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OUABAIN BINDING SITES AND THE (Na^+,K^+)-ATPase OF BRAIN MICROSOMAL MEMBRANES

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SUMMARY

Beef brain microsomes bound approximately 180–220 pmoles of [^{3}H]ouabain per mg of protein in the presence of either $MgCl_2$ and inorganic phosphate or ATP, $MgCl_2$ and NaCl. The ouabain-binding capacity and the ouabain–membrane complex were more stable than the (Na^+, K^+)-ATPase activity to treatment with agents known to affect the membrane integrity, such as, $NaClO_4$, sodium dodecyl sulfate, *p*-chloromercuribenzoate, urea, ultrasonication, heating, pH and phospholipase C.

The presence of binding sites that were normally inaccessible to ouabain in brain microsomes was demonstrated. These sites appeared after disruption of microsomes with 2 M $NaClO_4$ as evidenced by increased binding of [^{3}H]ouabain. These sites may be buried during the subcellular fractionation procedure and could be accessible in the intact cell.

INTRODUCTION

Much effort has been made to attempt to purify the (Na^+,K^+)-ATPase system from different sources with limited success[1–6]. Recent studies[7–15] have shown that [^{3}H]ouabain, a specific inhibitor of the (Na^+, K^+)-ATPase, can be bound to microsomal membranes containing this enzyme system. Previous studies in this laboratory involving binding of [^{3}H]ouabain to brain microsomes suggested that under certain conditions, there might exist an inactive form of the enzyme having high [^{3}H]ouabain binding capacity but low (Na^+, K^+)-ATPase activity[16]. It was suggested that this form might lack some critical membrane component or conformation necessary for full enzyme activity and it was hoped that ouabain binding could be used in the design of a purification scheme for the (Na^+, K^+)-ATPase in either the active or inactive forms.

In the present study we have further compared properties of the (Na^+, K^+)-ATPase with those of [^{3}H]ouabain binding in order to learn more about the relative

Abbreviation: PCMB, *p*-chloromercuribenzoate.

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stabilities of the two systems. The data suggest that under most experimental conditions the ouabain–membrane complex is more stable than is the (Na^+, K^+)-ATPase system.

METHODS

Beef brain microsomes were prepared by differential centrifugation in 0.32 M sucrose at pH 7.4 (ref. 17). Either freshly prepared or lyophilized microsomes were used. Aqueous solutions of labelled ouabain (10, 50 and 100 μM) were prepared by dilution of [^{3}H]ouabain (11.7 Ci/μmole, New England Nuclear) with stock 10^{-3} M unlabelled ouabain solution, (Sigma Chem. Co., St. Louis, Mo.) so that 1 pmole contained approximately 200 cpm. Radioactivity was measured with a Packard 3375 Liquid Scintillation counter using Bray's solution[18]. All other chemicals were of reagent quality.

Binding of ouabain to microsomes was performed under conditions described by Tobin and Sen[11]. In most experiments, ouabain binding was carried out in a standard reaction medium which contained 1 mM inorganic phosphate, 10 mM imidazole, and 5 mM $MgCl_2$, pH 7.4. In the experiments shown in Fig. 2, the medium contained 200 mM NaCl, 2 mM ATP, 4 mM $MgCl_2$, and 10 mM imidazole, pH 7.4. These solutions were described by the above authors as giving the maximum degree of ouabain binding. A reaction mixture containing 0.25–0.6 mg/ml protein in either 0.50 or 1.0 ml was incubated at 37 °C for 20 min (3 min with the ATP solution), then cooled in an ice bath and either centrifuged at 48 000 × g for 20 min in a Sorvall refrigerated centrifuge or dialyzed. All subsequent operations were at 4 °C. Ouabain concentrations above 15 nmoles/mg protein were considered to be saturating.

