The fluorescent maleimide derivatives, 2-(4'-maleimidylanilino)naphthalene 6-sulfonic acid (Mal-ANS) and N-(1-pyrene)-maleimide (Mal-pyrene), both alkylate sulfhydryl groups on the α subunit of the (Na,K)-ATPase to inhibit (Na,K)-ATPase and p-nitrophosphoryl phosphatase activities and phosphoenzyme formation. Reaction of the enzyme with Mal-pyrene, but not with Mal-ANS, also inhibits MgP- and Mg-ATP-Na-supported [3H]ouabain-binding to the enzyme.

Mal-pyrene and Mal-ANS react, in part, with different sulfhydryl groups on the enzyme protein. On the average, the sulfhydryl groups which react with Mal-pyrene are located in a more shielded or hydrophobic environment than are those which react with Mal-ANS. It is the reaction of Mal-pyrene with sulfhydryl groups, which are not accessible to Mal-ANS, that results in the decreased [3H]ouabain-binding capacity of the (Na,K)-ATPase.

The results indicate that phosphorylation of (Na,K)-ATPase is not required for Mg-ATP-Na-stimulated ouabain binding, and suggest that the ATP and sodium sites which modulate the interaction of ouabain with the (Na,K)-ATPase may be different from those which promote phosphorylation.

Enzyme Assays—(Na,K)-ATPase activity was measured at 37 °C using the spectrophotometric linked enzyme assay as described previously (14), in a medium containing 25 mM histidine-Cl, pH 7.4, 5 mM MgCl₂, 5 mM Na₂ATP (Boehringer), 100 mM NaCl, 10 mM KCl, 1 mM phosphoenolpyruvate, 0.4 mM NADH, and pyruvate kinase and lactate dehydrogenase (20 μg, Sigma). Greater than 99% of the ATPase activity was inhibited by the addition of 2 x 10⁻⁴ M ouabain. Potassium-dependent p-nitrophosphoryl phosphatase activity was measured at 37 °C by the continuous measurement of the liberation of p-nitrophenol at 410 nm in a medium containing 25 mM histidine-Cl, pH 7.4, 5 mM MgCl₂, 4 mM p-nitrophosphoryl phosphate (diluted salt, Calbiochem) and 4 mM KCl. Steady state phosphorylation was measured at both 3 and 37 °C as described previously (15), in the presence of 25 mM imidazole-Cl, pH 7.4, 3 mM MgCl₂, 20 mM NaCl or KCl, and 80 μM [γ-³²P]ATP (New England Nuclear, 45 mCi/mmol). [³²P]Ouabain binding was carried out at 37 °C in the presence of 50 mM Tris-Cl, pH 7.4, 5 mM MgCl₂, and either 5 mM inorganic phosphate or Tris-ATP (80 μM or 5 mM) and 100 mM NaCl with 0.1 or 2.5 mM [³²P]Ouabain, 100 mM CCl₄/mmol (New England Nuclear) as previously described (10, 15).

Reaction with Mal-ANS and Mal-pyrene and Fluorescence Measurements—Mal-pyrene, purchased from Molecular Probes, Inc., was dissolved to approximately 1 mM in ethanol. The actual concentration of the stock solution was calculated assuming ε₂₄₂ = 40,000 M⁻¹ cm⁻¹. The reaction of Mal-pyrene with (Na,K)-ATPase was monitored directly by measuring the increasing fluorescence intensity at 375 nm with an excitation wavelength of 342 nm using a Perkin-Elmer MPP 44A fluorescence spectrophotometer in the ratio mode at 24 °C. Mal-ANS, also from Molecular Probes, Inc., was dissolved in water and measured exactly as described previously (12). Mal-ANS was stored frozen and solutions of both probes were used within 48 h of preparation.

