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OUABAIN–RECEPTOR INTERACTIONS IN $(Na^+ + K^+)$-ATPase PREPARATIONS FROM DIFFERENT TISSUES AND SPECIES

DETERMINATION OF KINETIC CONSTANTS AND DISSOCIATION CONSTANTS

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SUMMARY

1. $[^3H]$Ouabain–receptor association in $(Na^+ + K^+)$-ATPase preparations from beef (kidney, brain, and heart), from dog (heart), and guinea pig (kidney) in the presence of $Mg^{2+} + P_i$ is a bimolecular reaction, while the dissociation is strictly a first-order process. For the ouabain–receptor subunit of the enzyme from beef kidney, rate constants at 37 °C for the association are $1.85 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and for the dissociation $0.94 \cdot 10^{-4} \cdot s^{-1}$. A dissociation constant of $0.47 \cdot 10^{-8}$ M was calculated from these rate constants and is determined from a Scatchard plot under equilibrium conditions. The dissociation constant determined in the presence of Na^+, Mg^{2+}, and ATP was $1.1 \cdot 10^{-8}$ M. Scatchard plots indicate one single type of receptor. The ouabain–receptor dissociation constants varied with the tissue source of $(Na^+ + K^+)$-ATPase. The considerable variation of the dissociation constant in guinea pig kidney ($1.6 \cdot 10^{-7}$ M) was preferentially caused by changes of the dissociation rate constants.

2. From the dissociation constant at 37 °C a ΔG° of -11 kcal·mole^{-1} is calculated for the ouabain–receptor subunit of the enzymes from beef kidney brain, and heart and from dog heart. For the beef kidney enzyme ΔH° is -5 kcal·mole^{-1} and ΔS° 21 cal·mole^{-1}·deg^{-1}. For the guinea pig subunit thermodynamic calculations reveal a ΔG° of -9 kcal·mole^{-1}, a ΔH° of -11 kcal·mole^{-1}, and a ΔS° of -4 cal·mole^{-1}·deg^{-1}.

3. Dissociation constants of the ouabain–receptor complex are 10- to 100-fold lower than the ouabain concentrations necessary for half-maximal inhibition of the $(Na^+ + K^+)$-ATPase. The stoichiometry of $[^3H]$ouabain-binding sites: phosphorylated intermediate varied between 4 (guinea pig kidney) and 1 (beef enzymes). It is assumed that the ouabain receptor and the ATP hydrolysing subunit are not tightly linked.

INTRODUCTION

$(Na^+ + K^+)$-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3), which is closely related to active Na^+ transport, is specifically inhibited by cardiac glyco-

sides[1,2]. In fact it may be involved in the mechanism of the positive inotropic action of cardioactive steroids[3,4]. Because of this, the interaction between the enzyme and cardiac glycosides has drawn much attention[2–24,28]. Nevertheless the knowledge about the interaction of ouabain with its receptor is still quite vague. According to Hansen[10] and Baker and Willis[19] the binding of ouabain (S) to its receptor (E) can be described by the mass law equation (Eqn 1):

$$E + S \rightleftharpoons ES \tag{1}$$

This equation implies that the process of ouabain binding is reversible. It has been reported that ouabain binding is irreversible[14], difficult in reversal[15,25], and readily reversible[6,12].

Available experimental data on the formation and dissociation of the ouabain–enzyme complex do not only differ because of different enzyme preparation being used but also due to the relatively unprecise parameters offered for the ouabain–enzyme interaction. The kinetics of ouabain binding and the half-lives of the various $(Na^+ + K^+)$-ATPase–ouabain complexes have been reported in terms of "a relatively slow process requiring several minutes to complete[16]" or $k_{0.5}$-values[13]. We therefore decided to measure the rate constants of association and dissociation of ouabain–enzyme complexes from different tissues and species.

This paper describes the direct measurement of the association and dissociation rate constants, the determination of the dissociation constant of the enzyme–ouabain complex by two different methods and some properties of the ouabain receptor in membranes of beef kidney, heart and brain, dog heart, and guinea pig kidney.