All dialyses were performed in Visking tubing 1 cm in diameter that had previously been boiled for 1 h successively in water, 5 mM EDTA (pH 7.4), and again in water. This tubing was stored in a 1-mM imidazole solution in the cold. Samples were dialyzed at 4 °C against 1000-fold excess of the appropriate medium for 2–3 h. The solutions were changed and dialysis was continued for another 12 h.

The (Na^+, K^+)-ATPase enzyme assay[17], the protein[19] and phospholipid phosphorus analyses[20] were carried out by standard methods. The specific activity of the beef brain microsomal (Na^+, K^+)-ATPase (activity in the presence of Na^+, K^+ and Mg^{2+} less the activity in presence of 10^{-4} M ouabain) was in the range of 10–18 μmoles phosphate formed per mg protein per h.

The initial titrations of ouabain-binding sites in intact microsomes were carried out by the procedure of Matsui and Schwartz[8] using the conditions of Tobin and Sen[11] with the exception that after centrifugation the resulting pellets were resuspended, washed with fresh buffer, and recentrifuged until the supernatant had reached background radioactivity. Usually three washes were required.

In experiments in which the enzyme was heat denatured the enzyme suspension was maintained at 60 °C. Portions were withdrawn at the indicated times and cooled in an ice bath. These were then used for the subsequent enzyme assays and binding studies. Thus these experiments measure the ability of the microsomes to bind ouabain as compared to those which measure the stability of the ouabain–microsome complex.

Portions of a microsomal suspension to which ouabain had previously been bound were dialyzed against solutions at the indicated pH, using glycine, acetate, or Tris standard organic buffers[21] for 4 h. The half escape time of the ouabain had been previously determined to be approximately 90 min under the conditions of dialysis used here. Data on the effects of chemical treatment on ouabain binding and the (Na^+, K^+)-ATPase specific activity were obtained by placing microsomes with bound ouabain (either saturating or 1% saturating) in a suspension such that the final concentrations of reagents were as indicated in Table I. These were kept at room temperature for 2 h and then dialyzed against the standard reaction solution overnight to remove both the ouabain that had been lost and added reagents. Following dialysis, the suspension was assayed for the amount of ouabain retained and, where appropriate, for (Na^+, K^+)-ATPase activity.

Treatment with purified phospholipase C was by the method described by Stahl[22]. At the conclusion of the enzymic digestion, usually 2 h, the reaction was stopped by centrifugation at $48\,000 \times g$ followed by washing the pellet with the standard reaction medium and recentrifuging. A second addition of ouabain was then made to a portion of the phospholipase C-treated sample at as close to the original ratio of ouabain to protein as possible. The unlabelled microsomes were treated in like fashion.

Ultrasonication was carried out with a Branson Sonifier at a setting of 5 using the microprobe adjusted to produce optimum cavitation. All solutions were immersed in an ice bath and sonication was carried out in bursts of 10 s alternating with 1-min cooling periods.

RESULTS

Ouabain binding and (Na^+, K^+)-ATPase of intact microsomes

In the presence of $MgCl_2$ and the phosphate-containing standard reaction medium, saturation appeared to be complete at approximately 220 pmoles ouabain bound per mg protein (Fig. 1). NaCl caused inhibition of ouabain binding in the

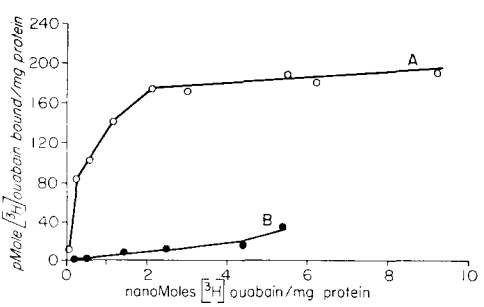
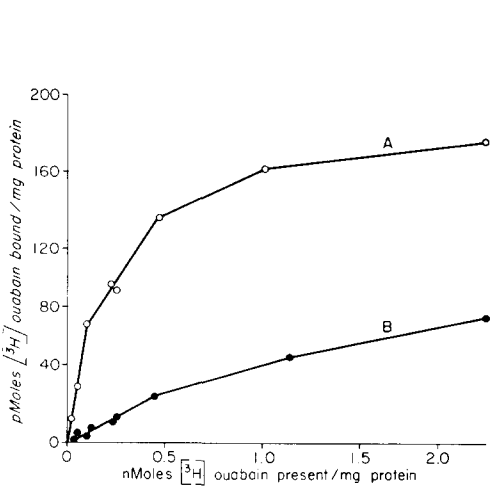


