# Studies on the Interaction of Ouabain and Other Cardioactive Steroids with Sodium-Potassium-Activated Adenosine Triphosphatase

R. W. Albers, G. J. Koval, and G. J. Siegel

*Laboratory of Neurochemistry, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland 20014*

(Received January 5, 1968)

SUMMARY

The combination of ouabain with ($Na^+ + K^+$)-ATPase is essentially irreversible at physiological temperature and pH. The rate of inhibition of the enzyme by different cardioactive steroids varies widely. The amount of steroid bound parallels the inhibition of enzyme activity. $Mg^{2+}$, $Na^+$, $K^+$, nucleotides, and orthophosphate markedly influence the rate of interaction. In the presence of nucleotides, the rate is accelerated by $Mg^{2+}$ and $Na^+$ and retarded by $K^+$. In the presence of orthophosphate, $Mg^{2+}$ increases, $K^+$ slows, and $Na^+$ markedly decreases the rate of interaction.

The ratio of ouabain binding to $Na^+$-dependent phosphorylation is 0.50 for *Electrophorus* electric organ ATPase. In contrast to the native enzyme, the ouabain-treated enzyme rapidly incorporates orthophosphate.

Cardioactive steroids appear to inhibit ($Na^+ + K^+$)-ATPase by reducing the difference between the conformational energies of the phosphorylated and nonphosphorylated forms of the enzyme.

## INTRODUCTION

Cardiac glycosides are powerful and specific inhibitors of active $Na^+$ transport and of NaATPase.[1] This fact is a substantial part of the argument that the two systems are functionally related (1). The molecular basis of CS[1] inhibition is thus of theoretical interest; it is also of interest because of the probability that both toxic and therapeutic effects of CS are mediated by this or a similar mechanism (1, 2).

The hydrolysis of ATP by NaATPase proceeds through several steps (3). On the basis of studies of the effects of magnesium ions and several inhibitors on NaATPase (4–6), we have suggested an enzymatic mechanism which can be readily incorporated into a model for coupled active $Na^+$ and $K^+$ transport (Fig. 1). In this model, a phosphorylated enzyme mediates the vectorial work of transport through allosteric transitions. Our present hypothesis is that the action of CS may be described in terms of this model. *N*-Ethyl maleimide, BAL-arsenite,[1] and oligomycin affect several properties of NaATPase in the same way. They activate a $Na^+$-dependent ATP-ADP exchange, increase the sensitivity of the system to $Na^+$, and abolish its response to $K^+$. The ATP hydrolysis is inhibited while the capacity of the enzyme to be phosphorylated by ATP is not quantitatively changed. These effects may be explained if it is assumed that the *cis* forms of the enzyme are stabilized by these inhibitions (Fig. 1) (6).

Cardiac glycoside inhibition is not

[1] The following abbreviations appear in the text: NaATPase = $Na^+$- and $K^+$-dependent Mg-ATP phosphohydrolase; CS = cardioactive steroids and the corresponding glycosides; Tris = tris(hydroxymethyl)amminomethane. BAL = 2,3-dimercaptopropanol.

accompanied by similar effects. Charnock and Post (7), Matsui and Schwartz (8), and Portius and Repke (9) have proposed that CS combine with the phosphorylated enzyme to inhibit the $K^+$-dependent dephosphorylation (Fig. 1, step 4). Antagonism between $K^+$ and CS is observed (1). Ahmed *et al.* have described this as reversible competition and derived a $K_I$ (10). However, the apparent reversal of CS inhibition by $K^+$ is difficult to demonstrate except at very low CS concentrations, and some workers have considered that the interaction may be less direct (1, 11).

The experiments reported here comprise our initial studies on the interaction of CS with NaATPase of *Electrophorus* electric organ. The electric organ preparation has a high specific activity and a low degree of contamination with other ATPases. ATPase activity and phosphorylation of the preparation by ATP are both more than 95% $Na^+$-dependent (6, 12). The properties of this enzyme are very similar to those of

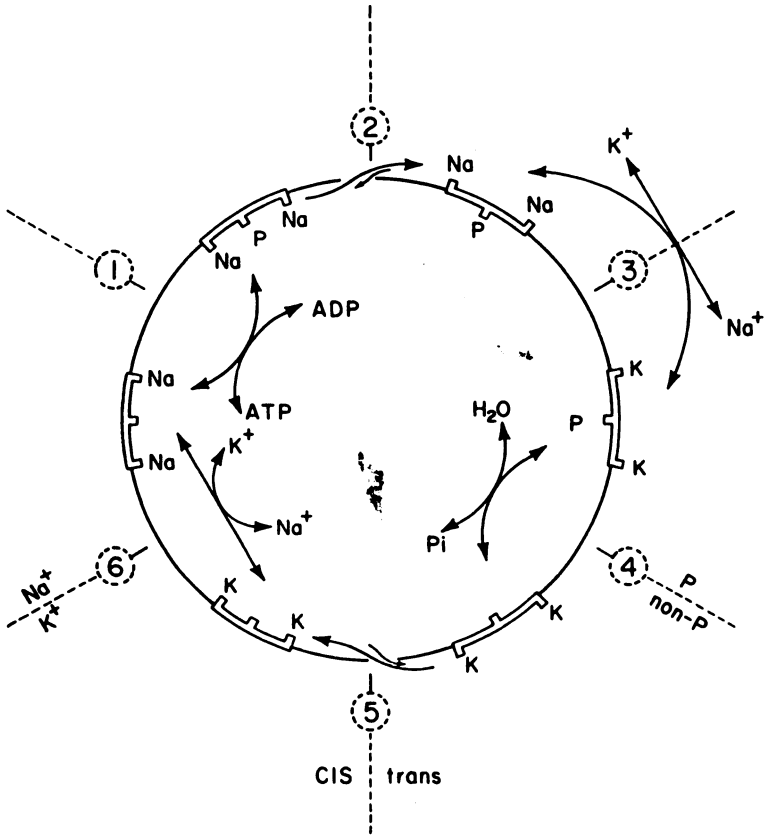


FIG. 1. *Hypothetical mechanism relating NaATPase to active transport of $Na^+$ and $K^+$*

The circle denotes a plasma membrane of which the enzyme is a component. The phosphorylation site is on the cytoplasmic surface of the membrane. The cation effector sites of the enzyme are considered identical with the active transport carrier. The enzyme is denoted as "*cis*" or "*trans*" when the carriers are adjacent or opposite the phosphorylation site.