For fluorescence intensity monitoring of the Mal-pyrene reaction with (Na,K)-ATPase, 25 μg of enzyme protein/ml were preincubated at 24 °C for 5 min with 10 μM Tris-Cl, pH 7.4, and other ligands as indicated. The Mal-pyrene labeling reaction was initiated by the addition of 2.5 μM Mal-pyrene in 25 μl of ethanol (final concentration of ethanol = 1%). In experiments where various concentrations of Mal-pyrene were added to the enzyme, the quantity of added ethanol was kept constant at 25 μl by varying the concentration of the Mal-pyrene stock solutions. The conditions used for measuring the time-dependent inhibition of (Na,K)-ATPase activity were essentially the same, except that a final concentration of 10 μM Mal-pyrene was compared with the reaction of the enzyme with N-(1-pyrene)maleimide which is bulkier and more hydrophobic than Mal-ANS. These two fluorescent maleimide analogues appear to react, in part, with different sulfhydryl groups on the α subunit of the (Na,K)-ATPase, and this difference in sulfhydryl group reactivity is reflected by the difference in the functional parameters of the modified enzymes.
added to 0.1 mg of enzyme protein/ml. Reactions of the (Na,K)-ATPase with Mal-ANS were carried out as described previously (12).

For florescence polarization measurements, the polarizer accessory for the MFP series (Perkin-Elmer) was used. Uncorrected fluorescence polarization was measured using the equation \( P = (I_p - I_p - I_o) / (I_p - I_o) \), where \( I_p \) and \( I_o \) are the fluorescence intensities of parallel and perpendicular components of polarized light.

Measurements of the collisional quenching of Mal-pyrene and Mal-ANS fluorescence were conducted by adding various concentrations of freshly prepared NaI to the sample cuvette and adding the same concentration of NaCl to the control cuvette. The ratio \( F/F_0 \) was calculated from the initial fluorescence intensity \( (F_0) \) and the fluorescence intensity \( (F) \) measured at each concentration of NaAl added to the probe-enzyme complex. The ratio was normalized by dividing by the \( F/F_0 \) value obtained by adding the same concentration of NaCl to the probe-enzyme complex.

The amounts of Mal-pyrene and Mal-ANS bound to the (Na,K)-ATPase preparation were calculated assuming \( c_{\text{nat}} = 20,000 \text{ M}^{-1} \text{ cm}^{-3} \) (Mal-ANS) and \( c_{\text{nat}} = 40,000 \text{ M}^{-1} \text{ cm}^{-3} \) (Mal-pyrene). In order to quantitate the amount of probe bound to each of the protein subunits and the incorporation of probe into the lipids, 8 mg of Mal-ANS or Mal-pyrene-labeled (Na,K)-ATPase were solubilized with 2% SDS in the presence of 25 mM Tris-Cl, pH 7.5, 0.01 mM 2-mercaptoethanol and applied to a Bio-Gel A-5 column (1.5 × 190 cm) equilibrated and eluted with 25 mM Tris-Cl, pH 7.4, 0.1% SDS, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 0.02% NaN₃. The fractions containing the subunits were concentrated by lyophilization and then quantitated by measuring the absorbance at 343 or 322 nm, as described previously for Mal-ANS (12).

Other Methods—Lipid phosphorous was determined according to Bartlett (18). SDS-polyacrylamide gel electrophoresis was conducted as described by Laemmli (17) on 7.5% polyacrylamide gels. It has been reported (18, 19) that the colorimetric protein assay described by Lowry et al. (20) overestimates the amount of (Na,K)-ATPase protein by approximately 40%. In addition, the molecular weights of the \( \alpha \) and \( \beta \) subunits, as well as the number of each of these subunits comprising a functional unit of the (Na,K)-ATPase, are in question (21–23). For determining the relative levels of Mal-ANS and Mal-pyrene labeling of (Na,K)-ATPase in the present study, the enzyme and subunit protein concentrations have been estimated by the method of Lowry et al. (20), using bovine serum albumin (Sigma) as standard. Further, it has been assumed that the functional unit of (Na,K)-ATPase is \( \alpha \beta_2 \) (Mᵣ = 278,000), with \( M \), (\( \alpha \)) = 95,000 and \( M \), (\( \beta \)) = 43,800.