Fig. 1. Titration of beef brain microsomes with increasing amounts of [^{3}H]ouabain in the standard medium (5 mM $MgCl_2$, 1 mM H_3PO_4, 10 mM imidazole, pH 7.4) with (B) or without (A) 0.1 M NaCl. The reaction tubes contained 0.25–0.6 mg protein per ml in a volume of either 0.5 or 1 ml. The reaction was carried out for 20 min at 37 °C, then the samples were cooled to 4 °C and centrifuged as described in the test. Samples presaturated with 1 mM unlabelled ouabain were used as background for each concentration shown. Values shown are from three experiments, each run in duplicate.

$MgCl_2$–P_i medium as described previously. In the presence of ATP, $MgCl_2$ and NaCl (Medium 2), the ouabain-binding sites appeared to be saturated at 180 pmoles ouabain per mg protein (Fig. 2). There was a marked reduction in binding in the presence of 10 mM KCl. Both ouabain-binding capacity and (Na^+, K^+)-ATPase activity of microsomal suspensions were diminished by preincubation at 60 °C (Fig. 3). The ability of microsomes to bind ouabain was clearly more resistant to heat inactivation than was (Na^+, K^+)-ATPase activity, which was almost completely inactivated at a time when ouabain-binding capacity was still 50% of that of control microsomes. However, a number of treatments, *e.g.* strong acid or base and 8 M urea, which completely inactivated the enzyme also completely inhibited [^{3}H]-ouabain binding (data not shown).



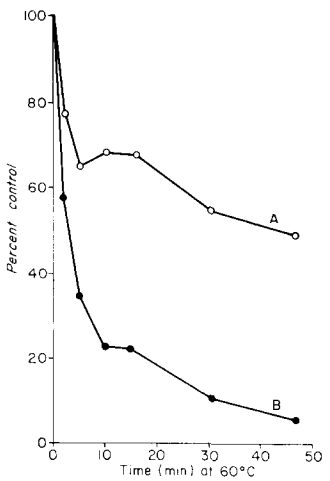


Fig. 2. Titration of beef brain microsomes with increasing amounts of [^{3}H]ouabain in Medium 2 (3 mM ATP, 4 mM $MgCl_2$, 100 mM NaCl and 10 mM imidazole, pH 7.4) with (B) or without (A) 10 mM KCl. The reaction was run for 3 min at 37 °C and the tubes were then cooled to 4 °C and centrifuged. Otherwise, conditions were identical to those in Fig. 1.

Fig. 3. The loss of ouabain-binding capacity and decrease in (Na^+, K^+)-ATPase specific activity upon incubation at 60 °C. Beef brain microsomes, 0.4 mg/ml in the standard medium were incubated at 60 °C. At the indicated intervals portions were withdrawn and cooled in ice. The (Na^+, K^+)-ATPase assay and ouabain binding were performed on each portion. Ouabain binding was carried out in standard medium for 20 min in the presence of [^{3}H]ouabain. Values shown are expressed as percentage of zero time control. The upper curve (A, ○—○) shows cpm ouabain per mg protein and the lower curve (B, ●—●) the (Na^+, K^+)-ATPase specific activity, expressed as percent of activity in absence of heating.