Reaction 1 is the $Na^+$-dependent ATP-ADP exchange (4–6). In the native enzyme, the phosphorylated *cis* enzyme is rapidly converted to the *trans* form (reaction 2). In the presence of $K^+$, reaction 3 activates the hydrolytic step (reaction 4). The *trans* enzyme becomes less stable as a result of the dephosphorylation and reverts to the *cis* form (reaction 5). The cycle is completed when $Na^+$ displaces $K^+$ from the *cis* enzyme (reaction 6).

mammalian NaATPase preparations. In certain cases we report parallel experiments with NaATPase from cat brain.

## METHODS

*Preparation of NaATPases.* The preparation of enzyme from *Electrophorus* electric organ has been described (13). The cat brain enzyme was prepared as follows.

Whole cat brain was minced and homogenized in 10 volumes of 0.32 M sucrose (pH 7.4 with KOH). Dispersion was accomplished with 10 strokes of a Teflon pestle (0.2 mm clearance) driven at 2300 rpm by a drill press. The crude homogenate was centrifuged for 10 min at 600 *g*, then 15 min at 10,000 *g*. The 10,000 *g* supernatant solution was centrifuged for 1 hr at 20,000 *g*, and the resulting pellet (pII) was treated with NaI as described by Nakao *et al.* (14), except that the washing solution contained no $Na^+$. Determinations of ATPase, $^{32}P$, and $^3H$-ouabain binding to particles were performed within 24 hr on material stored at 5°. All preparative procedures were at 0–5°. The ratio of total $(Na^+-K^+)$-ATPase to non-$Na^+$-dependent activity was increased from 2 to 5 by NaI treatment.

Protein was measured by the method of Lowry *et al.* (15).

*pH stat measurement of ATP hydrolysis.* In unbuffered solution at pH > 7.6, essentially 1 eq of $H^+$ is released upon hydrolysis of 1 mole of ATP (16). The base used as titrant must not alter the enzyme activity. 2-Amino-2-methyl-1-propanol is satisfactory. Its pK is about 9.9, so it is 99% ionized at pH 7.8–7.9.

The usual assay solution contained 2 mM ATP (Tris), 2.5 mM $MgCl_2$, 0.25 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid, 0.5 mg/ml bovine serum albumin, and 1 mM dithiothreitol. Two milliliters of this solution were equilibrated at 25° in the thermostatted reaction vessel of an automatic titrator (Radiometer). The solution was adjusted to pH 7.8–7.9 and subsequently the pH was controlled by the addition of 0.1 N aminomethylpropanol from a 50-$\mu$l syringe (Hamilton) driven by the micrometer syringe drive of the titrator. The reaction was initiated by the addition of 0.1–0.25 unit of NaATPase. NaCl and KCl were added subsequently according to the design of the experiment. Additions of activators and inhibitors were made in volumes of 1–10 $\mu$l.

*Assay of initial rates of ATP hydrolysis.* The rate of formation of ADP was measured by recording the rate of oxidation of NADH spectrophotometrically in the presence of excess phosphoenolypyruvate, pyruvokinase, and lactic dehydrogenase. The assay solution contained 0.2 unit/ml lactic dehydrogenase and pyruvokinase (Sigma type I lactic dehydrogenase), 0.8 mM phosphoenolpyruvate (cyclohexylamine salt), 1 mM ATP (Tris salt), 25 mM KCl, 3 mM $MgCl_2$ in 50 mM Tris-HCl buffered at pH 7.4. The reactions were measured at room temperature for 2 min after addition of 0.01–0.03 unit of NaATPase. NaATPase activity was calculated from the difference between this rate and the rate after addition of 5 $\mu$l of 5 M NaCl (62 mM). Since pyruvokinase requires $K^+$, $K^+$ activation of ATPase cannot be measured by this method. The commercial lactic dehydrogenase contains ammonium sulfate; however, the 4–5 $\mu$moles of $NH_4^+$ from this source do not inhibit the NaATPase.

*Microsome-bound $^3H$-ouabain.* Microsomes, 0.1 mg protein, were incubated with 0.1–0.2 mM $^3H$-ouabain (sp. act. 90 C/mole) in a ratio of 20–40 m$\mu$moles per milligram of protein in various media with final volumes of 20–25 $\mu$l. After indicated intervals at 24°, the mixture was diluted 70-fold in 1.0 ml ice cold water. This was filtered through Millipore (0.45 $\mu$ pore size) paper with suction and washed with 6 ml of cold water. The filter paper was dissolved directly in 10 ml of naphthalene dioxane scintillation counting solution (6) containing 10% (v/v) $H_2O$, and the radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Blanks were obtained by filtering similar quantities of $^3H$-ouabain under the same conditions but without microsomes and were subtracted from the experimental values. Prior experiments showed that the label was not lost from the microsomes during the dilution prior to filtration.

Except where noted, computations of ouabain binding were based on the amount of protein in the stock enzyme suspension, not corrected for the protein loss during the washing procedure.

*Preparation of "ouabain-enzyme."* For experiments which required larger batches of ouabain-treated enzyme, 10–50 mg of electric organ microsomes were incubated with 0.2 mM ouabain and 8 mM $MgCl_2$ at pH 7.5 in Tris-HCl for 90 min or until 95% inhibition of NaATPase was obtained. Then the microsomes were washed three times by suspension in 5 mM dithiothreitol and sedimentation at 100,000 *g*. When $^3$H-ouabain was used, this procedure reduced the radioactivity of the final supernatant solution to 0.5% of that in the pellet.

*Steady-state level of $^{32}$P-protein.* This was determined in 45 sec in the presence of 60 mM NaCl, 3 mM $MgCl_2$, 2 mM ($\gamma$-$^{32}$P-ATP) (sp. act. $6 \times 10^7$ cpm/$\mu$mole) as reported (6). The 4 times washed pellet was finally resuspended in 100 $\mu$l of 5% (w/v) trichloroacetic acid and transferred to the counting vial, where the acid was neutralized with 0.4 ml of 0.1 N NaOH before addition of naphthalene dioxane scintillation counting solution.

Unless otherwise noted, computations are based on the amount of protein in the stock enzyme suspension without correction for protein loss in the washing procedure.