RESULTS

Reaction of Probes with (Na,K)-ATPase—The addition of various concentrations of Mal-pyrene to 0.1 mg/ml of (Na,K)-ATPase resulted in a time-dependent increase in the fluorescence intensity at 375 nm, indicating incorporation of the probe into the enzyme preparation. With concentrations of Mal-pyrene lower than 5 \( \mu \)M, this time-dependent increase in the fluorescence intensity was proportional to the amount of Mal-pyrene incorporated into the enzyme preparation. The reaction of Mal-pyrene is similar to that described previously (12) for the reaction of Mal-ANS with this same enzyme preparation, except that the total incorporation of Mal-ANS into the (Na,K)-ATPase preparation was measured at 2.5-fold higher than that observed with Mal-ANS when the labeling reactions were carried out under identical conditions.

To determine whether the greater incorporation of Mal-pyrene was due to the specific labeling of the \( \alpha \) subunit, as was observed previously with Mal-ANS (12), or was due in part to nonspecific incorporation of Mal-pyrene into either the enzyme proteins or the associated lipids, the following experiments were carried out. Data summarized in Table I, were conducted with (Na,K)-ATPase (0.1 mg/ml) incubated with 2-mercaptoethanol prior to the addition of 10 \( \mu \)M Mal-pyrene (line 5). The reaction was continued for 30 min and the enzyme was then washed twice by centrifugation and resuspension to remove unbound probe. Since the precubication of the (Na,K)-ATPase with 2-mercaptoethanol prevents the alkylation of protein sulfhydryl groups by Mal-pyrene, the

| Additions | Mal-pyrene | Amount of Mal-pyrene/278,000 g of protein | Fluorescence polarization |
|-----------|------------|----------------------------------------|--------------------------|
| 1. None   | 10         | 6.8                                    | 0.158                    |
| 2. 5 mM MgCl₂ | 10   | 13.0                                   | 0.136                    |
| 3. 5 mM MgCl₂ | 10   | 20.2                                   | 0.109                    |
| 4. 5 mM MgCl₂ | 10   | 20.2                                   | 0.109                    |
| 5. 2-Mercaptoethanol + 5 mM MgCl₂ | 10 | 13.9e | 0.091 |
| 6. Extracted lipids + 5 mM MgCl₂ | 10 | 3.9 | 0.174 |
| 7. 2-Mercaptoethanol (no enzyme) | 10 | 0.008 | |
| 8. 2-Mercaptoethanol + glycerol (no enzyme) | 10 | 0.174 | |

"Moles of Mal-pyrene nonspecifically incorporated into the amount of extracted lipid (278 mol) associated with 1 mol of \( \alpha \beta_2 \) (278,000 g) protein.

results of this experiment indicated that 4.9 mol of Mal-pyrene/mol of (Na,K)-ATPase were nonspecifically incorporated into the enzyme preparation. The results of this experiment were identical with those obtained when Mal-pyrene was preincubated with a 5-fold excess of 2-mercaptoethanol prior to its addition to the enzyme suspension (data not shown). II) Lipid vesicles (0.1 \( \mu \)mol of phospholipid/ml) which were prepared from lipids extracted from the (Na,K)-ATPase preparation by chloroform/methanol were incubated with 10 \( \mu \)M Mal-pyrene for 30 min in the absence of ATP, with approximately 10 mM Tris-Cl, pH 7.4, at 3 °C (line 6). Since this (Na,K)-ATPase preparation contains approximately 1 \( \mu \)mol of lipid phosphorous/mg of protein, the amount of Mal-pyrene associated with 1 \( \mu \)mol of lipid phosphorous represents the amount of Mal-pyrene associated with the lipid moiety of 1 mg of protein of the enzyme preparation. In both Experiments I and II, 4 to 5 mol of Mal-pyrene were found to be associated nonspecifically with 1 mol of the (Na,K)-ATPase preparation, assuming that 1 mg of protein equals 3.8 nmol of (Na,K)-ATPase (\( \alpha \beta_2 \)).