Mechanical disruption by ultrasonication of microsomes had a varied effect on enzymatic activity and on the capacity to bind ouabain. Fig. 4 shows a representative experiment. These experiments were carried out at 1% of the fully saturated ouabain–membrane complex so that (Na^+, K^+)-ATPase activities could be determined concurrently on the same sample. After brief sonication of approximately 1 min both the enzymatic specific activity and the amount of ouabain bound may be increased by 10% or more. Unfortunately, this procedure was not consistently reproducible but was repeated successfully several times. After an initial increase

in enzymatic specific activity, further sonication resulted in a complete loss of enzymatic activity. Ouabain-binding capacity also decreased but was retained to a significant degree even at times when all enzymatic activity was lost.

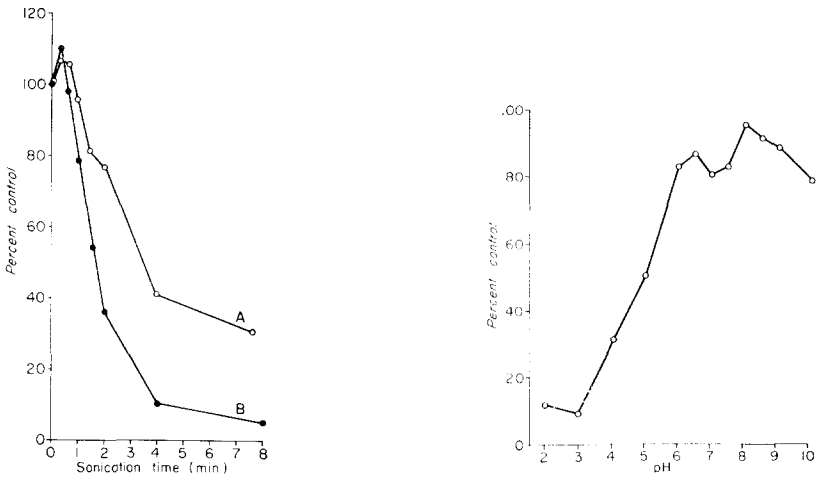


Fig. 4. The effect of ultrasonic disruption on the specific activity of the (Na^+, K^+)-ATPase and binding of ouabain. Sonication was for 10 s bursts with cooling between bursts. At the indicated times, portions were withdrawn and [^{3}H]ouabain was bound in the standard medium at 1% saturation and the excess was removed by dialysis. (Na^+, K^+)-ATPase activity and radioactivity were then determined. The data is expressed at percentage of zero time control (not sonicated). A (○—○), ouabain-binding; B (●—●), (Na^+, K^+)-ATPase activity.

Fig. 5. The pH stability of the ouabain brain microsome complex from a representative experiment. Ouabain was bound to microsomes at 5 nmoles ouabain per mg protein in the standard reaction medium. Portions were then dialyzed *vs* buffers for 4 h at 4 °C. The amount of ouabain retained was then determined and compared to that of the starting material.

(Na^+, K^+)-ATPase and ouabain-binding stability

Effects of pH. The ouabain–membrane complex, once formed was more resistant to incubation at low pH than was the (Na^+, K^+)-ATPase activity (Fig. 5). 50% of the complex was retained after dialysis of ouabain-labelled microsomes for 4 h against a buffer at pH 5.5. Under similar conditions below pH 5.5 less than 20% of the (Na^+, K^+)-ATPase activity found at pH 7.4 is retained[23]. However, the pH optimum for stability of ouabain binding was similar to that of the (Na^+, K^+)-ATPase.

Treatment with chemical agents. Several chemical agents were examined for their effects on ouabain-binding stability and on (Na^+, K^+)-ATPase activity (Table I). Beef brain microsomes were preexposed to ouabain at concentrations designed to produce either complete saturation or 1% saturation of available sites. Enzyme analysis was carried out on the same sample that had been treated with the lower concentration of ouabain. *p*-Chloromercuribenzoate (PCMB) totally inactivated the (Na^+, K^+)-ATPase, implicating a sulfhydryl group within the active site as previously reported[24]. This functional group does not appear to be directly involved with the binding of ouabain since 30–50% of bound ouabain was retained. Dithiothreitol also had no effect on ouabain binding but stabilized the enzymatic