MATERIALS AND METHODS

Preparation and quantitation of enzyme

$(Na^+ + K^+)$-activated ATPase from beef brain was prepared as described previously[26]. The enzymes of beef and guinea pig kidney were isolated by the method of Post and Sen[27] with the modification that heparin was not used. Cardiac $(Na^+ + K^+)$-ATPase preparations from beef and dog were isolated according to Matsui and Schwartz[9]. $(Na^+ + K^+)$-activated ATPase activity was measured with the coupled optical assay[26]. The reaction was continuously recorded and corrected for a Mg^{2+}-activated ATPase by inhibition of $(Na^+ + K^+)$-activated ATPase with 10^{-4} M ouabain. ATP was converted to the free acid by passage through Dowex (H^+-form) and neutralized with Tris. One enzyme unit is defined as the amount of enzyme hydrolyzing 1 μmole ATP per min at 37 °C. Protein was quantitated by the procedure of Lowry *et al.*[29].

Assay of [^3H]ouabain binding

Unless otherwise indicated 0.5–1.5 mg protein were incubated in a medium containing 100 mM imidazole–HCl buffer pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–phosphate and various amounts of [^{3}H]ouabain. Incubation was carried out in polypropylene tubes suited for the rotor 50 Ti of the spinco L50 ultracentrifuge. The reaction was started by adding enzyme and stopped by rapid cooling of the tubes

in liquid air. After thawing in ice the membrane-bound protein was sedimented at 80000 × *g* for 30 min at 0 °C. The supernatant was decanted and the centrifugation tubes were kimwiped thoroughly. 1 ml of 1 M NaOH was added to the pellet and it was heated in a water bath at 50 °C until the precipitate was dissolved. Then five drops of concentrated HCl were added and 10 ml of a scintillation fluid containing PPO, POPOP, toluene, ethanol, and Triton X-100 (ref. 30). The radioactivity was counted in a Packard Tri-Carb 3375. Specific [^{3}H]ouabain binding is obtained by subtracting from the total radioactive uptake the amount that is not displaced by high concentrations (10^{-4} M) of unlabelled ouabain.

Preparation of [^3H]ouabain–enzyme complex

The amount of enzyme protein needed for the experiment was incubated in a medium containing 50 mM imidazole–HCl pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–phosphate, and 1–10 μM [^{3}H]ouabain. This mixture was incubated for 30 min at 37 °C and centrifuged at 80000 × *g*. The pellet was resuspended in 0.01 M imidazole–HCl, pH 6.5, and assayed for radioactivity as described before.

Release of [^3H]ouabain from the [^3H]ouabain–enzyme complex

0.5–1.5 mg of protein of the [^{3}H]ouabain–enzyme complex were incubated in a medium containing 50 mM imidazole–HCl pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–phosphate and 10^{-4} M unlabelled ouabain in a total volume of 3 ml. The reaction was stopped by rapid cooling in liquid air and immediate centrifugation.

Preparation of [^{32}P]phosphoprotein

Phosphorylation of membrane protein was carried out by a modification of the method of Post *et al.*[31]: 0.5 ml of a reaction mixture containing 100 μmoles imidazole–HCl pH 7.25, 0.5 μmole $MgCl_2$, and 10 μmoles terminally labelled [^{32}P] ATP were incubated with 0.6–1 mg of ATPase protein in 0.5 ml at 0 °C. Final Na^+ or K^+ concentrations were 100 and 10 mM, respectively. 30 s after the start of the labelling reaction by injection of the enzyme, the reaction was terminated by addition of 5 ml 5% (w/v) trichloroacetic acid (0 °C) and centrifugation at 2000 × *g*. The precipitate was taken up in 5 ml of cold trichloroacetic acid again and centrifuged. This washing procedure was repeated three times. The final precipitate was dissolved in 1 ml Protosol (New England Nuclear) and counted in a liquid scintillation fluid containing 4 g of PPO and 100 mg of POPOP/l of toluene. The values in the presence of 10 mM K^+ were subtracted from those in the presence of 100 mM Na^+.

Materials

[^{3}H]Ouabain with a specific activity of 13 Ci/mmole was obtained from New England Nuclear, Dreieichenhain, Germany and [γ-^{32}P]ATP from the Radiochemical Center, Amersham (specific activity 300–5000 Ci/mole). The sodium salt of ATP was converted to the free acid by passage through Dowex 50 (H^+-form) and neutralized with Tris. Triton X-100 was a product of Koch–Light Laboratories Ltd, Colnbrook, England. All other chemicals were of analytical grade and obtained through Boehringer-Mannheim and E. Merck A.G., Darmstadt.