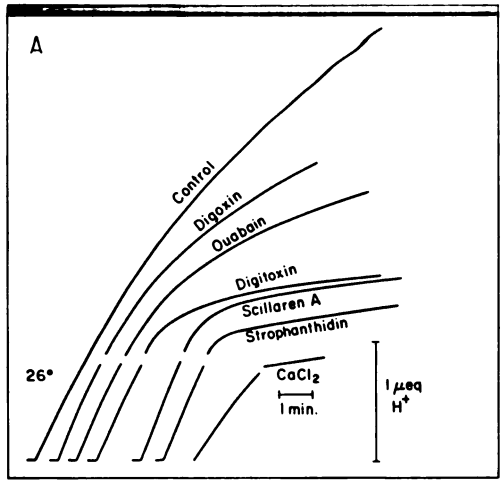


FIG. 2A. *Inhibition of Electrophorus electric organ $Na^+ - K^+$-ATPase by various cardioactive steroids as a function of time*

Rate of hydrolysis of ATP was measured by the pH-stat method. The reaction mixture contained 97 $\mu$g enzyme protein, 1 mg bovine serum albumin, 1 $\mu$mole dithiothreitol, 0.5 $\mu$mole EGTA, 4 $\mu$moles ATP, 50 $\mu$moles NaCl, 5 $\mu$moles KCl, 5 $\mu$moles $MgCl_2$ in a total volume of 2 ml at 26°. The pH was maintained at 7.8 by the addition of 0.10 M aminomethyl propandiol. At the time indicated by the interrupted curve, 20 $\mu$moles of cardioactive steroid was added. The right-hand curve is included from another experiment as a control for the rate of mixing. Conditions were similar except that 48 $\mu$g of enzyme protein was incubated at 30° and 1 $\mu$mole of $CaCl_2$ was added at the break.

## RESULTS

### *The Rate of Inhibition of ATP Hydrolysis*

*pH stat measurements.* The NaATPase reaction in the pH stat is not linear with time, partly because the enzyme denatures slowly in dilute solution and partly because the products are inhibitory. However, the effects of inhibitors may be usefully studied for 10 min or until about 30% of the ATP has been hydrolyzed if the results are compared with appropriate controls.

Addition of $10^{-3}$ M $CaCl_2$ during the course of the reaction decreases the rate instantaneously relative to the time resolution of the method (Fig. 2). Cardioactive steroids added during the course of the reaction produce a time-dependent

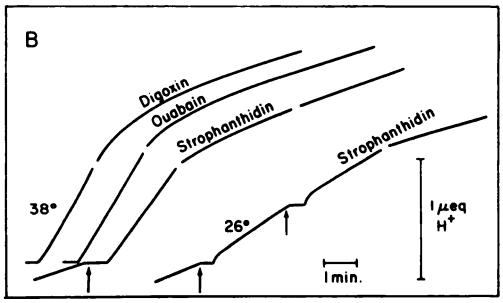


FIG. 2B. *Rate of inactivation of cat brain $Na^+ - K^+$-ATPase by cardioactive steroids*

Conditions were similar to those of Fig. 2A, except that 190 $\mu$g of cat brain protein was used. The amount of $Mg^{2+}$-ATPase can be estimated from the strophanthidin curves, in which the KCl was added at the times indicated by arrows.

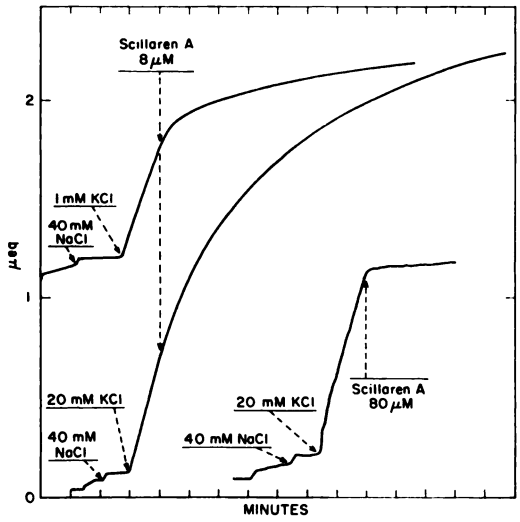


FIG. 3. *The influence of $K^+$ on rate of inactivation of eel $Na^+ - K^+$-ATPase by scillaren A*

Experimental conditions were similar to those of Fig. 2 except for the concentrations of $Na^+$ and $K^+$.

inhibition which progresses to essentially complete inhibition if the steroid concentration is sufficient (Figs. 2 and 3).

The time-dependence is a function of

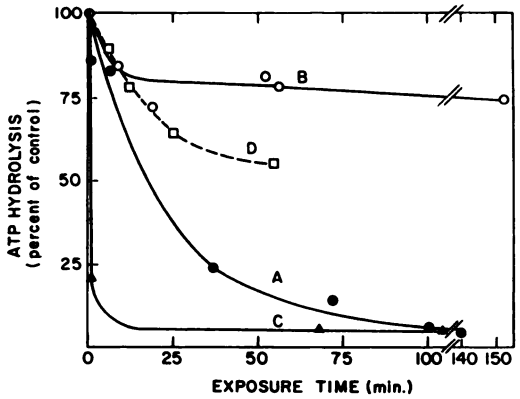


FIG. 4. *Inhibition of ATPase by exposure of microsomes to 0.2 mM ouabain (40 mμmoles/mg protein) for varying intervals at 24°*

After exposure, the enzyme was diluted 400-fold for determination of initial rates as in Table 1. Exposure media: ●—●, A, 3 mM $MgCl_2$, 60 mM Tris-HCl (pH 7.4); ○—○, B, medium A plus 60 mM NaCl; ▲——▲, medium B plus 2 mM ATP. Curve D (□- - -□) was a separate experiment in which enzyme was incubated in 30 mM Tris-HCl (pH 7.4) with 1.3 mM ouabain (150 mμmoles/mg protein).

the nature of the steroid, its concentration, and the concentrations of $Na^+$ and $K^+$.

*Effectors that modify the rate of enzyme-steroid interaction.* The spectrophotometric assay requires only 2–5 μg of electric organ

TABLE 1

*Cation and nucleotide effects on ouabain inhibition of $(Na^+ + K^+)$-ATPase*

Microsomes were exposed to ouabain in 0.03 M Tris HCl (pH 7.4) alone or in media containing 3 mM $MgCl_2$, 40 mM NaCl and/or nucleotide, as indicated. In experiment A, the nucleotide concentration was 2 mM; in B, 1.4 mM. Tris EDTA, 0.1 mM, was present in all. The treated enzyme was diluted 400-fold for assay of initial rates of $Na^+$-stimulated ATP hydrolysis. Ouabain concentration was 0.2 mM in A and B; 0.05 mM in C. Protein concentration was 2.2 mg/ml in A and B; 4.4 mg/ml in C.

