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MEMBRANE ($Na^+ + K^+$)-ATPase OF CANINE BRAIN, HEART AND KIDNEY

TISSUE-DEPENDENT DIFFERENCES IN KINETIC PROPERTIES AND THE INFLUENCE OF PURIFICATION PROCEDURES

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Summary

Effects of commonly used purification procedures on the yield and specific activity of ($Na^+ + K^+$)-ATPase (Mg^{2+}-dependent, $Na^+ + K^+$-activated ATP phosphohydrolase, EC 3.6.1.3), the turnover number of the enzyme, and the kinetic parameters for the ATP-dependent ouabain-enzyme interaction were compared in canine brain, heart and kidney. Kinetic parameters were estimated using a graphical analysis of non-steady state kinetics. The protein recovery and the degree of increase in specific activity of ($Na^+ + K^+$)-ATPase and the ratio between ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase activities during the successive treatments with deoxycholate, sodium iodide and glycerol were dependent on the source of the enzyme. A method which yields highly active ($Na^+ + K^+$)-ATPase preparations from the cardiac tissue was not suitable for obtaining highly active enzyme preparations from other tissues. Apparent turnover numbers of the brain ($Na^+ + K^+$)-ATPase preparations were not significantly affected by the sodium iodide treatment, but markedly decreased by deoxycholate or glycerol treatments. Similar glycerol treatment, however, failed to affect the apparent turnover number of cardiac enzyme preparations. Cerebral and cardiac enzyme preparations obtained by deoxycholate, sodium iodide and glycerol treatments had lower affinity for ouabain than renal enzyme preparations, primarily due to higher dissociation rate constants for the ouabain · enzyme complex. This tissue-dependent difference in ouabain sensitivity seems to be an artifact of the purification procedure, since less purified cerebral or cardiac preparations had lower dissociation rate constants. Changes in apparent association rate constants were minimal during the purification procedure. These results indicate that the presently used purification procedures may alter

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the properties of membrane ($Na^+ + K^+$)-ATPase and affect the interaction between cardiac glycosides and the enzyme. The effect of a given treatment depends on the source of the enzyme. For the in vitro studies involving purified ($Na^+ + K^+$)-ATPase preparations, the influence of the methods used to obtain the enzyme preparation should be carefully evaluated.

Introduction

($Na^+ + K^+$)-ATPase preparations (Mg^{2+}-dependent, $Na^+ + K^+$-activated ATP phosphohydrolase, EC 3.6.1.3) obtained from various animal sources have remarkably similar characteristics with regard to the substrate specificity and the activation by Na^+, K^+ and Mg^{2+} (see ref. 1). In addition, specific antibodies for ($Na^+ + K^+$)-ATPase obtained with porcine or canine kidney cross-react and inhibit ($Na^+ + K^+$)-ATPase obtained from other sources [2,3]. Thus, the enzyme molecules obtained from various sources appear to have common features. Reported turnover numbers, however, of various ($Na^+ + K^+$)-ATPase preparations range from 1900 to 12850 [4—7]. The difference in the turnover number of various enzyme preparations might be due to the difference in the source of the enzyme. A progressive decrease, however, in the turnover number during a purification process [6] indicates that the purification treatments also alter the characteristics of ($Na^+ + K^+$)-ATPase. Such an alteration might then have a great influence on the data obtained with cardiac ($Na^+ + K^+$)-ATPase, a putative receptor for the positive inotropic action of cardiac glycosides [1,8,9]. Because of low ($Na^+ + K^+$)-ATPase and high Mg^{2+}-ATPase activities, studies with cardiac enzymes have been generally performed with extensively purified preparations.

In contrast to the substrate specificity and ligand activation, the kinetics of the interaction between cardiac glycosides, such as ouabain, and ($Na^+ + K^+$)-ATPase are dependent on the source of enzyme preparations [8,10—13]. The observed difference in kinetic parameters, however, might be partially due to the methods employed for the preparation of ($Na^+ + K^+$)-ATPase. A difference in kinetic parameters for the ouabain-enzyme interaction in the presence of Mg^{2+} and inorganic phosphate has been reported with different enzyme preparations obtained from the same tissue [14]. Although the ouabain binding to ($Na^+ + K^+$)-ATPase observed under such a ligand condition may not represent the drug-enzyme interaction occuring in vivo [15], the results indicate an alteration of ouabain binding sites due to the purification procedure. Purification procedures would affect the kinetics of the glycoside-enzyme interaction if the release of bound glycoside from ($Na^+ + K^+$)-ATPase is controlled by a lipid barrier [15], since several steps of the purification procedures are aimed at the alteration and disruption of the membrane lipids.

Thus, the present study was initiated to compare the kinetic properties of ($Na^+ + K^+$)-ATPase obtained from canine brain, heart and kidney and to examine the influence of purification procedures on these properties. Since the interaction between cardiac glycosides and ($Na^+ + K^+$)-ATPase is a relatively slow process [16], a non-steady state kinetic analysis modified from our previous study [17] was employed.

Materials and Methods

Chemicals. [^{3}H]Ouabain (specific radioactivity, 13.2 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Ouabain octahydrate and Tris-ATP were obtained from Sigma Chemical Co., St. Louis, Mo. Other chemicals used were of reagent grade.