RESULTS

Binding of [^3H]ouabain to the enzyme

The formation of a [^{3}H]ouabain–enzyme complex is a time- and temperature-dependent process following second order kinetics (Fig. 1). In agreement with the findings of Hansen[10] the amount of [^{3}H]ouabain maximally bound per mg of enzyme protein is linearly related to the enzyme activity for a given species and tissue (Fig. 2). It was repeatedly found that heart and brain microsomes from beef and dog can bind a maximum of 130 pmoles [^{3}H] ouabain /enzyme unit. Kidney enzyme preparations from beef or guinea pig, however, can bind a maximum of 390 pmoles ouabain/ enzyme unit. From these data it is possible to calculate the initial concentration of receptor sites (and hence, the rate constant k_{+1} of [^{3}H]ouabain–receptor association). The strictly bimolecular nature of the association process (Fig. 1) is confirmed by the fact that constant k_{+1} values are obtained when the amount of ouabain bound per min is fitted into the second order equation

$$k_{+1} = \frac{2.303}{(a-b)\cdot t} \cdot \log \frac{b(a-x)}{a(b-x)}$$

where a is the initial concentration of ouabain, b is the initial concentration of the free receptor, and x is the amount of the product (*i.e.* ouabain–receptor complex) after reaction time t. At 37 °C the association rate constant of ouabain binding to beef kidney microsomes is $1.85 \cdot 10^{-4}$ $s^{-1} \cdot M^{-1}$ (Fig. 1B). All enzyme preparations tested (beef kidney, heart and brain, dog heart, and guinea pig kidney) followed second order kinetics for the rate of ouabain receptor association. The values for k_{+1} were found to be quite similar (Table I) for the different species and tissue microsomes.

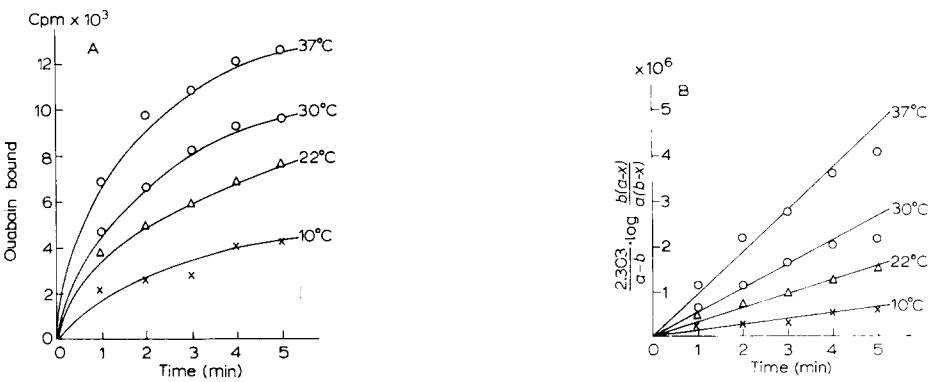


Fig. 1. Formation of the [^{3}H]ouabain–enzyme complex. (A) Time and temperature-dependent binding of [^{3}H]ouabain (initial concentration $105 \cdot 10^{-9}$ M) to beef kidney ($Na^+ + K^+$)-ATPase preparation ($464 \cdot 10^{-9}$ M, initial concentration of free receptor) in the presence of 50 mM imidazole–HCl, pH 7.25, 3 mM $MgCl_2$, and 3 mM Tris–phosphate. Each point was done in duplicate. (B) The experimental points along the curve in A are substituted into the second order equation:

$$k_{+1} \cdot t = \frac{2.303}{a-b} \log \frac{b(a-x)}{a(b-x)}$$

Dissociation of [^3H]ouabain–enzyme complex

[^{3}H]ouabain specifically bound to the different ($Na^+ + K^+$)-ATPase preparations from different species is displaced by high concentrations of unlabelled ouabain. The rate of dissociation is a strictly first-order process (Fig. 3) for all enzyme preparations tested. It is highly temperature dependent. At 37 °C the dissociation rate constant for beef kidney enzyme is $0.94 \cdot 10^{-4}$ s^{-1}. At 0 °C the ouabain–enzyme complexes of beef kidney, heart, and brain as well as that of dog heart are stable for several h and can be washed in the centrifuge repeatedly without losing much [^{3}H]ouabain or regaining catalytic activity. At higher temperatures the labelled ouabain–enzyme complex dissociates increasingly, which is noticeable when unlabelled ouabain is added.

TABLE I

KINETIC CONSTANTS FOR SPECIFIC INTERACTION OF OUABAIN WITH ($Na^+ + K^+$)-ATPase PREPARATIONS FROM BEEF, DOG AND GUINEA PIG

All values were measured at 37 °C in the presence of 3 mM $MgCl_2$ and 3 mM Tris–phosphate.