| Exposure medium | Percent inhibition: 5 min; 24° | 10 min; 0° |
|---|---|---|
| A. Buffer | 0 | 2 |
| $Na^+$ | 11 | — |
| $Mg^{2+}$ | 57 | — |
| $Na^+$, $Mg^{2+}$ | 17 | 2 |
| ATP | 0 | — |
| $Na^+$, ATP | 94 | 26 |
| $Mg^{2+}$, ATP | 88 | 26 |
| $Na^+$, $Mg^{2+}$, ATP | 94 | 90 |
| ADP | 0 | — |
| $Na^+$, ADP | 31 | — |
| $Mg^{2+}$, ADP | 92 | — |
| $Na^+$, $Mg^{2+}$, ADP | 95 | 87 |
| B. Buffer | 0 | 0 |
| UTP, $Mg^{2+}$, $Na^+$ | 88 | 83 |
| GTP, $Mg^{2+}$, $Na^+$ | 93 | 40 |
| CTP, $Mg^{2+}$, $Na^+$ | 90 | 85 |
| ATP, $Mg^{2+}$, $Na^+$ | 94 | 90 |
| | 15 min, 26° | |
| C. Adenosine, $Mg^{2+}$, $Na^+$ | 21 | |
| 5′-AMP, $Mg^{2+}$, $Na^+$ | 12 | |
| ATP, $Mg^{2+}$, $Na^+$ | 90 | |

enzyme protein. Thus a drug which reacts slowly with the enzyme can be incubated with a high concentration of the enzyme in a small volume. The time course of the drug-enzyme interaction can be followed by diluting a small portion of the incu-

TABLE 2

*Irreversible inhibition of eel ATPase by ouabain*

A. Electroplax microsomes, 1.3 mg protein, were treated with ouabain, 0.12 μmole/mg protein (1.2 mM), in 2 mM ATP, 3 mM $MgCl_2$, 50 mM NaCl, in a volume of 0.14 ml for 45 min at pH 7.4 0°. The enzyme was then suspended in 10 ml of wash solution (5 mM dithiothreitol, 1 mM $MgCl_2$) and centrifuged for 30 min at 0°, 100,000 *g*. This procedure was performed 3 times. Controls contained no ouabain. Initial rates were determined as in Table 1.

B. Electroplax microsomes, 1.3 mg protein, were treated for 60 min at 0°, pH 7.4, in 0.2 ml of indicated media. Concentrations: ouabain, 0.23 μmoles/mg protein (1.5 mM), 2 mM ATP, 3 mM $MgCl_2$, 50 mM NaCl. Samples were assayed and washed as in Table 2A.

| Pretreatment | Specific activity (μmoles ATP/mg/min) | | | |
|---|---|---|---|---|
| | Before wash | After wash | | |
| | | I | II | III |
| A. Ouabain, $Na^+$, $Mg^{2+}$, ATP | 0.208 | 0.354 | 0.294 | 0.369 |
| $Na^+$, $Mg^{2+}$, ATP | 5.04 | 6.16 | 3.54 | 3.87 |
| B. $Na^+$, $Mg^{2+}$, ATP | 4.60 | — | 6.30 | — |
| Ouabain alone | 1.71 | — | 0.98 | — |
| Ouabain, $Na^+$, $Mg^{2+}$, ATP | 0.20 | — | 0.46 | — |

TABLE 3

*Stability of microsome-bound $^3H$-ouabain*

Microsomes were labeled with $^3H$-ouabain as described in the text, then washed and collected by ultracentrifugation. The labeled microsomes in A, B, and C contained 0.655 mμmole of ouabain per milligram of protein. They were exposed to the indicated media and collected by washing on Millipore filters of 0.45-μ pore size. The concentrations used were 0.05 M Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 60 mM NaCl, 2 mM ATP, 1 mM ouabain.

In experiment D, the labeled microsomes contained 0.503 mμmole of ouabain per milligram of protein. The counts per minute are given for a 15-μl sample containing 76.5 μg of protein. From control experiments, 30–40% of the protein passes through the Millipore. The amount retained is reproducible for a given enzyme suspension.

| Conditions | $^3H$-ouabain bound (cpm) | Percent of control |
|---|---|---|
| A. Incubated at 26° for 30 min in indicated media. Washed with water on Millipore | | |
| $H_2O$ | 1630 | 65 |
| Tris-HCl, ouabain | 1718 | 69 |
| Tris-HCl, $MgCl_2$, NaCl, ATP, ouabain | 1715 | 69 |
| Tris-HCl | 1855 | 74 |
| Trichloroacetic acid, 5% (w/v) | 35 | — |
| B. Incubated at 100° for 3 min in $H_2O$; washed with water on Millipore | 51 | — |
| C. Filtered and washed on Millipore with indicated solutions. No incubation | | |
| $H_2O$ | 1862 | 74 |
| Tris-HCl, $MgCl_2$, NaCl, ATP, ouabain | 1747 | 70 |
| Untreated control for A, B, and C | 2830 | 100 |
| D. Incubated in 10 volumes of methanol at 5° for 15 min. Centrifuged. Radioactivity measured in the methanol supernatant and subtracted from the control | 135 | 4 |
| Untreated control for D | 3205 | 100 |

bation mixture several hundredfold in the spectrophotometric assay reagent. Sufficient dilution will essentially stop further interaction during the 1 or 2 min required for the assay. If ouabain is diluted to $<4 \times 10^{-6}$ M, its effect on the initial velocity is negligible. Subsequently described experiments demonstrate that dissociation of the drug-enzyme complex does not occur to a significant extent as a result of the dilution.

Table 1 presents data obtained in this manner. Interaction of ouabain with NaATPase is extremely slow in metal-free Tris buffer at pH 7.4. $Na^+$ and $Mg^{2+}$ each accelerate the interaction. However, $Na^+$ and $Mg^{2+}$ in combination are much less effective than $Mg^{2+}$ alone (see also Fig. 4). ATP and ADP are without effect in the absence of metal activators. These nucleotides are synergistic with both $Mg^{2+}$ and $Na^+$ and the combination of $Na^+$, $Mg^{2+}$, and ATP is more effective than any pair. In contrast to the high specificity for ATP of the hydrolysis, exchange, and phosphorylation reactions (12, 14, 17), other nucleoside triphosphates are nearly as effective as ATP in accelerating the ouabain interaction rate. 5'-AMP and adenosine are ineffective in combination with $Na^+ + Mg^{2+}$.

### *Stability of the Ouabain-NaATPase Complex*

*Attempts to reactivate NaATPase by repeated washing.* After incubating with 1.2 mM ouabain, $Na^+$, $Mg^{2+}$ and ATP, NaATPase activity was reduced to 4.1% of the control enzyme incubated without ouabain (Table 2A). After the enzyme was washed three times by sedimentation and resuspension, the ouabain-treated enzyme had 9.5% as much activity as the control. The incomplete inhibition obtained in the absence of activators was also reversed by this washing procedure (Table 2B).