Purification of ($Na^+ + K^+$)-ATPase. Frozen dog tissues were obtained from Pel-Freez Biologicals, Rogers, Arkansas. ($Na^+ + K^+$)-ATPase preparations were obtained from brain, heart or kidney according to a method developed by Pitts et al. [18] for the purification of cardiac enzymes. In short, a 9000 × *g* sediment of a tissue homogenate was treated with 0.1% deoxycholate. The particulate fraction released from large particles by the deoxycholate treatment (i.e., the small particles which do not sediment at 9000 × *g* after the treatment, but sediment at 100 000 × *g*) was successively treated with NaI, deoxycholate (second deoxycholate treatment) and glycerol. In the present study, tissues were homogenized in a solution containing 0.25 M sucrose/1 mM Tris · EDTA/ 5 mM histidine-HCl/10 μM dithiothreitol, instead of a 0.25 M sucrose solution containing 1 mM Tris · EDTA in the original method.

The final suspension of a glycerol-enzyme containing 20% (v/v) glycerol, 25 mM imidazole · HCl and 1 mM Tris · EDTA was dialyzed against the same solution at 2°C for 24 h. After the dialysis, the enzyme suspension was centrifuged at 50 000 rev./min in a Beckman Ti-50 rotor for 120 min. The sediment was resuspended in a solution containing 0.25 M sucrose/50 mM histidine-HCl/1.0 mM Tris · EDTA and stored at −20°C. The glycerol-suspended enzyme preparations lost their ($Na^+ + K^+$)-ATPase activity during the storage at −20°C even after the dialysis (see Results). In contrast, the sucrose-suspended enzyme preparations could be stored for more than 2 months at the same temperature without a noticeable change in both ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase activities.

ATPase assay. Enzyme preparations at various stages of the purification were assayed for ATPase activities and [^{3}H]ouabain binding. Interfering substances such as deoxycholic acid or NaI were removed by the centrifugation and the resuspension of the resulting sediment prior to the assay.

The ATPase activity was assayed from the amount of inorganic phosphate released from ATP during a 10 min incubation of 20 μg of enzyme protein in a total volume of 1.0 ml at 37°C in the presence of 100 mM NaCl, 15 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris · HCl buffer (pH 7.5) [10]. Mg^{2+}-ATPase activity assayed in the absence of NaCl and KCl and in the presence of 0.1 mM ouabain was subtracted from the total ATPase activity assayed in the presence of NaCl and KCl and in the absence of ouabain to calculate the ($Na^+ + K^+$)-ATPase activity. The amount of inorganic phosphate was assayed as described earlier [19]. All assays were performed in triplicate.

The assay of [^{3}H]ouabain binding. The specific binding of [^{3}H]ouabain to ($Na^+ + K^+$)-ATPase was assayed in the presence of 5 mM $MgCl_2$, 100 mM NaCl, 5 mM Tris-ATP and 50 mM Tris · HCl buffer (pH 7.5). Enzyme preparation containing 450 μg of protein was incubated in 15 ml of the above mixture in the presence of various concentrations (0.025—0.25 μM) of [^{3}H]ouabain at 37°C. At 1 min intervals, 1.0-ml aliquots were taken and filtered through nitro-

cellulose filters (Millipore Corporation, Bedford, Mass.; type AA, pore size, 0.8 μm), and the bound [^{3}H]ouabain retained on the filter was assayed with liquid scintillation counting [12]. Non-specific ouabain binding was estimated by a concurrent assay performed in the absence of ATP. Since 100 mM NaCl was present in the incubation mixture, the binding of [^{3}H]ouabain observed in the absence of ATP was less than 5% of that observed in the presence of ATP. The specific (ATP-dependent) ouabain binding was calculated as the difference in the binding observed in the absence and presence of ATP. Such an ATP-dependent binding has been shown to be a specific binding of ouabain to the cardiotonic steroid binding sites on the enzyme [20,21], which results in an enzyme inhibition [16,21].

The ouabain binding velocity was calculated at every minute for 10 min from the slope of a curved fitted to data points by least squares method using a polynomial approximation-curve fitting technique [22] as described in a previous paper [17]. From the ouabain binding velocity, kinetic parameters for the interaction between ouabain and ($Na^+ + K^+$)-ATPase were calculated (for details, see Results).

Estimation of turnover numbers. Apparent turnover numbers were calculated as the ratio between ($Na^+ + K^+$)-ATPase activity (in μmol ATP hydrolyzed/mg protein per min) and the enzyme concentration (in μmol enzyme/mg protein). These values are expressed as $(min)^{-1}$. In the present study, the enzyme concentration was estimated from the maximal concentration of ouabain binding sites. A true turnover number should be calculated from the concentration of phosphorylation sites (active centers) which is generally estimated from the formation of the ADP- and K^+-sensitive phosphoenzyme observed in the presence of Na^+, Mg^{2+} and 0.05 mM γ-^{32}P-labeled ATP. Phosphoenzyme concentration, however, was not used in the present study because the value obtained with such a low concentration of ATP may not represent the concentration of the total phosphorylation sites on the enzyme [23]. Although the numbers of phosphorylation sites and ouabain binding sites might not be equal, the concentration of the ouabain binding site should be proportional to the concentration of the enzyme molecules and, hence, to the concentration of phosphorylation sites. Thus, the change in turnover numbers due to purification procedures should be accurately estimated using the concentration of the ouabain binding sites.