To determine if a time-dependent hydrolysis of bound Mal-pyrene occurred, as reported previously by Lux and Gerard (24), the emission spectra of freshly prepared (Na,K)-ATPase-Mal-pyrene complex and an \( \alpha \)-Mal-pyrene complex, which had been isolated from this labeled enzyme and stored for 2 months, were compared. The two emission spectra were virtually identical with respect to emission maxima and ratios of the peak heights (data not shown), indicating that hydrolysis of protein-bound Mal-pyrene had not taken place.

Location of Bound Probes—The limiting values of the fluorescence polarization of Mal-pyrene plus 2-mercaptoethanol, at room temperature, in buffer (n = 1 cp) and in glycerol (n = 1,000 cp) were 0.009 and 0.174, respectively (Table I). The fluorescence polarization of the nonspecifically labeled

"Downloaded from http://www.jbc.org/ by guest on March 24, 2020"
(Na,K)-ATPase or lipid vesicles, 0.049 and 0.091, respectively, is substantially lower than the fluorescence polarization (0.136) of the Mal-pyrene-enzyme complex which was formed in the absence of 2-mercaptoethanol. This indicates that the Mal-pyrene molecules which are nonspecifically associated with the enzyme preparation and/or intercalated into the lipid moiety have a higher degree of freedom of motion than do those molecules which are specifically bound to the enzyme proteins.

To determine the amounts of Mal-pyrene and Mal-ANS covalently bound to the (Na,K)-ATPase protein subunits, the labeled enzymes were solubilized with SDS and chromatographed on a Bio-Gel A-5m column to separate the α and β subunits and the lipids (Fig. 1). Since both Mal-pyrene and Mal-ANS absorb in the 280 nm region, the peaks of relative absorbance at 280 nm shown in Fig. 1A represent both protein and probe absorbance. The protein content of the column fractions was estimated by the method of Lowry et al. (20), and the relative absorbance at 750 nm of the colorimetric assay for protein of these fractions is shown in Fig. 1B. The relative fluorescence of the fractions (Fig. 1C) reveals the presence of a large quantity of Mal-pyrene, but not Mal-ANS, in the lipid-containing fractions which elute behind the β subunit. As shown in Table II, approximately 50% of the Mal-pyrene which is associated with the (Na,K)-ATPase preparation is eluted with the lipid fraction.

For both Mal-pyrene and Mal-ANS, most of the covalently bound probe is located on the α subunit of the enzyme. A small quantity of both probes (approximately 1 mol/mol) appears to be associated with the fractions containing the β subunit of the enzyme (Fig. 1). This observation appears to be inconsistent with our earlier conclusion (12) that Mal-ANS labeled only the α subunit of the (Na,K)-ATPase. The fluorescence intensity of the β subunit-containing fractions is so low with both probes that it can only be detected by monitoring the column fractions with a fluorescence spectrophotometer, as was done in Fig. 1. However, when the β subunit fractions are pooled, concentrated by lyophilization, and submitted to SDS-polyacrylamide gel electrophoresis, there is no detectable Mal-pyrene or Mal-ANS fluorescence associated with the β subunit protein band. When α subunit fractions, which contain an amount of fluorescence equivalent to that contained in the β subunit column fractions, are electrophoresed under the same conditions, a distinct fluorescent band which corresponds to the protein band is readily apparent. These results suggest that the low level of fluorescence which co-chromatographs with the β subunit on Bio-Gel A-5m (Fig. 1) is not due to Mal-ANS or Mal-pyrene being covalently bound to the β subunit. However, the possibility that the β subunit may be minimally labeled by Mal-ANS or Mal-pyrene cannot be completely ruled out at this time.

The relative locations of the Mal-ANS and Mal-pyrene molecules incorporated into the (Na,K)-ATPase preparation were determined by measuring the collisional quenching of the fluorescence intensity of the probe-enzyme complexes by iodide ions (25). As calculated from the curves shown in Fig. 2A, the apparent collisional quenching constant (Kq) of the Mal-pyrene-(Na,K)-ATPase complex is 2.0 M⁻¹, compared to 3.6 M⁻¹ for Mal-pyrene nonspecifically incorporated into the enzyme, and 16.6 M⁻¹ for Mal-pyrene dissolved in 10 mM Tris-Cl, pH 7.4, plus 1 mM 2-mercaptoethanol. This indicates that