TABLE I

RETENTION OF BOUND OUABAIN AND (Na^+, K^+)-ATPase DURING CHEMICAL TREATMENT

Retention of bound ouabain after chemical treatments. Ouabain, either at saturating or 1% saturating levels, was bound to brain microsomes in standard medium as stated in Methods and the excess was removed by dialysis. These preparations were then suspended in standard medium containing the indicated additions. All samples were maintained at room temperature for 2 h and then dialyzed a second time to remove both the ouabain released and the chemical agent. The (Na^+, K^+)-ATPase assay was performed on the 1% saturated sample. All values are reported as percentage of control sample and represent ranges from 2–3 experiments.

Treatment	*Ouabain retained (%)*		
	Saturating ouabain (5 μM)	*1% saturation with ouabain (0.05 μM)*	*(Na^+, K^+)-ATPase (% retained)*
Satd media	100	100	100
5 mM PCMB	65–78	33–49	0
10 mM PCMB	0–5	19–37	0
10 mM dithiothreitol	95–100	88–96	114–126
10 mM EDTA	63–72	83–85	100–109
10 mM EDTA + 10 μM unlabelled ouabain	60–71	—	—
0.05% sodium dodecyl sulfate	56–58	81–86	20–51
0.10% sodium dodecyl sulfate	0–4	2–20	0
1.3 M urea	68–84	81–97	39–60
2.7 M urea	27–49	57–63	6–17
4.5 M urea	5–8	5–20	0
7.2 M urea	0	9–12	0

activity. The chelating agent EDTA consistently released 30–40% of bound ouabain. Incubation with unlabelled ouabain in high concentration removed the same proportion of the label. Tobin and Sen[11] working with kidney cortex microsomes have reported total exchange of radioactive bound ouabain for unlabelled ouabain at 37 °C in the presence of EDTA. The anionic detergent sodium dodecyl sulfate at a concentration of 0.05% (w/v) caused a greater than 60% loss in enzymatic activity, but only 40% loss of the bound ouabain. However, at a concentration of 0.1%, this reagent also resulted in the total loss of both bound ouabain and enzymatic activity. Urea caused a greater loss in enzymic activity than bound ouabain but at 7.2 M both were lost.

Treatment with phospholipase C. Work in this laboratory[22] as well as others[1,25] has shown that treatment of microsomes with phospholipases causes a decrease in (Na^+, K^+)-ATPase activity implying the necessity of phospholipids for enzymatic activity. To determine what effect this treatment would have on the ouabain-binding characteristics microsomes labelled with [^{3}H]ouabain were treated with purified phospholipase C (Table II). After this treatment, the amount of ouabain retained per mg of protein was reduced by $25 \pm 12\%$ (Table IIB). Under similar conditions, (Na^+, K^+)-ATPase activity is reduced by up to 70%[22]. In most experiments when

unlabelled brain microsomes were digested with phospholipase C prior to binding of [^{3}H]ouabain, approximately 70% as much ouabain could be bound as was possible in untreated microsomes (Table IIC). Further, ouabain that was lost during the phospholipase C treatment could be rebound if [^{3}H] ouabain was again added (Table IID).

TABLE II

EFFECT OF PHOSPHOLIPASE C TREATMENT ON [^{3}H]OUABAIN BINDING

Treatment with purified phospholipase C was carried out as described in Methods. Purified phospholipase C was freshly prepared for each experiment. (B) The microsomes were labelled under saturating conditions, dialyzed and treated with phospholipase C. (C) Ouabain binding was carried out after phospholipase treatment. (D) Microsomes treated as in (B) were re-exposed to labelled ouabain following phospholipase C treatment. The values shown are expressed as percentage of a control run in parallel with the enzymic digestion. Values are mean ±S.D. for 4 experiments. All suspensions were dialyzed overnight *vs* the standard media.

