different conditions. Which complex is predominent when ouabain binds to Na^+, K^+-ATPase in intact heart, and therefore which complex is relevant to *in vivo* situation, is yet to be determined.

DR. OKITA: I would like to make one comment in relation to your heart preparations. You have to realize that in physiological saline solution, you have about 5 or 6 mM potassium, and I would like to suggest that you are forming the E_2 type of phosphoenzyme. In your last studies, in which you show a fast release of ouabain from the enzyme, I wonder whether you are getting the release from E_1 form and still not accounting for the release from E_2 form.

DR. AKERA: The dissociation was observed in the presence of 7.5 mM potassium, and therefore I feel it reasonably simulates the dissociation in physiological environment.

DR. OKITA: But when you do your washout, are you sure that you are washing out from the E_2 form of the complex?

DR. AKERA: In those studies we did not wash out the enzyme. We simply stopped the reaction by adding cold ouabain, and on top of that, we added potassium chloride, which converted the ouabain-enzyme complex to stable form. Thus, the reported dissociation rates are the slowest rates observable with guinea pig heart enzyme.

DR. SIEGEL: What was the nature of the preparations in which you studied the dissociation? Were these all sodium iodide-treated microsomes?

DR. AKERA: Yes.

DR. SIEGEL: Can you estimate what proportion of your ouabain binding can be specifically related to inhibition of the ATPase.

DR. AKERA: In these rat brain preparations nonspecific or ATP-independent binding is approximately 2% of the total binding, and therefore 98% of the ouabain binding is specific binding to the enzyme that is related to the enzyme inhibition. Incidentally, the reported values are ATP-dependent portion of the binding, which is total minus nonspecific binding.

DR. SIEGEL: What proportion of the potassium-affected release might or might not be actually related to an enzyme site? Further, is this phenomenon really

dependent on the nature of the tissue preparation, which is of course always different from the *in vivo* state? Is there some kind of a nonspecific entrapment phenomenon?

DR. AKERA: The nature of the ATP-dependent ouabain binding has been stoichiometrically studied by Drs. Hansen, Jensen, and Nørby (1971). It appears that ATP-dependent binding is a specific binding to the sodium potassium ATPase, which results in an enzyme inhibition. The release of ouabain results in a reactivation of the enzyme activity (Akera and Brody, 1971). Ouabain release and enyzme reactivation appear linearly correlated. ATP-independent binding, which was subtracted from the total binding in reported values, represents nonspecific binding or entrapment phenomenon, so to speak. But again this is an insignificantly small portion under the experimental conditions. Therefore, I believe that most of the potassium-affected release is the release from cardiac glycoside binding sites on the enzyme.