Enzyme preparation	*Association rate* k_{+1} ($M^{-1} \cdot s^{-1}$)	*Dissociation rate* k_{-1} (s^{-1})	*Dissociation constant* K_D (M)	
			From k_{-1}/k_{+1}	*From Scatchard plot*
Beef kidney	$1.85 \cdot 10^4$	$0.94 \cdot 10^{-4}$	$0.51 \cdot 10^{-8}$	$0.47 \cdot 10^{-8}$
Beef heart	$3.61 \cdot 10^4$	$0.99 \cdot 10^{-4}$	$0.28 \cdot 10^{-8}$	$0.31 \cdot 10^{-8}$
Beef brain	$4.63 \cdot 10^4$	$1.33 \cdot 10^{-4}$	$0.28 \cdot 10^{-8}$	—
Dog heart	$5.45 \cdot 10^4$	$2.31 \cdot 10^{-4}$	$0.42 \cdot 10^{-8}$	$0.43 \cdot 10^{-8}$
Guinea pig kidney	$1.75 \cdot 10^4$	$2.45 \cdot 10^{-3}$	$1.5 \cdot 10^{-7}$	$1.62 \cdot 10^{-7}$

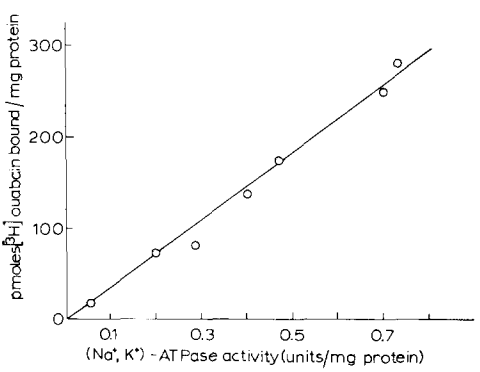


Fig. 2. Amount of ouabain maximally bound to beef kidney enzyme. Kidney enzyme protein (1.45 mg protein) was partially heat denatured for 0–30 min at 50 °C. Afterwards catalytic activity (enzyme units/mg protein) and maximal binding capacity were determined at 37 °C in the presence of 2 μM [^{3}H]ouabain, 3 mM $MgCl_2$, 3 mM Tris–phosphate, 50 mM imidazole–HCl pH 7.25.

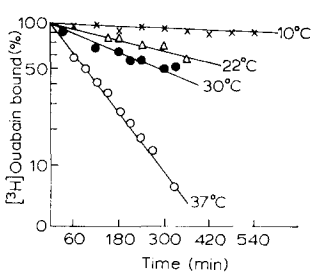
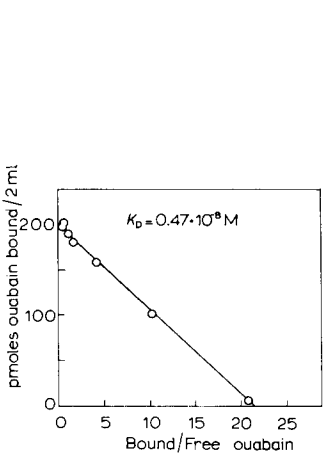


Fig. 3. Rates of dissociation of [^{3}H]ouabain from beef kidney microsomes. 2.1 mg of [^{3}H]-ouabain–enzyme complex (preparation see Materials and Methods) were incubated for the indicated time and temperatures in 3 mM $MgCl_2$, 3 mM Tris–phosphate, 50 mM imidazole–HCl, pH 7.25 and 10^{-3} M unlabelled ouabain, total volume 3 ml.

If the dissociation constant varies considerably, as it is the case with the guinea pig kidney enzyme, this variation is mainly caused by changes in the dissociation rate constant (Table I). This finding is in agreement with Tobin *et al.*[12], who investigated the rate of dissociation of the ouabain–enzyme complex from kidney, heart, and brain tissue from guinea pig, dog and cat enzymes. The [^{3}H]ouabain–enzyme complex of guinea pig kidney dissociates most rapidly at 37 °C as indicated by the relatively high dissociation rate constant of $2.54 \cdot 10^{-3}$ s^{-1}.