Conditions of microsomes at time of phospholipase C treatment	[^{3}*H*]*Ouabain binding* (*% of control*)	*Phospholipid phosphorus content* (*μmoles/mg protein*)
A. Control (no phospholipase C)	100	0.514±0.058
B. Prelabelled with [^{3}H]ouabain	75±12	0.162±0.030
C. Unlabelled	70±9	0.172±0.038
D. As in B, in the second addition of [^{3}H]ouabain	96±8	0.162±0.030

TABLE III

EFFECT OF MEMBRANE PERTURBATIONS ON TOTAL AVAILABLE OUABAIN-BINDING SITES

Brain microsomes were presaturated with 1 mM unlabelled ouabain in the standard medium and the excess was removed by 16 h dialysis. [^{3}H]Ouabain (5 nmoles/mg protein) was added and portions were treated as shown and allowed to remain for 2 h at room temperature. The unbound [^{3}H]ouabain was removed by a second dialysis. In a parallel experiment, microsomes without presaturation bound 170 pmoles of [^{3}H]ouabain per mg protein while those presaturated bound 52 pmoles/mg protein. For the experiments shown the presaturated microsomes bound 36±4% of the maximum possible. The values shown are expressed as percentage of this control value (±S.D. for 3 experiments).

Treatment	[^{3}*H*]*ouabain binding* (*% of control*)
Sonicated 30 s	114± 8
2 M $NaClO_4$	176±26
0.05% sodium dodecyl sulfate	60±14
2 M urea	58± 9

Exposure of additional ouabain-binding sites

Ouabain binding after presaturation with unlabelled ouabain. To determine whether it would be possible to expose ordinarily inaccessible sites on the microsomes the following experiments were performed. Unlabelled ouabain (10^{-4} M) was added to brain microsomes in standard medium and dialyzed overnight (Table III). These microsomes were then subjected to a number of relatively mild perturbing treatments; brief ultrasonication, or exposure to 2 M $NaClO_4$, 0.05% sodium dodecyl sulfate or 2 M urea. [^{3}H]Ouabain was then added. The suspensions were dialyzed a second time to remove the excess labelled ouabain and other added agents. With no further treatment, microsomes bound 60 pmoles [^{3}H]ouabain per mg protein, which may either represent new sites, or more likely sites to which unlabelled ouabain had been weakly bound and dissociated during dialysis.

Microsomes sonicated for 30 s showed a slight increase in ouabain binding over the control indicating some exposure of additional sites. Longer periods of sonication have been shown to cause a dramatic decrease in both ouabain-binding capacity and (Na^+, K^+)-ATPase specific activity (Fig. 4). Treatment with either 0.05% sodium dodecyl sulfate or 2 M urea caused a net decrease of binding sites over controls demonstrating the generally strong disruptive effects of these reagents and contrasts with the milder membrane perturbation achieved with $NaClO_4$ (ref. 16).

DISCUSSION

Correlation of (Na^+, K^+)-ATPase activity to [^{3}H]ouabain binding

The capacity of brain microsomes to bind [^{3}H]ouabain has been thought to be a valid marker for the membranous (Na^+, K^+)-ATPase. The present data suggest that there are fewer requirements necessary for the binding of ouabain than are required for full enzymatic activity and once formed the ouabain–membrane complex is also more stable than is the (Na^+, K^+)-ATPase. Ouabain binding may qualitatively reflect the changes in enzymatic character. Thus, ouabain did not bind to the fully denatured enzyme, but did bind to a lesser degree to the enzyme reversibly inactivated by treatment with phospholipase C. Under conditions such as extremes of pH or temperature, that caused a rapid loss of enzymatic activity, ouabain binding capacity also decreased, but usually to a lesser extent.

It might be suggested that a certain type of matrix must surround or be within the environment of the active site of the (Na^+, K^+)-ATPase before the enzyme can function, but is not an absolute requirement for ouabain binding. Such a matrix probably includes phospholipid since treatment with purified phospholipase C appreciably decreases enzyme activity but only slightly decreases binding capacity. This is in agreement with the work of Taniguchi and Iida[25]. Their investigations showed that 70% of the phospholipids may be removed without affecting ouabain binding. In our study there was a decrease in the number of ouabain molecules bound after phospholipase C treatment but these sites could still accept and bind ouabain if it was made available subsequent to phospholipase C treatment. This indicates that the phospholipids removed are not directly involved in the binding of ouabain to microsomes.