Miscellaneous. Protein concentration was determined by the method of Lowry et al. [24] with bovine serum albumin as the standard. Statistical analyses of data were performed with Student's *t*-test. Criterion for the statistical significance is a *P* value of less than 0.05.

Results

Purification of ($Na^+ + K^+$)-ATPase. The method of Pitts et al. [18] was developed to obtain highly active ($Na^+ + K^+$)-ATPase preparations from the cardiac tissue. In the present study, however, this method was used for the preparation of ($Na^+ + K^+$)-ATPase from the brain, heart and kidney without any modification for each tissue, in order to obtain comparable data. The yield of the final glycerol-treated enzyme was quite low in the heart, and significantly

TABLE I

PURIFICATION OF $(Na^+ + K^+)$-ATPase FROM CANINE BRAIN, HEART AND KIDNEY BY THE METHOD OF PITTS ET AL. [18]

The values are the mean of three experiments. After the first deoxycholate treatment, the enzyme preparations were suspended in EDTA solution. After the NaI treatment, the enzyme preparations were suspended in imidazole buffer solution.

Fraction	Protein (%)			$(Na^+ + K^+)$-ATPase			Mg^{2+}-ATPase			$(Na^+ + K^+)/(Mg^{2+})$ ratio		
	Brain	Heart	Kidney	Brain	Heart	Kidney	Brain	Heart	Kidney	Brain	Heart	Kidney
				(μmol P_i/mg protein per h)								
Homogenates in sucrose	100	100	100	11.3	4.3	11	6.3	17	17	1.8	0.25	0.65
After 1st deoxycholate treatment	9	3.26	21.6	44.7	13	38	23.7	18	26	1.9	1.2	1.5
After NaI treatment	4.7	0.57	4.7	33.5	30.8	76.4	0.4	7.7	9.5	83.8	4	8
Glycerol-enzyme	0.7	0.09	0.67	95	105	182	1.6	8.9	6.7	59.3	18	27

higher in the brain and kidney (Table I). The specific ($Na^+ + K^+$)-ATPase activity (enzyme activity per mg protein) of brain enzyme preparations was unexpectedly low, indicating that the method is optimized for the cardiac tissue, but is not suitable for the brain. Brain ($Na^+ + K^+$)-ATPase preparations with higher activity may be obtained with less complex and less time consuming methods (for example, ref. 15). An improved method for cardiac ($Na^+ + K^+$)-ATPase preparations is also available [25]. Nevertheless, the original method of Pitts et al. [18] was used in the following study, because the purpose of the present work was to investigate the effects of purification procedures on the properties of ($Na^+ + K^+$)-ATPase. This method employed three commonly used procedures for the purification of ($Na^+ + K^+$)-ATPase, namely deoxycholate treatment, NaI treatment and glycerol treatment.

The deoxycholate treatment caused a marked increase in the specific enzyme activity. NaI treatment caused a marked decrease in Mg^{2+}-ATPase activity and a slight increase in ($Na^+ + K^+$)-ATPase activity, except for the brain enzyme. In the brain tissue, the specific activity of ($Na^+ + K^+$)-ATPase was not increased by the NaI treatment. The ratio between ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase activities, however, was significantly increased due to a marked reduction in Mg^{2+}-ATPase activity. Glycerol treatment further increased the specific activity of ($Na^+ + K^+$)-ATPase without a marked effect on Mg^{2+}-ATPase. These results generally confirm those by Pitts et al. [18].

The stability of glycerol-treated enzyme preparations obtained from kidney was tested. During the storage of frozen suspension at −20°C, the enzyme preparation dialyzed and suspended in a 20% (v/v) glycerol solutions (freezing point, −7°C) containing 25 mM imidazole and 1 mM Tris · EDTA lost approximately half of its activity in 70 days (Table II). Thus, the final enzyme preparations were suspended in a sucrose solution in the following studies.

Determination of kinetic constants for the ouabain-enzyme interaction. The binding of ouabain to ($Na^+ + K^+$)-ATPase proceeds as a second order reaction with respect to ouabain and the binding site on the enzyme [26], or the pseudo first order reaction when the changes in the ouabain concentration may be ignored [27]. Although the actual reaction may be a complex one involving several parallel and sequential pathways [17], the kinetic behavior may be

TABLE II

THE STABILITY OF GLYCEROL-SUSPENDED RENAL ENZYME PREPARATIONS AT −20°C

The values are the mean of two enzyme preparations. The enzyme preparations were prepared as described by Pitts et al. [18] and suspended in a 20% (v/v) glycerol solution containing 1 mM Tris · EDTA.