Dissociation constants of the ouabain–enzyme complex

From the ratio of the dissociation rate constant k_{-1} and the association rate constant k_{+1} the dissociation constant of the enzyme–ouabain complex can be calculated. At 37 °C, applying this method, a dissociation constant of the enzyme ouabain complex of $0.51 \cdot 10^{-8}$ M for the enzyme preparation from beef kidney is obtained (Table I). This value agrees well with that calculated from a Scatchard plot[32] of the ouabain–enzyme interaction at equilibrium (Fig. 4, Table I). Using this different method a value of $0.47 \cdot 10^{-8}$ M is found. The agreement in the dissociation constants obtained by both methods furthermore indicates that the kinetic experiments are almost giving true rate constants. Beef kidney microsomes have a single high-affinity receptor site as indicated by the straight line in the Scatchard plot. A Scatchard plot with a straight line could be obtained with all enzyme preparations but that of beef brain (Fig. 5). Apparently with beef brain microsomes there is some interaction among the bound ouabain molecules with increasing concentrations (cooperative effect). This cannot be found, when the association and dissociation rate constants are determined, because these experiments are performed at a constant glycoside concentration. For several determinations of the association rate constants



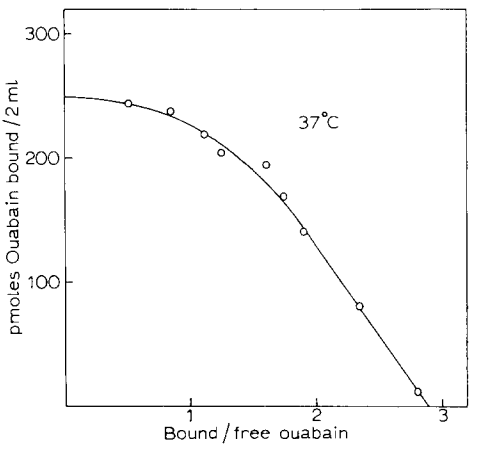


Fig. 4. Scatchard plot of ouabain binding to $(Na^+ + K^+)$-ATPase from beef kidney. 1.4 mg of enzyme protein were incubated with varying amounts of [^{3}H]ouabain ($15.4 \cdot 10^{-9}$ M–$0.5 \cdot 10^{-4}$ M) until equilibrium was reached (30 min) at 37 °C, total volume 2 ml. Each point was done in triplicate.

Fig. 5. Scatchard plot of ouabain binding to $(Na^+ + K^+)$-ATPase preparation from beef brain. 0.75 mg of enzyme protein were incubated as in Fig. 4 until equilibrium was reached (60 min). Each point was done in triplicate. For explanation see text.

of beef brain enzyme, ouabain concentrations varying from $20 \cdot 10^{-9}$–$200 \cdot 10^{-9}$ M were chosen. In all cases the k_{+1} values were identical.

It should be mentioned that the maximal number of binding sites increases if the microsomes are incubated with high concentrations of [^{3}H]ouabain for more than 3 h. Apparently the unspecific binding increases with time and the membranes are damaged.

Binding of ouabain to cell membranes under physiological conditions probably occurs by Na^+-dependent phosphorylation of the enzyme from ATP. It was therefore of interest to measure the dissociation constant of the enzyme–ouabain complex under these conditions. In the presence of 3 mM $MgCl_2$, 3 mM Tris–ATP, and 100 mM NaCl a dissociation constant of $1.1 \cdot 10^{-8}$ M was found for the enzyme preparation from beef kidney. This value obtained under equilibrium conditions is in the same range as that with Mg^{2+} and P_i.

Thermodynamic properties of ouabain binding to microsomes

For the enzyme from beef kidney the rate of dissociation is slightly more sensitive to temperature than is the rate of association (Fig. 6A). This was found to be true for all enzymes but that of guinea pig kidney, where the rate of dissociation is much more temperature sensitive. This disproportionate change of the rate constants with temperature results in a slightly temperature-dependent dissociation constant. This finding is more evident from an Arrhenius plot of the equilibrium constant as a function of temperature (Fig. 6B). The free energy ΔG° of ouabain binding to the enzyme from beef kidney as calculated from the equilibrium constant ($2 \cdot 10^8$ M) at 37 °C is -11 kcal·mole^{-1}. From the dependence of the equilibrium constant on temperature (Fig. 6B), the calculated ΔH° is -5 kcal·mole^{-1}. The entropy change is 24 cal·mole^{-1}·deg^{-1}. As shown in Table II quite similar thermodynamic parameters were found for the enzymes of beef and dog. It is remarkable for the binding of ouabain to beef and dog enzymes that the negative free energy ($-\Delta G^\circ$) is considerably greater than the heat energy ($-\Delta H^\circ$), which should indicate a considerable increase in entropy by the binding process. The difference between ΔG°