![Graph](http://www.jbc.org/)

**TABLE II**

**Distribution of Mal-ANS and Mal-pyrene in the Probe-(Na,K)-ATPase complexes**

| Probe | (Na,K)-ATPase | α | β | 2(α + β) | Lipid* | mol/probe/mol |
|-------|---------------|---|---|---------|--------|---------------|
| 1. Mal-ANS (10 μM) | 8.9 | 3.7 | 0.8 | 9.9 | 0 |
| 2. Mal-ANS (50 μM) | 17.5 | 5.9 | 1.6 | 10.0 | 0 |
| 3. Mal-ANS (100 μM) | 22.5 | 5.5 | 0.9 | 12.8 | 13.3 |
| 4. Mal-pyrene (50 μM) | 30.6 | 8.5 | 0.9 | 18.8 | 14.6 |

*Moles incorporated into the amount of phospholipid (278 mol) associated with 1 mol of (Na,K)-ATPase (278,000 g of protein).
the Mal-pyrene molecules which are bound to the (Na,K)-ATPase are located in a more shielded or more hydrophobic environment than are the Mal-pyrene molecules dissolved in aqueous buffer or incorporated into lipids.

The collisional quenching of Mal-ANS fluorescence with NaI revealed a different pattern of quenching (Fig. 2B). The apparent collisional quenching constants for the Mal-ANS molecules which are bound to the (Na,K)-ATPase preparation, suggest that the Mal-pyrene molecules which are bound to the enzyme are in a more hydrophobic environment than are the Mal-ANS molecules.

**Effect of Ligands**—The rate of reaction of Mal-pyrene with (Na,K)-ATPase, as measured by an increase in the fluorescence intensity at 375 nm, is influenced by the ligands present in the reaction medium in essentially the same manner as that reported previously for Mal-ANS (12). The apparent binding constant for the Mg\(^{2+}\)-induced enhancement of Mal-pyrene fluorescence was 0.77 mM, compared to 0.70 mM reported previously for the reaction of Mal-ANS with this enzyme, suggesting that the same Mg\(^{2+}\) site(s) is regulating the interaction of the enzyme with both probes.

The location of the additional six sulfhydryl groups on the (Na,K)-ATPase which are labeled by Mal-pyrene in the presence of 5 mM MgCl\(_2\) was investigated using steady state fluorescence polarization of the Mal-pyrene-enzyme complex. As shown in Table I, the fluorescence polarization of Mal-pyrene-enzyme complex which had been labeled in the presence of 5 mM MgCl\(_2\) was 0.136, compared to 0.158 for the complex formed in the presence of 10 mM Tris-Cl, pH 7.4, alone. This suggests that the additional sulfhydryl groups, which are labeled in the presence of MgCl\(_2\), may be located in a more fluid environment than those which are labeled in the absence of MgCl\(_2\), thereby lowering the average polarization value.

It is also apparent from the data in Table I, that the fluorescence polarization of the Mal-pyrene-(Na,K)-ATPase complex is highest when the probe concentration and, therefore, the number of sulfhydryl groups labeled is the lowest (line 4). With higher concentrations of Mal-pyrene (lines 2 and 3), the fluorescence polarization decreases indicating, on the average, a higher degree of freedom of rotation around the covalent bond. Since the relative quantities of covalently bound probe and nonspecifically incorporated probe are equal for 2, 10, and 30 μM Mal-pyrene (data not shown), the observed concentration dependence of the fluorescence polarization is probably not due to the amount of nonspecifically incorporated Mal-pyrene. Rather, the high degree of fluorescence polarization at 2 μM Mal-pyrene suggests that those sulfhydryl groups which have the highest affinity for Mal-pyrene are located within either crevices or regions of the (Na,K)-ATPase protein where the bound probe molecules can be immobilized by hydrophobic interaction with protein.