*Stability of enzyme-bound $^3$H-ouabain.* Microsomes were labeled with $^3$H-ouabain and collected by sedimentation as described under Methods. In the experiments of Table 3, electric organ microsomes bound 0.655 mμmole of ouabain per milligram of protein. The ouabain-enzyme complex is relatively stable to incubation in water or neutral Tris buffer at 26°. The bound radioactive steroid, $10^{-7}$ M, does not exchange with $10^{-3}$ M nonradioactive ouabain, either in neutral Tris or in Tris + ($Na^+$, $Mg^{2+}$, and ATP). However, the complex

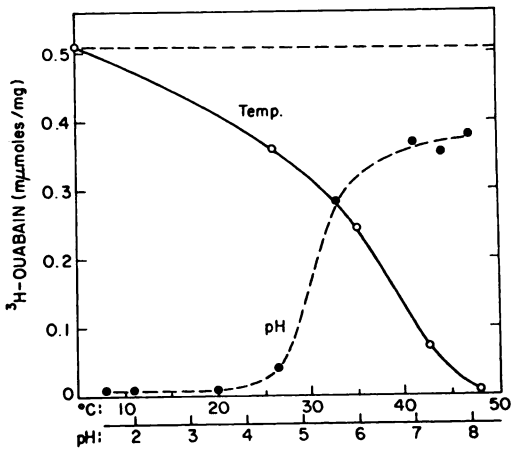


FIG. 5. *Stability of bound ouabain as a function of temperature and pH*

Electric organ microsomes were labeled with $^3$H-ouabain as described in Table 5. Portions containing 0.1 mg protein were incubated in 30-μl volumes, then washed on 0.45-μ filters (see Methods). The temperature stability data were obtained by incubating the samples for 40 min in water. The pH stability data were obtained by incubating the samples for 30 min at 26° in buffers fo Tris-HCl, Tris-acetate, or dilute trichloroacetic acid.

is dissociated by exposure to acid, boiling, or extraction with methanol. A more detailed study of the stability of the complex as a function of pH and temperature is presented in Fig. 5.

### *Effect of Ouabain Binding on the Extent of Phosphorylation of the Enzyme by ATP*

Since phosphorylation of NaATPase by ATP is a rapid reaction, even at 0°, whereas conditions may be chosen to make the rate of interaction with ouabain slow, it is possible to measure the extent to which the enzyme may be phosphorylated as a function of the extent of the ouabain interaction (Fig. 6). The $^{32}$P incorporation decreases as the $^3$H-ouabain binding increases. However, when hydrolysis has been reduced to 6% of the control rate,

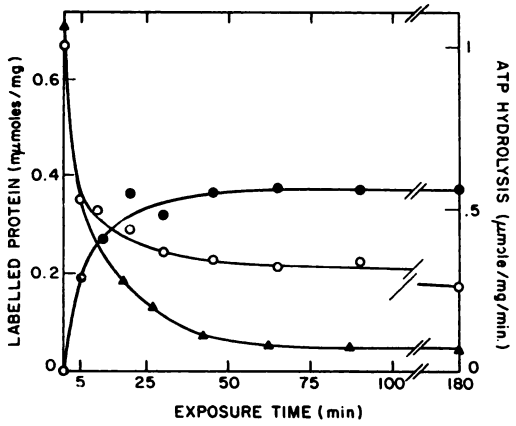


FIG. 6. *Comparison of $^3$H-ouabain labeling of microsomes with inhibition of ATP hydrolysis and of $^{32}$P-incorporation*

Microsomes were exposed to 0.2 mM $^3$H-ouabain (40 mμmoles/mg protein) in 3 mM $MgCl_2$, 60 mM Tris-HCl (pH 7.4) at 24°. At indicated intervals, appropriate portions were removed for assay of initial rates of ATP hydrolysis and steady-state level of $^{32}$P-protein and for filtration on Millipore. Maximum $^{32}$P-protein in the absence of $Na^+$ was 10% of the total $^{32}$P bound by untreated enzyme. O—O, protein-bound $^{32}$P; ●—●, microsome-bound $^3$H-ouabain; ▲——▲, $Na^+$-activated ATP hydrolysis.

$^{32}$P incorporation was only reduced to 25% of the control. The enzyme bound 0.37 mμmole of ouabain per milligram of protein after exposure times longer than 45 min.

TABLE 4

*Maximum binding of $^{32}$P- and $^3$H-ouabain*

Preparation A was made from electric organ tissue which had been stored in liquid nitrogen for 10 months; the microsomes had been stored for 4 months. Preparation B was made from tissue stored 6 days; these microsomes were stored 5 days in liquid nitrogen before this experiment.

Labeling with $^3$H-ouabain and AT$^{32}$P are described in Methods. Protein was measured on aliquots of the preparations after the labeling procedure.

| Enzyme preparation | $^{32}$P incorporated | $^3$H-ouabain bound | $^3$H/$^{32}$P |
|---|---|---|---|
| *Electrophorus* enzyme A | 1.30[a] | 0.655 | 0.503 |
| *Electrophorus* enzyme B | 1.01 | 0.503 | 0.498 |
| Cat brain | 0.136 | 0.140 | 1.03 |

[a] Values are expressed as millimicromoles per milligram of proteins.

Phosphorylation declined from an initial 0.65 mμmole/mg to about 0.2 mμmole/mg. Thus one molecule of bound ouabain appears to have prevented the phosphorylation of 1.2–1.4 sites on the enzyme.

TABLE 5

*Effect of various agents on ouabain binding*

Electric organ microsomes containing 0.1 mg of protein were incubated for 1 min at 25° in media containing 0.1 mM $^3$H-ouabain, 60 mM Tris-HCl buffer (pH 7.4), and other components as indicated. Unless specified, compounds added were 2 mM. The reaction was terminated by dilution and filtration on Millipore as described in the text.

A. 3 mM $MgCl_2$ + 60 mM NaCl

| Addition | mμmoles ouabain/mg protein |
|---|---|
| — | 0.08 |
| EDTA | 0.08 |
| Tris-$PO_4$ | 0.09 |
| ATP | 0.49 |
| ADP | 0.48 |
| CTP | 0.52 |
| GTP | 0.48 |
| UTP | 0.48 |

B. 3 mM $MgCl_2$

| Addition | mμmoles ouabain/mg protein | |
|---|---|---|
| | −NaCl | +50 mM NaCl |
| — | 0.05 | 0.03 |
| Tris-$PO_4$ | 0.22 | 0.05 |
| Tris-ADP | 0.10 | 0.33 |
| Tris-ATP | 0.28 | 0.41 |

C. 3 mM $MgCl_2$ + 2.5 mM Tris-$PO_4$

| mM KCl | mμmoles ouabain/mg protein |
|---|---|
| 0 | 0.26 |
| 0.5 | 0.23 |
| 5 | 0.16 |
| 25 | 0.14 |
| 50 | 0.13 |
| 100 | 0.10 |

D.

| mM $MgCl_2$ | mμmoles ouabain/mg protein |
|---|---|
| 0 | 0.04 |
| 1.25 | 0.16 |
| 2.5 | 0.20 |
| 5.0 | 0.20 |
| 10.0 | 0.21 |
| 2.5[a] | 0.42 |

[a] Incubated 10 min at 25°

### *The Relationship of Enzyme Activity to Ouabain Binding and Phosphorylation*

The electric organ NaATPase activity is not stable to incubation at 38°. Residual phosphorylation, ouabain binding, and enzyme activity were measured as a function of time of incubation at 38° (Fig. 7). All three properties of the microsomal preparation decreased, 30%, 40%, and 30%, respectively, after 2 hours.