Short periods of sonication may cause an increase in enzyme activity, while

prolonged periods drastically decrease it. The effects of ultrasonication on (Na^+, K^+)-ATPase are compatible with those described with erythrocytes by Askari and Frantantoni[26]. Ouabain binding is affected in a similar way but appears to be more stable (Fig. 4). This type of treatment does not rupture the primary or secondary structures of the membrane proteins[27]. Since it also appears that the major portion of the phospholipids are not directly involved in ouabain binding, one effect of ultrasonication must be to disrupt the necessary environment within the membrane.

Membrane disruption with the detergent sodium dodecyl sulfate affected ATPase activity and ouabain binding in a similar fashion. Chan[28] has reported that at 0.25 mM sodium dodecyl sulfate causes a stimulation of (Na^+, K^+)-ATPase activity but loss of cation specificity and ouabain sensitivity. Levels of sodium dodecyl sulfate above 0.4 mM bring about inhibition of enzyme activity. The levels of sodium dodecyl sulfate used in this study were between 0.05–0.1% or 3.9–8.1 mM. These are above the critical micelle concentration[29] and it should be noted that the ionic strength of the solution is very critical to the critical micelle concentration of sodium dodecyl sulfate. At the lower concentration used in this study the loss in enzyme activity was twice as great as the loss of ouabain-binding capacity. At the level of 8.1 mM sodium dodecyl sulfate all enzyme activity was lost and less than 20% of the binding capacity remained. The higher concentrations caused a total loss in both. Treatment with urea also demonstrated that the ouabain binding was more stable than the enzymic activity but it was greatly reduced as the urea concentration was increased.

The PCMB effects are consistent with the work of Fahn *et al.*[24], which indicated that *N*-ethylmaleimide, a sulfhydryl reagent, abolishes the (Na^+, K^+)-ATPase activity but does not interfere with the phosphorylation of the enzyme. In our experiments all enzymic activity was lost after exposure to 5 mM PCMB but greater than 50% of the bound ouabain was retained. At 10 mM PCMB the ouabain binding was greatly affected which may indicate that a second sulfhydryl is required for the structural integrity of the ouabain-binding unit.

It was observed that most of the bound ouabain would not exchange with a 1000-fold excess of unlabelled ouabain. Tobin and Sen[11] reported a half exchange rate of 3 min at 37 °C using a guinea pig kidney preparation. The difference of organ, species and method or preparation are probably sufficient to account for these observed differences. Yoda and Hokin[30] working with a beef brain preparation concluded that the ouabain binding was irreversible.

Thus, the alterations produced by mechanical, enzymatic and a series of chemical treatments suggest that the (Na^+, K^+)-ATPase and the ouabain binding are related. In nearly all cases the ouabain-binding capacity and the ouabain–membrane complex are more stable than the enzyme activity but these decrease appreciably when the membranes are denatured. The present work also suggests that the number of binding sites to which ouabain has access can be altered. Within any preparation under neutral conditions there will be a finite number of binding sites that can be determined. Unfortunately, if any disruptive method is used two things may happen. A portion of the original sites will be destroyed and additional sites that had been buried will be uncovered .The microsomal fraction contains bits of outer membrane, endoplasmic reticulum, Golgi bodies and other membrane fragments. It is obviously not a homogeneous collection of similar subcellular

organelles. During preparation many of these fragments close up or interact with each other by entropic forces to give an enclosed vesicular structure[31]. By the process of preparing the microsomes an ouabain-binding site or a site of the (Na^+, K^+)-ATPase that may have been exposed in the intact cell might be buried in the resulting particles. A subsequent publication will describe further the exposure and alteration of ouabain-binding sites in membranes.

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