The effects of other ligands on the rate of Mal-pyrene...
reaction with the (Na,K)-ATPase are illustrated in Fig. 3 and are essentially the same as those observed previously with Mal-ANS (12). As with Mal-ANS, comparison of the rates of fluorescence enhancement with 5 mM MgCl₂ and 3 mM ATP (Curve A), 2 mM MgCl₂ (Curve D), 5 mM MgCl₂, 3 mM ATP, and 100 mM NaCl (Curve C) indicates that some of the sulfhydryl groups of the E·ATP form of the enzyme, but not of the E₂P form, are protected from reaction with Mal-pyrene.

**Effects of Mal-pyrene and Mal-ANS on Enzymic Activity**—
The effects of Mal-pyrene alkylation of the (Na,K)-ATPase are, in many respects, very similar to the effects observed previously with Mal-ANS (12). The reaction of both probes resulted in a time- and concentration-dependent loss of (Na,K)-ATPase activity and a parallel reduction in the steady state levels of phosphoenzyme formation and (Na,K)-ATPase activity (12) (Fig. 4). In addition, the loss of (Na,K)-ATPase activity in both cases was multiphasic, indicating that the loss of activity is due to the alkylation of multiple classes of sulfhydryl groups.

The effects of Mal-pyrene and Mal-ANS reaction on the potassium-dependent p-nitrophenyl phosphatase activity are not the same. With Mal-pyrene, the loss of phosphatase activity paralleled the loss of (Na,K)-ATPase activity and the steady state level of phosphoenzyme formation (Fig. 4). As reported previously (12), the loss of (Na,K)-ATPase and phosphatase activities with Mal-ANS was parallel only to a level of approximately 50% inhibition, with the remaining phosphatase activity being much more resistant to inhibition than was the (Na,K)-ATPase activity.

The effects of Mal-pyrene and Mal-ANS on the interaction of ouabain with the (Na,K)-ATPase are markedly different. Alkylation of the (Na,K)-ATPase with Mal-pyrene resulted in a significant decrease in the [³H]ouabain-binding capacity of the enzyme. When the binding assay was carried out in the presence of 5 mM MgCl₂, 5 mM ATP, and 100 mM NaCl (Mg-ATP-Na), the loss of [³H]ouabain-binding sites with increasing labeling by Mal-pyrene was almost parallel to the loss of (Na,K)-ATPase activity (Fig. 5A). Binding in the presence of 5 mM MgCl₂ and 5 mM inorganic phosphate (Mg·P) was also reduced, but to a lesser extent than was the Mg·ATP-Na-supported binding. Nonspecific labeling of the enzyme preparation with Mal-pyrene as described above (Experiment I and line 5 of Table I) had no effect on either (Na,K)-ATPase activity or the [³H]ouabain-binding capacity of the enzyme. In contrast, the effects of Mal-ANS alkylation on ouabain binding are much less apparent (Fig. 5B). We reported previously (12) that the ouabain-binding capacity of the (Na,K)-ATPase, measured in the presence of MgCl₂ and inorganic phosphate, was only slightly reduced by Mal-ANS. We have now determined that Mal-ANS alkylation also causes only a slight reduction (approximately 14%, Fig. 5B) in the level of Mg·ATP-Na-supported ouabain binding. This is particularly interesting because the (Na,K)-ATPase activity of this Mal-ANS-labeled enzyme was 81% inhibited. Since the inhibition of phosphoenzyme formation from ATP parallels the inhibition of (Na,K)-ATPase activity (Fig. 8 of Ref. 12 and line 2 of Table IV), it appears that a large proportion of the Mal-ANS-enzyme complex which cannot be phosphorylated by ATP in the presence of MgCl₂ and NaCl is still capable of binding ouabain in the presence of MgCl₂, NaCl, and ATP. To verify that the above measured equilibrium levels of [³H]ouabain binding to the Mal-ANS-labeled (Na,K)-ATPase represent inorganic phosphate- and ATP-stimulated binding, the rates of binding with various ligands were determined. As shown in