Days	($Na^+ + K^+$)-ATPase	Mg^{2+}-ATPase	($Na^+ + K^+$)/(Mg^{2+}) ratio
	(μmol P_i/mg protein per h)		
1	187	6.7	27.9
5	161	5.3	30.4
30	115	2.5	45.8
50	113	2.9	40.0
70	85.7	1.0	85.7

described as follow using an overall apparent association (k_1) and an overall dissociation (k_{-1}) rate constants.

$$E + Ou \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot Ou \quad (1)$$

where E, Ou and E · Ou are free enzyme, unbound ouabain and the ouabain · enzyme complex, respectively. The time course of the specific binding of [^{3}H]-ouabain to ($Na^+ + K^+$)-ATPase can be seen in Fig. 1, which shows data for brain enzyme as an example. Similar curves were obtained with other concentrations of [^{3}H]ouabain or with enzyme preparations obtained from heart or kidney (data not shown).

Since the binding of ouabain to ($Na^+ + K^+$)-ATPase follows a pseudo first order kinetics [17,27], the binding velocity (V) at a given time (t) can be expressed as follows [17]:

$$\ln V = \ln V_{init} - (k_1 \cdot [Ou] + k_{-1}) \cdot t \quad (2)$$

where V_{init} is the initial binding velocity and [Ou] is the concentration of free ouabain. Plots of the binding velocity in a logarithmic scale against the time of incubation yielded a straight line for each ouabain concentration (Fig. 2), confirming the pseudo first order kinetics for the ouabain-enzyme interaction.

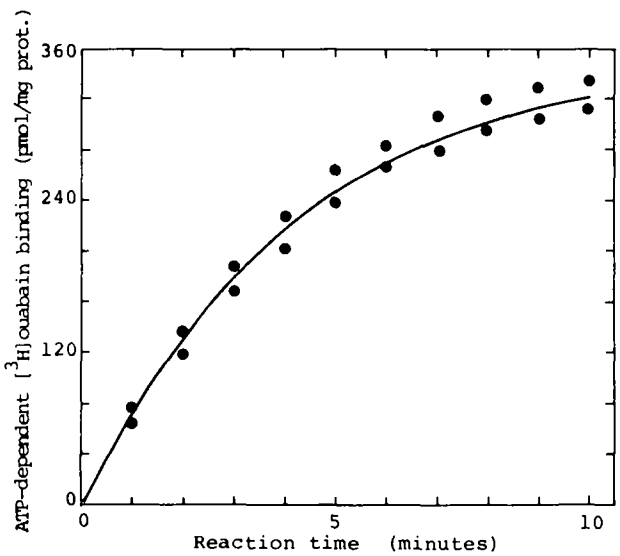

Fig. 1. Time course of [^{3}H]ouabain binding to glycerol-treated ($Na^+ + K^+$)-ATPase obtained from canine brain. ($Na^+ + K^+$)-ATPase preparations (30 μg of protein per ml) were incubated with [^{3}H]ouabain (final concentration; 0.1 μM) in the presence of 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris · HCl buffer (pH 7.5) at 37°C. At indicated times, aliquots of the incubation mixture were taken and the amount of bound ouabain was assayed. The solid curve represents the following equation fitted to experimental data by the least squares method [17,22].

$$[E - Ou] = \frac{[E] \cdot [Ou]}{[Ou] + k_{-1}/k_1} \left(1 - e^{-(k_1 \cdot [Ou] + k_{-1}) \cdot t}\right)$$

Parameters to yield the best fit are: k_1 = 21.6 $mM^{-1} \cdot s^{-1}$; k_{-1} = 0.19/100 s; and [E] = 653 pmol/mg protein or 19.6 nM. [Ou] = 0.1 μM.

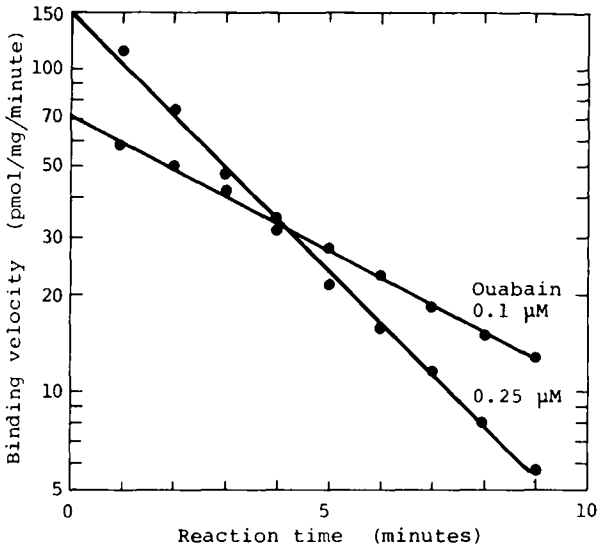

Fig. 2. Time course of [^{3}H]ouabain binding velocity: semilogarithmic plot. The binding of [^{3}H]ouabain to ($Na^+ + K^+$)-ATPase was assayed under the condition of experiments shown in Fig. 1. The binding velocities were calculated from the tangent of curves fitted by the least squares method shown in Fig. 1.

From the ordinate intercept of the regression line, the initial velocity may be determined.