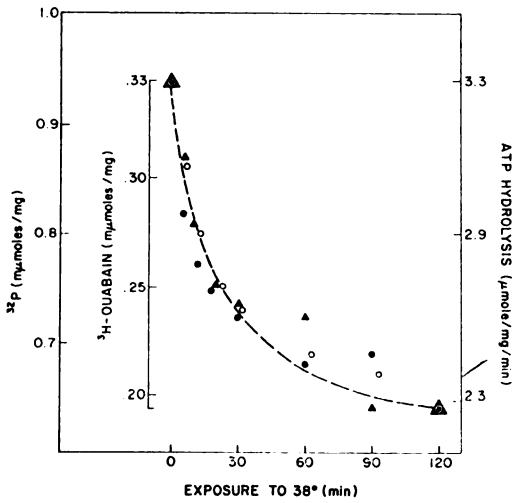


FIG. 7. *Rate of heat inactivation of ATPase, $^{32}$P-binding, and $^{3}$H-ouabain labeling activities*

Microsomes, 5 mg protein/ml in 50 mM Tris-HCl (pH 7.4), were incubated at 38°. At various intervals, appropriate portions were removed for assay of residual activities as described in the text. Maximum $^{32}$P-binding in the absence of $Na^+$ was 10% of the total at zero time. ●—●, $^{32}$P-protein; O—O, microsome-bound $^{3}$H-ouabain; ▲——▲, initial rates of $Na^+$-activated ATP hydrolysis.

### *Stoichiometry of the Ouabain-Enzyme Interaction*

*Experiments with nonradioactive ouabain and scillaren* A. Most preparations of electric organ microsomes incorporate about 1 mμmole of phosphate from ATP per milligram of protein, of which 95% is sodium dependent. Inhibition of NaATPase is demonstrable in the presence of less than $10^{-7}$ M cardioactive steroid. If enzyme concentrations of the order of 1 mg/ml are used, one may expect that at concentrations less than $10^{-6}$ M, inhibition will be limited by the amount of ouabain available. This is the rationale of the experiment of Fig. 8 which relates fractional inhibition to the ratio (total steroid) : (total microsomal protein). Conditions were chosen to promote maximum binding of the steroids. At concentrations of steroid which inhibited 70% or less, the relation between total CS and inhibition is nearly linear. The linear part of the curve extrapolates to 1.9 mμmoles/mg for ouabain and 3.0 mμmoles/mg for scillaren A.

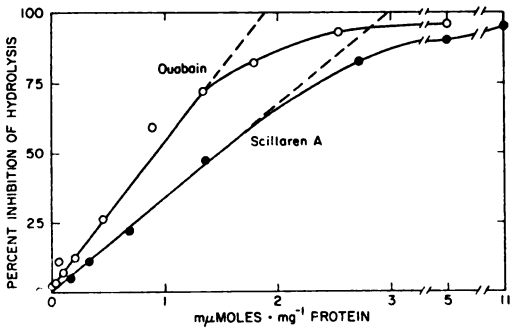


FIG. 8. *Irreversible inhibition of electroplax ($Na^+$ + $K^+$)-ATPase after preincubation with glycosides*

Eel microsomes, 0.06 mg protein, were treated with glycosides ($3 \times 10^{-8}$M to $2 \times 10^{-5}$M) in media of constant volume containing 40 mM NaCl, 2.7 mM $MgCl_2$, 30 mM Tris-HCl (pH 7.4) plus 1.7 mM ATP in the case of ouabain or ADP in that of scillaren A at 24° for 4 hr. Scillaren experiments were in 0.12 M dimethylsulfoxide. The treated enzyme was diluted 400-fold for assay: $Na^+$-increments in ATP hydrolysis over the first minute were obtained at 24° in 50 mM Tris-HCl (pH 7.4) 1 mM ATP, 25 mM $MgCl_2$, and 80 mM NaCl added last. These were compared to controls assayed in glycoside concentrations corresponding to those of diluted experimental samples.

*Experiments relating $^{3}$H-ouabain binding to $^{32}$P incorporation.* Several of the experiments already described allow one to calculate the ratio of bound ouabain to phosphate incorporation. In Fig. 6, the ratio of maximal ouabain bound to maximal $^{32}$P is 0.37/0.70 = 0.53. From Fig. 7, the ratio calculated from total binding with the control sample is 0.33/0.92 = 0.36, and that calculated from the decrement after 2 hr at 38°, 0.14/0.27 = 0.52. In these ex-

periments, protein was measured only in the stock enzyme suspension.

The data from two preparations of electric organ enzyme are presented in Table 4. In these experiments, connections were obtained for protein loss during the washing and filtration steps of the labeling procedures. These losses were about 30% in both the ouabain and phosphate procedures. The data confirm a ratio of one ouabain bound per two phosphorylation sites in the case of electric organ NaATPase.

Comparable data from a cat brain NaATPase yield a ratio of one (Table 4). This preparation has less than 0.1 the specific activity of the eel enzyme, and no adequate control has been devised for nonspecific ouabain binding.

### *Effect of Orthophosphate on Ouabain Binding*

Schwartz and Matsui have found that inorganic phosphate increases the binding of ouabain to heart muscle NaATPase and that $Na^+$ opposes this effect.[2] Table 5, A and B, extends this observation to the electric organ NaATPase. Table 5C demonstrates that $K^+$ retards interaction with ouabain in the presence of phosphate as it does in the presence of $Na^+$ and ATP (Fig. 3). Table 5D indicates that $Mg^{++}$ is required for the phosphate activation of ouabain binding.

### *Effect of Ouabain on Orthophosphate Incorporation into Microsomes*

Electric organ microsomes, containing 0.1 mg protein, were incubated 10 min at 24° in 30 $\mu$l containing 0.1 mM ouabain, 0.5 mM Tris-$^{32}PO_4$ ($3 \times 10^5$ cpm/$\mu$mole), 3 mM $MgCl_2$ and 60 mM Tris-HCl (pH 7.4). These microsomes incorporated 0.35 m$\mu$mole of $^{32}P$ per milligram of protein which was stable to acid and, after acid denaturation, did not exchange with nonradioactive phosphate in the washing solution. Control samples, incubated in the same way except without ouabain, incorporated 0.04 m$\mu$mole of $^{32}P$ per milligram of protein.