![Fig. 5. Effects of Mal-ANS and Mal-pyrene on [³H]ouabain binding and (Na,K)-ATPase activity. (Na,K)-ATPase (0.1 mg/ml) was incubated at room temperature in the presence of 10 mM Tris-Cl, pH 7.4, and 5 mM MgCl₂ and varying concentrations of Mal-pyrene (A) or Mal-ANS (B). After 20 min the reactions were terminated by the addition of 1 mM 2-mercaptoethanol, and aliquots were assayed for (Na,K)-ATPase activity (●) and equilibrium levels of [³H]ouabain binding in the presence of MgCl₂ and inorganic phosphate (〇), and MgCl₂, ATP, and NaCl (△) as described under "Materials and Methods."](http://www.jbc.org/)

![Table III](http://www.jbc.org/)

| Table III | The effect of ligands on the rate of [³H]ouabain binding to Mal-ANS-labeled (Na,K)-ATPase |
|-----------|------------------------------------|
| (Na,K)-ATPase (0.1 mg/ml) was labeled with 10 μM Mal-ANS for 100 min in the presence of 10 mM Tris-Cl, pH 7.4, and 5 mM MgCl₂. After removal of unbound probe by centrifugation and resuspension, the rates of [³H]ouabain binding to 10 μg of control or labeled (Na,K)-ATPase were measured at 37°C in the presence of 0.1 μM ouabain, 50 mM Tris-Cl, pH 7.4, and 5 mM MgCl₂ (Mg), 5 μM MgCl₂ and 5 mM inorganic phosphate (Mg·P), 5 mM MgCl₂ and 80 μM ATP (Mg·ATP), and 5 mM MgCl₂, 100 mM NaCl, and 80 μM ATP (Mg·ATP-Na). |
| [³H]ouabain binding | Mg·P | Mg·ATP | Mg·ATP-Na |
|----------------------|------|--------|-----------|
| Control               | 20   | 1.2    | 9.3       | 1.5       |
| Mal-ANS-labeled       | 50   | 2.3    | 21        | 3.3       |
Table III, although the rates of binding to the Mal-ANS-enzyme complex are consistently two times slower than those to the control enzyme, the degrees of stimulation of the rates of binding by inorganic phosphate, ATP, and ATP plus sodium were the same for both control and Mal-ANS-labeled (Na,K)-ATPase. It might be argued that the binding measured in the presence of added magnesium and ATP was due to the presence or the generation of inorganic phosphate. It should be noted, however, that NaCl decreases the rate of ouabain binding in the presence of magnesium and inorganic phosphate. For example, in the presence of 5 mM MgCl₂ and 5 mM inorganic phosphate the addition of 100 mM NaCl increases the ε₁/₂ for binding to control enzyme from 1.6 to 38 min. If the concentration of inorganic phosphate is lowered to 5 μM (2.5 times the concentration of inorganic phosphate contamination present in the 80 μM ATP used here), the addition of 100 mM NaCl essentially prevents the binding of ouabain (data not shown). Since the rate of binding to the Mal-ANS-labeled (Na,K)-ATPase is six times faster in the presence of Mg·ATP·Na than it is with Mg·ATP (Table III), it is apparent that the interaction of ouabain with the Mal-ANS-enzyme complex is regulated by ATP and by ATP + sodium, even though this modified enzyme cannot be phosphorylated by ATP.