Eqn. 2 also indicates that the plot of the slope of regression lines on Fig. 2, namely ($k_1 \cdot [Ou] + k_{-1}$), against the concentration of ouabain should yield a straight line with a slope of k_1 and the ordinate intercept of k_{-1}. Such a plot

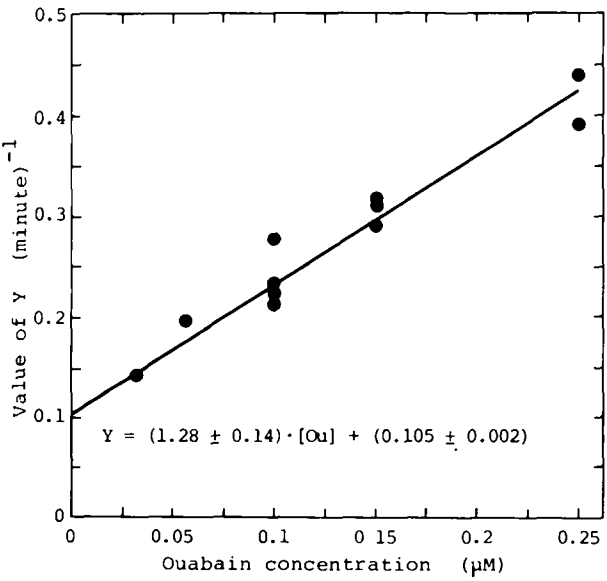

Fig. 3. Determination of apparent association and overall dissociation rate constants. A linear regression line was fitted to plots shown in Fig. 2 for each concentration of ouabain, and the slope of the regression line is plotted against the concentration of ouabain. Each point represents the slope of a regression line on a plot similar to Fig. 2. Ordinate (Y) is the negative slope of regression lines times 2.303 (for the conversion to natural logarithm).

(Fig. 3), in fact, yielded a straight line. From the slope of the regression line and the ordinate intercept, the apparent association and the overall dissociation rate constants were obtained.

The concentration of the ouabain binding sites on ($Na^+ + K^+$)-ATPase can be determined from the initial binding velocity (V_{init}) as follows:

$$V_{init} = k_1 \cdot [E] \cdot [Ou] \quad (3)$$

where [E] is the concentration of the free enzyme. The plot of V_{init} against the ouabain concentration yielded a straight line passing through the point of origin (Fig. 4). Since the slope of the regression line is equal to $k_1 \cdot [E]$, where k_1 may be determined as above, the enzyme concentration can be calculated (Table III). Enzyme concentrations estimated with this method were used for the following calculation of the apparent turnover numbers.

Apparent turnover numbers of ($Na^+ + K^+$)-ATPase. Apparent turnover numbers (molecular activity) of glycerol-treated ($Na^+ + K^+$)-ATPase preparations obtained from canine brain, heart and kidney were calculated from the ($Na^+ + K^+$)-ATPase activity and the ouabain binding site concentrations (Table III). The apparent turnover number was approximately 3900/min for the cardiac enzyme preparations, and significantly lower in enzyme preparations obtained from the brain or kidney.

In order to determine whether apparent turnover numbers were altered during the purification process, turnover numbers of brain ($Na^+ + K^+$)-ATPase were estimated at various stages of the purification procedure (Table IV). The results indicate that the apparent turnover numbers decreased from approximately 7200/min to 2700/min during the purification. It should be noted that the decreases in apparent turnover numbers occurred with deoxycholate and

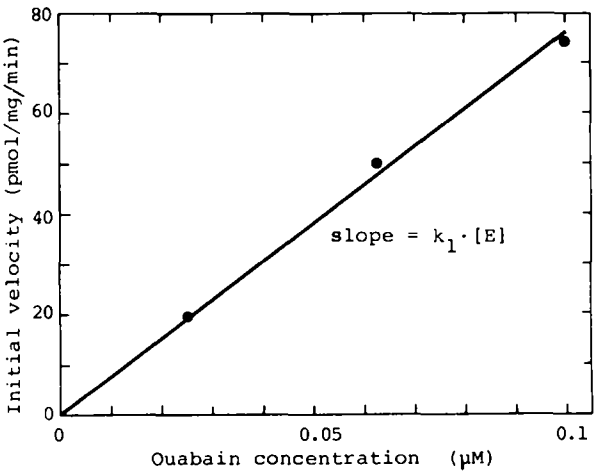

Fig. 4. The relationship between initial velocities for [^{3}H]ouabain binding and the concentration of [^{3}H]-ouabain. Glycerol-treated ($Na^+ + K^+$)-ATPase preparations obtained from dog brain (30 μg of protein per ml) were incubated with various concentrations of [^{3}H]ouabain under the condition described in the legend to Fig. 1. The initial velocity of [^{3}H]ouabain binding reaction was estimated from the ordinate intercept of plots shown in Fig. 2.

TABLE III

KINETIC PARAMETERS OF GLYCEROL-TREATED ($Na^+ + K^+$)-ATPase PREPARATIONS OBTAINED FROM VARIOUS CANINE TISSUES

Values are mean ± S.E.M. of 3 (brain), 6 (heart) and 4 (kidney) enzyme preparations. Apparent turnover numbers were calculated as ($Na^+ + K^+$)-ATPase activity (pmol P_i/mg protein per min) per ouabain binding site (pmol/mg protein) for each enzyme preparation, and then averaged.