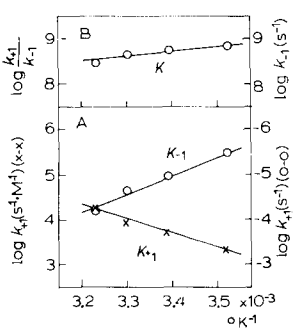


Fig. 6. (A) Arrhenius plot of the rate constants of association (×—×) and dissociation (○—○) for beef kidney enzyme–ouabain interaction. The association rate constants are calculated from the slopes in Fig. 1B. The rate constants of dissociation were determined from the slopes of Fig. 3. (B) Temperature dependence of the equilibrium constant of the ouabain–receptor complex from beef kidney. The equilibrium constant was calculated from the ratio of the rate constants (k_{+1}/k_{-1}) of Fig. 6A.

and ΔH° is accounted for by this entropy change. In contrast to the aformentioned enzymes, ouabain binding to the enzymes from guinea pig kidney is probably due to the ΔH° of -10 kcal·mole^{-1} (Table II).

Comparison of dissociation constants ot the ouabain–receptor complex with the inhibitory action of ouabain on the $(Na^+ + K^+)$*-ATPase*

The time-dependent binding of ouabain to its receptor site[11] is accompanied by a decrease of $(Na^+ + K^+)$-ATPase activity[11,18] as well as transport activity[17,20,33]. In accordance with this finding a decrease of the ouabain concentrations necessary

TABLE II

THERMODYNAMIC PROPERTIES OF [^{3}H]OUABAIN BINDING TO $(Na^+ + K^+)$-ATPase PREPARATIONS FROM BEEF, DOG, AND GUINEA PIG ORGANS

All values are calculated for 37 °C.

Enzyme preparation	ΔG° *kcal·mole*$^{-1}$	ΔH° *kcal·mole*$^{-1}$	ΔS° *cal·mole*$^{-1}$·*deg*$^{-1}$
Beef kidney	−11	−5	21
Beef heart	−11	−4	24
Beef brain	−11	−2	31
Dog heart	−11		
Guinea pig kidney	− 9	−10	−4

TABLE III

COMPARISON OF THE DISSOCIATION CONSTANTS OF OUABAIN-RECEPTOR COMPLEX AND OUABAIN CONCENTRATIONS CAUSING HALF MAXIMAL INHIBITION OF $(Na^+ + K^+)$-ATPase

Microsomal $(Na^+ + K^+)$-ATPase activity was either assayed without preincubation in an optical coupled test at various ouabain concentrations or the microsomes were preincubated for 1 h at 37 °C in 100 mM imidazole–HCl, pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–phosphate, and various ouabain concentrations. Then 10 µl of enzyme suspension were taken out and assayed in an optical test. The kinetics of ATP hydrolysis was continuously followed photometrically at 366 nm and simultaneously recorded. For details see Materials and Methods.

Enzyme preparation	*Half maximal inhibition of* $(Na^+ + K^+)$*-ATPase (M)*		*Ouabain–receptor* K_D *(M)*	I_{50} *equil* K_D
	Without preincubation (I_{50})	*With preincubation* (I_{50} *equil*)		
Beef kidney	$5 \cdot 10^{-7}$	$5 \cdot 10^{-7}$	$0.5 \cdot 10^{-8}$	100
Beef brain	$6 \cdot 10^{-7}$	$5 \cdot 10^{-7}$	$0.3 \cdot 10^{-8}$	168
Beef heart	$2 \cdot 10^{-7}$	$3 \cdot 10^{-8}$	$0.3 \cdot 10^{-8}$	10
Dog heart	$1.6 \cdot 10^{-7}$	$5 \cdot 10^{-8}$	$0.4 \cdot 10^{-8}$	12.5
Guinea pig kidney	$2 \cdot 10^{-6}$	—	$1.5 \cdot 10^{-7}$	—