[2] Personal communication.

In another experiment, electric organ microsomes were reacted with ouabain, as described in Methods, and then exposed to 0.6 mM $^{32}P_i$, 3 mM $MgCl_2$, and 45 mM Tris-HCl (pH 7.5). Under these conditions the same amount of $^{32}P$ ($0.299 \pm 0.004$ m$\mu$mole/mg) was incorporated during 0.5 and 1 min at 5° and 10 min at 25°. This level of incorporation was not changed by the presence of 0.1 M KCl.

TABLE 6

*Incorporation of $^{32}P_i$ into ouabain-treated microsomes*

Ouabain-treated microsomes from electric organ (79 $\mu$g of protein) were incubated for 10 min at 0° with the indicated concentration of orthophosphate as the Tris salt, 3.3 mM $MgCl_2$ and 45 mM Tris-HCl (pH 7.5) in 30 $\mu$l. Each tube contained $1.28 \times 10^6$ cpm $^{32}P_i$. The preparation of the ouabain-microsomes and the procedure for measuring phosphate incorporation are described in Methods. The microsomes were the same preparation as "A" in Table 4.

| $P_i$, mM | Phosphate (m$\mu$moles per mg microsomal protein) |
|---|---|
| 0.04 | 0.16 |
| 0.20 | 0.30 |
| 1.0 | 0.56 |
| 5.0 | 0.85 |

Table 6 demonstrates that as the phosphate concentration is increased, the level of incorporation approaches the level of $Na^+$-dependent incorporation from ATP (Table 4).

## DISCUSSION

The binding of digoxin to a membrane preparation containing NaATPase activity was first demonstrated by Matsui and Schwartz (8) using $^3H$-digoxin and a preparation from heart muscle. They demonstrated that digoxin binding was increased from 2 $\mu\mu$moles to 30 $\mu\mu$moles per milligram of protein by the addition of $Na^+$, $Mg^{2+}$, and ATP together. The nucleotide effect was shown to be nonspecific, and the activating effect of $Na^+$ was diminished by $K^+$. These observations are qualitatively consistent with ours. Evidence that ouabain binds to a NaATPase preparation from

rat brain was reported by Ahmed and Judah (18).

We have attempted to evaluate the quantitative relationship of ouabain binding sites to $Na^+$-dependent phosphorylation sites and to NaATPase activity. In the absence of pure enzyme preparations these relationships are provisional; however, they may be tested by comparing the properties of enzymes from different sources and of different specific activities. Catalytic center activities at 37° have been estimated from a summary of current data (3). The majority of preparations will hydrolyze between 5000 and 15,000 moles of ATP per minute per equivalent of $Na^+$-dependent $^{32}P$ incorporation. Of the preparations for which CS binding data are available, the values are 13,600 for heart NaATPase, 14,700 for electric organ ATPase, and (from Table 4, assuming $Q_{10} = 2.3$) 5800 for cat brain NaATPase. The corresponding ratios of CS binding to $^{32}P$ incorporation are 0.9 (8), 0.5, and 1.0. Thus while the specific activities of these enzymes vary by 20-fold, the range of catalytic center activities is 2.5 and of steroid/phosphate ratios is 2. The correlation of both $Na^+$-dependent $^{32}P$ corporation and CS binding with NaATPase activity is good although the data are limited. At present we cannot decide whether the ratio of CS/$^{32}P$ binding in electric organ is significantly different from that in the mammalian enzymes. The distinctive effects of cations and nucleotides on rates of CS binding may be useful in further study of this problem. A comparison of Fig. 8 and Table 4 demonstrates the existence of nonspecific CS binding by electric organ microsomes which is not stable to the washing procedure. Similarly, the calculations of Ahmed and Judah (18) indicate that rat brain NaATPase binds 2.5 ouabain per phosphate under conditions which do not distinguish between reversible and irreversible binding.

The CS/$^{32}P$ binding ratio will be of interest in studies of the substructure of the enzyme. Two laboratories have applied X-ray inactivation technique to estimate the molecular weight of erythrocyte NaATPase: The estimates are 500,000 (19), and 1,000,000 (20). A preparation from guinea pig brain, solubilized with detergent, has an estimated molecular weight of 670,000 which was derived from gel filtration data (21). If the lowest estimate of 500,000 is taken as the combining unit for ouabain in electric organ NaATPase, the purity of the preparations reported in Table 4 is greater than 25%.

We are primarily interested in the CS interaction for the information that may be derived about the mechanism of NaATPase and $Na^+$ transport. The results of earlier studies (4–6, 13, 17) have been rationalized in terms of the model given in Fig. 1. We may infer from the physiological requisites for active transport that NaATPase is more complex than any of the models of allosteric enzymes which have received theoretical treatment (22–26). Energy from one molecule of ATP is transformed to vectorial work on two or three Na and K ions (27–29). This distribution of energy would seem to require the interaction of nonidentical protomers (22). Also in contradistinction to its role in most enzymes, the allosteric transition when equated with the operation of a transport carrier does not simply regulate catalytic activity, but constitutes the primary function of the system. Each molecule of ATP hydrolyzed corresponds to one cycle of the allosteric transition. Jencks has discussed "the case of the oscillating enzyme" (Fig. 7 of ref. 30), which has many of the features of the model of Fig. 1. However, in the present case the oscillations are driven by a chemical reaction, whereas in Jencks' model the oscillations catalyze the reaction.

Several kinetic studies have indicated that NaATPase has allosteric properties (10, 31, 32). Such studies are complicated by the requirement of multiple activators and the likelihood of competitive effects between $Na^+$ and $K^+$. However, distinct homotropic activation by $Na^+$ is evident in the phosphorylation of NaATPase by AT$^{32}P$ in the absence of $K^+$ (6, 17).

Although the action of CS has been proposed to relate to $K^+$ activation (7, 10), these studies have not recognized the irreversibility of CS binding, which invalidates the concept of a $K_I$ for these inhibitors.

The slow rate of combination of CS with NaATPase may result from a steric barrier to the CS effector site. Combinations of other effectors may accelerate or retard CS interaction as they loosen or tighten this barrier. However, once formed, the CS enzyme complex is extremely stable: it does not equilibrate with CS in the medium (Table 3A).

An emerging principle of tertiary protein structure is that the separation of nonpolar segments of peptide chains into the interior of the molecule is an important force for stability (33). Monod *et al.* suggest that this is also important in stabilizing oligomers (22). Lipids per se may be integral components of some enzyme systems, particularly of those associated with membranes (34).