Source of enzyme	($Na^+ + K^+$)-ATPase	Mg^{2+}-ATPase	Ouabain binding site (pmol/mg protein)	Turnover number $(min)^{-1}$
	(μmol P_i/mg protein per h)			
Brain	100.3 ± 3.2	6.1 ± 2.4	602 ± 35	2730 ± 95 *
Heart	85.8 ± 6.2	8.2 ± 2.2	372 ± 30	3910 ± 254
Kidney	105 ± 30	4.9 ± 1.5	729 ± 221	2440 ± 58 *

* Significantly different from corresponding values for cardiac enzymes.

glycerol treatments, but not with the NaI treatment. Similar experiments were not performed with cardiac or renal enzymes since high Mg^{2+}-ATPase activities in homogenates of these tissues precluded an accurate estimation of ($Na^+ + K^+$)-ATPase activity and turnover numbers. The decrease in the apparent turnover number of brain ($Na^+ + K^+$)-ATPase during the purification process was due to a greater increase in ouabain binding site concentration than that in specific ($Na^+ + K^+$)-ATPase activity (Table IV).

The cardiac ($Na^+ + K^+$)-ATPase after the NaI treatment had an apparent turnover number of approximately 4000/min (data not shown). Thus, it appears that the glycerol treatment alters the apparent turnover number of ($Na^+ + K^+$)-ATPase obtained from the brain tissue, but fails to alter it in the cadiac tissue.

Kinetic parameters for the ouabain-enzyme interaction. Apparent association and overall dissociation rate constants for the interaction of ouabain with ($Na^+ + K^+$)-ATPase were estimated as described above from the time course of the [^{3}H]ouabain binding reaction using glycerol-treated enzyme preparations obtained from brain, heart and kidney (Table V). Brain and heart enzymes had similar apparent association rate constants and similar overall dissociation rate constants, and hence similar dissociation constants (reciprocal indices of affin-

TABLE IV

EFFECTS OF PURIFICATION PROCEDURES ON ($Na^+ + K^+$)-ATPase ACTIVITY, OUABAIN BINDING SITE CONCENTRATION, AND APPARENT TURNOVER NUMBER OF CANINE BRAIN ENZYME

Values are the mean ± S.E.M. of 4 preparations except for the final glycerol-enzyme preparations in which values are the mean ± S.E.M. of 3 preparations. After the deoxycholate treatment, the enzyme preparations were suspended in EDTA solution. After the NaI treatment, the enzyme preparations were suspended in imidazole buffer solution.

Fraction	($Na^+ + K^+$)-ATPase activity (μmol P_i/mg per h)	Ouabain binding site (pmol/mg)	Apparent turnover number $(min)^{-1}$
Homogenate in sucrose	11.4 ± 0.2	26.4 ± 0.8	7190 ± 220
After 1st deoxycholate treatment	43.4 ± 0.9	189 ± 16 *	3970 ± 390 *
After NaI treatment	51.4 ± 2.1 *	211 ± 4	4075 ± 75
Glycerol-enzyme	100.3 ± 3.2 *	602 ± 35 *	2730 ± 95 *

* Significantly different from corresponding values for the preceding step ($P < 0.05$).

TABLE V

KINETIC PARAMETERS FOR THE INTERACTION BETWEEN OUABAIN AND GLYCEROL-TREATED ENZYME PREPARATIONS OBTAINED FROM VARIOUS CANINE TISSUES

Values are the mean ± S.E.M. of 4 experiments. Apparent association and overall dissocation rate constants were obtained from the time course of [^{3}H]ouabain binding velocity. Dissociation constant was calculated as the ratio between the overall dissociation rate constant and the apparent association rate constant.

Source of enzyme	Apparent association rate constant ($mM^{-1} \cdot s^{-1}$)	Overall dissociation rate constant ($(100\ s)^{-1}$)	Dissocation constant (nM)
Brain	21.3 ± 2.3	0.174 ± 0.036	81.7
Heart	19.9 ± 2.7	0.180 ± 0.030	90.0
Kidney	10.4 ± 2.0 *	0.026 ± 0.002 *	25.0 *

* Significantly different from corresponding values for brain and heart enzymes ($P < 0.05$).

ity). ($Na^+ + K^+$)-ATPase preparations obtained from the kidney, however, had a lower apparent association rate constant and a markedly lower overall dissociation rate constant. As the result, the kidney enzyme had higher affinity for ouabain (i.e., the lower dissociation constant) than ($Na^+ + K^+$)-ATPase obtained from the brain or heart.