for half-maximal inhibition of $(Na^+ + K^+)$-ATPase is reported to occur after preincubation of the enzyme with ouabain[8,13]. After this preincubation equilibrium of ouabain binding to its receptor can be assumed. In order to correlate the ouabain–receptor interactions with the action of the glycoside on the catalytic activity of $(Na^+ + K^+)$-ATPase, the ouabain concentrations giving half-maximal inhibition of ATP hydrolysis (I_{50}) after 2 h of preincubation were determined (Table III). In agreement with previous work[8,13] I_{50} values of the enzymes of beef heart, dog heart and guinea pig kidney decreased after preincubation. No effect of preincubation was seen on I_{50} values for the enzymes of beef brain and kidney. Table IV demonstrates that the ouabain bound during the preincubation period remains in a stable form during the optical assay. This is not the case with the guinea pig kidney enzyme as can be concluded from the recording of the time dependent activation of ATP hydrolysis after preincubation in the optical assay mixture. Although the I_{50} values may decrease by a factor of 10 after preincubation of the enzyme with ouabain, they are still far from the dissociation constants (Table III). It is also evident from this table that the dissociation constants of the ouabain–receptor complex of different tissues and species show relatively small variations, whereas the action of ouabain on the catalytic activity even after preincubation varies considerably. This is more evident from a comparison of the I_{50} equil/K_D ratios of the different enzymes. Similar discrepancies have also been reported to occur in comparisons of I_{50} values and the K_i values of the action of ouabain on $(Na^+ + K^+)$-activated ATPase[13].

TABLE IV

[^{3}H]OUABAIN BINDING AND $(Na^+ + K^+)$-ATPase ACTIVITY DURING THE OPTICAL ASSAY OF THE CATALYTIC ACTIVITY

Beef brain microsomes (8.5 mg protein, specific activity 3.18 units/mg protein) were labelled with [^{3}H]ouabain by incubation in 50 mM imidazole–HCl pH 7.25, 3 mM $MgCl_2$, 3 mM Tris–phosphate, and $1.5 \cdot 10^{-6}$ M [^{3}H]ouabain for 30 min at 37 °C. After centrifugation for 30 min at $80000 \times g$ the sediment was resuspended in 0.01 M imidazole–HCl, pH 6.5; 0.67 mg protein of this enzyme–ouabain complex was incubated in 2 ml of the optical test solution (see Materials and Methods) at 37 °C. After different intervals (0–10 min) the incubation was interrupted by freezing in liquid air. At the same time an aliquot was withdrawn and added to another reaction mixture for the determination of $(Na^+ + K^+)$-ATPase activity. The [^{3}H]ouabain bound to the enzyme was determined as described in Materials and Methods.

Time of incubation (min)	*$(Na^+ + K^+)$-ATPase activity (unit/mg protein)*	*[^{3}H]Ouabain bound (pmoles/0.67 mg protein)*
0	0.0145	277
1	0.0145	264
2	—	262
3	—	263
4	0.029	260
6	—	258
8	—	256
10	0.036	247
12	0.040	—

Stoichiometry of the ouabain–enzyme interaction

In order to get additional information on the quantitative relationship between the catalytic site of $(Na^+ + K^+)$-ATPase and the ouabain binding site of the enzyme, we measured the ouabain binding capacity and the maximal [^{32}P]phosphoprotein intermediate formation (Table V). Both, ouabain-binding capacity[10] and the maximal amount of phosphorylated intermediate[31,34] have been shown to be directly related to the $(Na^+ + K^+)$-ATPase activity of these membranes. As can be seen from Table V, beef kidney microsomes incorporated about three times as much [^{32}P]P_i from [γ-^{32}P]ATP as the enzymes from heart, brain or guinea pig kidney per enzyme unit. This finding which has also been described for enzymes from other sources[35], indicates that the apparent turnover may be different in enzymes isolated from different animals and organs. Ouabain-binding capacity can be shown to vary with the source of enzyme, too (Table V). This finding supports similar observations with heart enzymes from beef, dog and rat and rat kidney[21]. As can be seen from the [^{3}H]/[^{32}P] ratios, most enzyme preparations bind one ouabain per phosphorylation site except guinea pig kidney enzyme, which has four ouabain sites per phosphorylation site.

TABLE V

COMPARISON OF OUABAIN-BINDING CAPACITY AND MAXIMAL ^{32}P INCORPORATION

Enzyme preparation	*No. of determinations*	*Ouabain–receptor capacity*,***	*^{32}P incorporated**	*Apparent turnover****	[^{3}H]/[
Beef kidney	7	390	292	3 430	1.3
Beef brain	10	130	87	11 500	1.5
Beef heart	6	130	117	8 550	1.1
Dog heart	3	130	—	—	—
Guinea pig kidney	9	390	90	11 100	4.2

* Expressed as pmoles per enzyme unit.