The rate of interaction of cardioactive steroids is inversely related to the number of hydroxyl and sugar substituents (Fig. 2). This relationship and the ease of removal of bound ouabain by methanol extraction indicate that the interaction may be lipophilic. Since ouabain binding is also destroyed by heat or acid treatment of the enzyme, lipoprotein or nonpolar regions of a peptide chain may be involved. The major structural characteristic of cardioactive steroids is the *cis*-configuration of the C-D ring juncture. This forms a rather rigid nonpolar concavity on the $\alpha$-surface of the ring. Portius and Repke postulate that the steroid develops hydrophobic bonds with a complementary protein surface (9). They also propose that the $\alpha$-$\beta$ unsaturated carbonyl of the lactone ring forms a hydrogen bond with the phosphorylated enzyme. However, our data show that the enzyme is inhibited by ouabain at a comparable rate in the presence of either ATP or ADP (Table 1) and thus, whether phosphorylated or not.

The combination of ouabain with NaATPase modifies the enzyme so that it can be phosphorylated by orthophosphate (Table 6).[3] This observation is probably the key to understanding the action of cardioactive steroids on this enzyme. The native enzyme does not incorporate measurable amounts of orthophosphate (35). In the presence of 5 mM orthophosphate, the extent of phosphorylation of the ouabain-treated enzyme is comparable to the maximal phosphorylation of the native enzyme by ATP (Table 4). There is, thus, reason to assume that the phosphorylation sites will prove to be chemically identical. This point is currently under investigation. Preliminary experiments demonstrate that the pH-stability curve of $^{32}P$ incorporated into ouabain-treated enzyme from $^{32}P$ is similar to that of $^{32}P$ incorporated into native enzyme from $AT^{32}P$.

In terms of the model (Fig. 1), $K^+$ catalyzes step 4, i.e., reduces the activation energy of this step. However, $K^+$ does not catalyze $P_i$ incorporation into the native enzyme (35), is not required for $P_i$ incorporation into ouabain-treated enzyme, nor does it change the extent of phosphorylation of ouabain-treated enzyme by $P_i$. Thus, although ouabain abolishes the $K^+$ effect on step 4, this is not sufficient explanation of its action. It must, in addition, reduce the free energy difference between the phosphorylated and the nonphosphorylated forms of the enzyme. If ouabain does not change the chemical nature of the phosphate acceptor, the free energy change must be attributed to the conformational potential of the system: the combination of cardioactive steroid with NaATPase may remove a constraint on the structure of the enzyme which is normally a consequence of phosphorylation.

REFERENCES

1. I. M. Glynn, *Pharmacol. Rev.* **16**, 381 (1964).
2. K. Repke, *Drugs and Enzymes, Proc. 2nd Intern. Pharmacol. Meeting, Prague, 1963* p. 65. Czech. Medical Press, Prague, 1965.

[3] Ouabain treatment also induces orthophosphate incorporation into NaATPase prepared from calf brain (G. E. Lindenmayer, A. H. Laughter, and A. Schwartz, personal communication).

3. R. W. Albers, *Ann. Rev. Biochem.* **36**, 727 (1967).
4. S. Fahn, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **241**, 1882 (1966).
5. S. Fahn, M. R. Hurley, G. J. Koval and R. W. Albers, *J. Biol. Chem.* **241**, 1890 (1966).
6. G. J. Siegel and R. W. Albers, *J. Biol. Chem.* **242**, 4972 (1967).
7. J. S. Charnock, A. Rosenthal and R. Post, *Australian J. Exptl. Biol.* **41**, 675–686 (1963).
8. H. Matsui and A. Schwartz, *Federation Proc.* **26**, 398 (1967).
9. H. J. Portius and K. Repke, *Arzneimittel-Forsch.* **14**, 1073 (1964).
10. K. Ahmed, J. D. Judah and P. G. Scholefield, *Biochim. Biophys. Acta* **120**, 351–360 (1966).
11. J. F. Hoffman, *Am. J. Med.* **41**, 666 (1966).
12. R. W. Albers and G. J. Koval, *Life Sci.* **1**, 219 (1962).
13. R. W. Albers, S. Fahn and G. J. Koval, *Proc. Natl. Acad. Sci. U.S.* **50**, 474 (1963).
14. T. Nakao, Y. Tashima, K. Nagano and M. Nakao, *Biochem. Biophys. Res. Commun.* **19**, 755 (1965).
15. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
16. I. Green and W. H. F. M. Mommaerts, *J. Biol. Chem.* **202**, 541 (1953).
17. S. Fahn, G. J. Koval and R. W. Albers, *J. Biol. Chem.* in press.
18. K. Ahmed, and J. D. Judah, *Can. J. Biochem.* **43**, 877 (1965).
19. M. Nakao, N. Mizuno, K. Nagano, T. Nakao, M. Fujita and Y. Tashima, *Abstr. 7th Intern. Congr. Biochem. Tokyo, 1967* Vol. 2, p. 203. The Science Council of Japan, Ueno Park, Tokyo, 1967.
20. G. Kepner and R. Macey, *Biochem. Biophys. Res. Commun.* **23**, 202 (1966).
21. F. Medzihradsky, M. Kline and L. Hokin, *Arch. Biochem. Biophys.* **121**, 311 (1967).
22. J. Monod, J. Wyman and J. Changeux, *J. Mol. Biol.* **12**, 88 (1965).
23. J. Changeux, J. Thiery, Y. Tung and C. Kittel, *Proc. Natl. Acad. Sci. U.S.* **57**, 335 (1967).
24. D. E. Atkinson, *Ann. Rev. Biochem.* **35**, 85 (1966).
25. C. Frieden, *J. Biol. Chem.* **242**, 4045 (1967).
26. J. E. Haber and D. E. Koshland, Jr., *Proc. Natl. Acad. Sci. U.S.* **58**, 2087 (1967).
27. A. K. Sen and R. L. Post, *J. Biol. Chem.* **239**, 345 (1964).
28. R. Whittam and M. E. Ager, *Biochem. J.* **97**, 214 (1965).
29. P. J. Garrahan and I. M. Glynn, *J. Physiol. (London)* **192**, 217 (1967).
30. W. P. Jencks, *in* "Current Aspects of Biochemical Energetics" (N. Kaplan and E. Kennedy, eds.), p. 273. Academic Press, New York, 1966.
31. R. F. Squires, *Biochem. Biophys. Res. Commun.* **19**, 27 (1965).
32. J. D. Robinson, *Biochemistry* **6**, 3250 (1967).
33. H. F. Fisher, *Proc. Natl. Acad. Sci. U.S.* **51**, 1285 (1964).
34. S. Fleischer, B. Fleischer and W. Stoeckenius, *J. Cell Biol.* **32**, 193 (1967).
35. R. L. Post, A. K. Sen and A. S. Rosenthal, *J. Biol. Chem.* **240**, 1437 (1965).