A marked tissue-dependent difference in kinetically-determined overall disso-

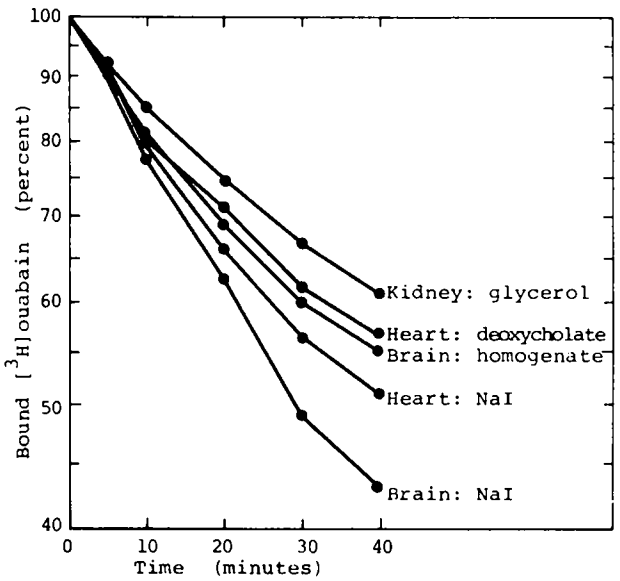

Fig. 5. Time course of the release of [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase. Enzyme preparations (30 μg of protein per ml) were incubated with [^{3}H]ouabain (0.1 μM) in the presence of 100 mM NaCl, 5 mM $MgCl_2$, 5 mM Tris-ATP and 50 mM Tris · HCl buffer (pH 7.5) at 37°C. After a 10 min incubation, excess non-labeled ouabain (final concentration, 0.1 mM) was added to terminate the binding of [^{3}H]ouabain, and subsequent release of bound [^{3}H]ouabain was monitored. (Non-labeled ouabain added at time zero). The amount of bound [^{3}H]ouabain was expressed as a percentage of the value immediately before the addition of non-labeled ouabain. Non-specific binding observed in the absence of ATP was subtracted. Deoxycholate, deoxycholate-treated enzyme preparations suspended in an EDTA solution; NaI, NaI-treated enzyme preparations suspended in an imidazole buffer solution: glycerol, glycerol-treated enzyme preparations suspended in a sucrose solution.

TABLE VI

KINETIC PARAMETERS FOR THE INTERACTION BETWEEN OUABAIN AND BRAIN ($Na^+ + K^+$)-ATPase: EFFECTS OF PURIFICATION PROCEDURES

Values are the mean ± S.E.M. of 4 experiments. After the 1st deoxycholate treatment, the enzyme preparations were suspended in EDTA solution. After the NaI treatment, the enzyme preparations were suspended in an imidazole buffer solution.

Fraction	Apparent association rate constant ($mM^{-1} \cdot s^{-1}$)	Overall dissocation rate constant ($(100\ s)^{-1}$)	Dissociation constant (nM)
Homogenate in sucrose	27.7 ± 4.0	0.110 ± 0.030	39.7
After 1st deoxycholate treatment	31.3 ± 2.8	0.103 ± 0.023	32.9
After the NaI treatment	27.0 ± 5.0	0.120 ± 0.015	44.0
Glycerol-enzyme	21.3 ± 2.3	0.174 ± 0.036 *	81.7 *

* Significantly different from corresponding values for the preceding step ($P < 0.05$).

ciation rate constants predicts that the release of ouabain from the renal enzyme should be slower than that of ouabain from the cerebral or cardiac enzyme after the termination of the ouabain binding reaction. In order to test this prediction, the rates of the release of [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase was monitored at 37°C. The enzyme preparation was incubated with [^{3}H]ouabain in the presence of Na^+, Mg^{2+} and ATP at 37°C for 10 min as described in Methods. After the 10 min incubation, the binding of [^{3}H]ouabain was terminated by the addition of excess non-labeled ouabain (final concentration, 0.1 mM) and the subsequent release of bound [^{3}H]ouabain was observed by taking aliquots at indicated times and estimating the amount of bound, undissociated [^{3}H]ouabain by the filtration and liquid scintillation techniques. The rates of the release of ouabain from NaI-treated enzyme preparations were similar for brain and heart, whereas that of ouabain from renal enzyme preparations was markedly slower (Fig. 5). These observations are in good agreement with kinetically-determined overall dissociation rate constants.

The rate of the release of [^{3}H]ouabain from ($Na^+ + K^+$)-ATPase in brain homogenates and in deoxycholate-treated cardiac enzyme preparations was markedly slower than corresponding values in NaI-treated enzyme preparations (Fig. 5). In these crude preparations (brain homogenate or deoxycholate-treated cardiac enzyme), the rate of ouabain release was similar to that of glycerol-treated renal enzyme preparations. These findings indicate that the purification procedures alter the kinetics of the interaction between oubain and ($Na^+ + K^+$)-ATPase.

Alterations of kinetic parameters for the ouabain-enzyme interaction was further confirmed by the estimation of the apparent association and the overall dissociation rate constants during the purification of brain ($Na^+ + K^+$)-ATPase (Table VI). Changes in the apparent association rate constants were minimal during the purification process. Overall dissociation rate constants, however, were significantly increased by the glycerol treatment of the enzyme. As the result, dissociation constant, or the affinity of ($Na^+ + K^+$)-ATPase for ouabain, was markedly altered by the glycerol treatment.

Discussion

Kinetic parameters reported in this paper compare favorably with those reported earlier [4—7,11—16,26,27] considering the difference in experimental conditions. In the present study, these values were obtained with a non-steady state kinetic analysis of the ATP-dependent [^{3}H]ouabain binding reaction. The results indicate that the renal ($Na^+ + K^+$)-ATPase is more sensitive to ouabain than cerebral or cardiac enzymes, primarily due to the difference in the dissociation rate constants. Concomitantly, the rate of the release of [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase was slower with renal enzymes than with cerebral or cardiac enzymes. These tissue-dependent differences in ouabain sensitivity, however, may be artifactual. Less purified cerebral or cardiac enzyme preparations had lower dissociation rate constants for ouabain; closer to that of the renal enzyme.