** Calculated from Scatchard plots (37 °C) as maximal number of binding sites.

*** This calculation assumes that ^{32}P incorporation is not temperature dependent, since this represents the total number of phosphorylation sites. If all hydrolysis proceeds through a phosphoryl enzyme, the apparent turnover number represents moles of ATP hydrolysed/enzyme phosphate per min[35].

DISCUSSION

Ouabain binding to different enzyme preparations has been intensively studied with respect to the kinetics[4,6,12,13,16] and the modification of the binding process by ligands and substrates of the $(Na^+ + K^+)$-activated ATPase[4–6,8,11,16]. Ouabain binding has also been used to identify parts of the Na^+-transport system under conditions where the catalytic activity of the enzyme has been lost[22–24]. Nevertheless there was only a rather imprecise knowledge about the quantitative interaction of the cardioactive steroid with its receptor.

In agreement with Tobin and Sen[6] and Tobin *et al.*[12] it could be established

that the binding between the $(Na^+ + K^+)$-ATPase and [^{3}H]ouabain is reversible (Fig. 3). Therefore it became possible to determine the dissociation constant from the k_{-1}/k_{+1} ratio by the calculation of the rate constants for the association and dissociation of ouabain. This was done in way analogous to that reported by Cuatrecasas[36] for the insulin–receptor interaction. The rate of the formation of the ouabain–receptor complex obeys second order kinetics (Fig. 1B), while the dissociation follows first order kinetics (Fig. 3). This finding supports the concept of the ouabain–receptor interaction set up by Hansen[10] and by Baker and Willis[19] (Eqn 1). Additional support for the correctness of Eqn 1 arises from the fact, that only one type of ouabain receptor is found in most tissues, as can be concluded from the linear Scatchard plot (Fig. 4). The dissociation constants calculated from the rate constants and from Scatchard plots agree well. They vary between $3 \cdot 10^{-9}$ M and $1.7 \cdot 10^{-7}$ M with five different enzyme preparations from three species (Table I). The variation in the dissociation constants for the guinea pig kidney enzyme was primarily caused by a variation in the dissociation rate constant[12] (Table I). Although most of the data reported in this paper were obtained under conditions where ouabain binding was stimulated by Mg^{2+} and P_i, similar dissociation constants were found, when ouabain binding was stimulated in the presence of Na^+, Mg^{2+}, and ATP.

The binding and release of ouabain is a temperature-dependent process. From the dissociation constants at 37 °C the free energy ΔG° of ouabain could be calculated. It varies in five different enzyme preparations between -11 kcal·mole^{-1} and -9 kcal·mole^{-1}. It is interesting to notice that the ΔH° values and ΔS° increased when the dissociation constant decreased (Table I, II). However, it is not yet possible to speculate on the significance of these data with respect to ouabain–receptor interaction in different membranes.

The studies of ouabain binding to ox brain cell membranes revealed a curved line in the Scatchard plot (Fig. 5). This may indicate a homotrope cooperative effect of ouabain on its receptor in this organ. Similar curved lines in Scatchard plots were observed in studies of antimycin binding[37], and of [^{35}S]atractyloside[38] and [^{35}S]gummiferin[39], both inhibitors of the adenine nucleotide carrier, to mitochondrial membranes. These lines became straight, however, when the membranes were lipid depleted. It remains to investigate, whether the cooperativity in ouabain binding reflects a general phenomenon of nervous tissue and whether the cooperativity disappears after lipid depletion.

The dissociation constants of the ouabain–receptor complex in the five different enzyme preparations lay in each case below the I_{50} values of the inhibition of the $(Na^+ + K^+)$-ATPase by the glycoside. The tightness of coupling between ouabain receptor and ATP hydrolyzing subunit was best in heart enzymes as is evident from a comparison of I_{50} equil/K_D ratios for the different enzymes (Table III). A constant and relatively low ratio should be obtained, when both subunits were linked tightly. Therefore it appears that the catalytic and the ouabain–receptor subunits are only loosely linked to each other. Support for this suggestion arises also from an investigation of the relationship between ouabain-binding capacity and catalytic activity of $(Na^+ + K^+)$-ATPase, which was found to differ with the enzymes from various tissue sources (Table V). Furthermore the stoichiometry of ouabain binding and phosphorylation sites varied between species: whereas the enzymes from beef bind only one ouabain per phosphorylation site, the enzyme from guinea pig kidney

binds 4 moles ouabain/1 mole [^{32}P]phosphoprotein formed. It should be mentioned that ($Na^+ + K^+$)-activated ATPase from electric eel organ binds only one ouabain per two phosphorylation sites[11].

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