Glycerol treatment increased the dissociation rate constant for the complex of brain enzyme with ouabain. Association rate constant, however, was only minimally affected by the purification procedure. These findings are consistent with the concept that the release of ouabain from ($Na^+ + K^+$)-ATPase is regulated by a lipid barrier, whereas the rate of ouabain binding is primarily determined by the concentration of the phosphoenzyme [17,28,29], and that the glycerol treatment affects the membrane lipids associated with ($Na^+ + K^+$)-ATPase. Influence of detergent treatments or lipolysis with phospholipase A on the level of [^{3}H]ouabain binding has been also reported [30]. In this context, however, the lack of marked effect of the deoxycholate treatment on the overall dissociation rate constant was unexpected. Perhaps, the effects of a particular treatment on membrane lipids are dependent on the concentration of the agent and the duration of the treatment. Moreover, the influence of the treatment by a given concentration of deoxycholate or glycerol seems to depend on the source of the enzyme. Glycerol treatment which affected the turnover number of ($Na^+ + K^+$)-ATPase in the brain or kidney failed to affect that of the enzyme in the heart. Recently, Zubler-Faivre and Dunant [31] reported that an extensive purification process failed to affect the turnover number of ($Na^+ + K^+$)-ATPase in the electric organ of *Torpedo marmorate*. The differential effects of a given purification procedure on ($Na^+ + K^+$)-ATPase in different tissues are consistent with the present finding that the method of Pitts et al. [18] which is optimized for the cardiac tissue was not suitable for the brain or kidney tissue. Thus, the purification procedure should be carefully selected for each enzyme source. An over-treatment influences the properties of ($Na^+ + K^+$)-ATPase.

The interaction of digitalis derivatives with cardiac ($Na^+ + K^+$)-ATPase is of particular interest because these cardiotonic substances specifically inhibit cardiac ($Na^+ + K^+$)-ATPase in low concentrations [32]. In order to determine whether the cardiac ($Na^+ + K^+$)-ATPase could be the positive inotropic receptor for digitalis, extensive studies have been performed exploring the relationship between the binding of digitalis derivatives to cardiac ($Na^+ + K^+$)-ATPase and the inotropic action of these compounds (see refs. 1, 9, 33). Usuaully, a correlation was observed between these two events. Most studies, however, were performed with extensively purified enzyme preparations, and therefore uncertain-

ties exist with regard to the quantitative relationship between the drug-enzyme interaction observed in vitro and the drug-enzyme interaction which occurs in the beating heart.

Cardiac ($Na^+ + K^+$)-ATPase requires extensive purification for ATPase assay because of relatively low ($Na^+ + K^+$)-ATPase and high Mg^{2+}-ATPase activities. Thus, cardiac homogenates have been used only in studies involving the estimation of the rates of glycoside binding and release, or the estimation of the concentration of easily accessible glycoside binding sites on the enzyme [15,21, 34—36]. ($Na^+ + K^+$)-ATPase activity or the total concentration of the glycoside binding sites on the enzyme cannot be estimated without a disruption of membrane lipids in this tissue [37].

Deoxycholate and glycerol treatments, both aimed at the alteration and disruption of the membrane lipids, decreased the apparent turnover number of ($Na^+ + K^+$)-ATPase. Such a decrease in the apparent turnover number is due to the effects of these treatments to increase the concentration of ouabain binding sites to a greater degree than to increase the ($Na^+ + K^+$)-ATPase activity. These findings are consistent with an earlier report by Kyte [6] that the turnover number of ($Na^+ + K^+$)-ATPase decreases as the enzyme purification progresses. One possible explanation for this phenomenon is that these treatments expose ouabain binding sites and catalytic sites on the enzyme differentially. This explanation, however, does not appear to be the case since both ($Na^+ + K^+$)-ATPase and [^{3}H]ouabain binding reactions have similar lipid requirements and similar obligatory steps, such as the binding of ATP, Na^+ and Mg^{2+} to the enzyme under the present experimental conditions. After the phosphorylation of the enzyme, ($Na^+ + K^+$)-ATPase reaction requires the binding of K^+, whereas [^{3}H]ouabain binding reaction requires the binding of ouabain to the enzyme. Both K^+ and ouabain bind to the enzyme at sites on the enzyme exposed to the extracellular space, and hence differential exposure of the binding sites by deoxycholate or glycerol treatment seems unlikely. An alternative explanation is that these treatments change the characteristics of membrane lipids associated with ($Na^+ + K^+$)-ATPase such that the [^{3}H]ouabain binding is favored, whereas the turnover of the enzyme is hindered. The reduction of the dephosphorylation rate [38] or the fluidization of the membrane lipids [39] would bring about such a condition. Studies on the tissue-dependent differences in effects of deoxycholate or glycerol treatments on the properties of membrane lipids associated with ($Na^+ + K^+$)-ATPase might shed light on these problems.

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