As described above (Fig. 2), the collisional quenching of the fluorescence of the Mal-ANS- and Mal-pyrene-labeled (Na,K)-ATPase fractions indicated that at least some of the sulfhydryl groups which are labeled by Mal-pyrene are different from those which are labeled by Mal-ANS. To determine if it is the labeling of different sulfhydryl groups by Mal-pyrene that is responsible for the greater inhibition of ouabain binding, (Na,K)-ATPase was labeled sequentially with Mal-ANS and Mal-pyrene. The concentrations of the probes and the incubation times were selected so that the degree of inhibition of (Na,K)-ATPase activity was equivalent with both Mal-ANS and Mal-pyrene. As shown in Table IV (line 2) the Mal-ANS-treated enzyme fraction appears to consist of approximately 10% native enzyme, 17% totally inactive enzyme (with respect to ATP hydrolysis and ouabain binding), and 83% that has been modified such that ATP- and inorganic phosphate are capable of supporting ouabain binding even though the enzyme cannot be phosphorylated by ATP. (Na,K)-ATPase that has been labeled only with Mal-pyrene (line 3) appears to contain 10% native enzyme, 62% totally inactive enzyme, and approximately 28% (38-11%) modified enzyme which can bind ouabain in the presence of magnesium and inorganic phosphate, but cannot be phosphorylated by ATP. When the Mal-ANS-(Na,K)-ATPase (line 2) was subsequently treated with Mal-pyrene, exactly as the enzyme in line 3 was, the [³H]ouabain-binding capacity of the resultant double-labeled enzyme was reduced to 20% and 36% of control in the presence of Mg·ATP·Na and Mg·Pi, respectively. Additional double labeling experiments, similar to that shown in Table IV, which were carried out with varying concentrations of Mal-ANS and Mal-pyrene and slightly different incubation times revealed that Mal-ANS labels approximately 4 sulfhydryl groups/α subunit, and that an additional 3-5 sulfhydryl groups/α subunit are labeled when the Mal-ANS-enzyme complex is exposed to Mal-pyrene. These results indicate that it is the alkylation of additional sulfhydryl groups on the α subunit, which are not accessible to Mal-ANS, that is responsible for the significant inhibition of [³H]ouabain binding by Mal-pyrene.

Since preincubation of the (Na,K)-ATPase with ouabain altered neither the rates nor the apparent magnitude of Mal-pyrene reaction with the enzyme (Fig. 3), these additional Mal-pyrene-labeled sulfhydryl groups would not appear to be located within the ouabain binding site. Furthermore, in separate experiments (Na,K)-ATPase was preincubated with 5 μM ouabagenin in the presence of Mg·Pi, and Mg·ATP·Na binding conditions, to protect the ouabain binding site from modification, and then exposed to Mal-pyrene. The labeled enzyme was washed by repeated centrifugation and resuspension to remove unbound Mal-pyrene and to dissociate ouabagenin from the enzyme. The amount of Mal-pyrene which bound to the (Na,K)-ATPase, the level of [³H]ouabain binding and the (Na,K)-ATPase activity were the same, whether or not the enzyme had been incubated with ouabagenin before and during the exposure to Mal-pyrene (data not shown).

**DISCUSSION**

The primary observations of this study are: 1) that the pyrene and ANS derivatives of maleimide react, in part, with different sulfhydryl groups on the α subunit, and have markedly different effects on the interaction of ouabain with the (Na,K)-ATPase; and 2) that phosphorylation of the (Na,K)-ATPase is not a prerequisite for the binding of Mal-ANS plus sodium-stimulated [³H]ouabain to the enzyme.

Both Mal-ANS and Mal-pyrene alkylate sulfhydryls on the α subunit which are essential for the binding of ouabain to its receptor site on the enzyme. From these experiments, it is not possible to determine either the location or the number of these essential sulfhydryl groups, but the results of the double-labeling experiments, in which (Na,K)-ATPase was reacted first with Mal-ANS and then with Mal-pyrene, suggest that 5 or less sulfhydryls/α subunit are involved. Since pretreatment of the enzyme with ouabain or ouabagenin does not decrease the rate or the magnitude of Mal-pyrene reaction with (Na,K)-ATPase, nor does that pretreatment protect the enzyme against inactivation by Mal-pyrene, it seems reasonable to assume that the sulfhydryl groups which are essential for ouabain binding are not located within the ouabain binding site of the (Na,K)-ATPase. The results do suggest that the interaction of ouabain with the (Na,K)-ATPase is regulated by a distinct region(s) of the enzyme protein containing sulfhydryl groups which are accessible to Mal-pyrene, but not to Mal-ANS.

On the average, the sulfhydryl groups which are alkylated by Mal-pyrene appear to be located in more shielded or more...
hydrophobic regions of the α subunit than are those which are alkylated by Mal-ANS. As reported previously with Mal-ANS (12), the interaction of Mal-pyrene with sulphydryl groups on the (Na,K)-ATPase is markedly enhanced by the presence of magnesium. The apparent binding constant for magnesium (0.77 mM) is similar to that (0.95 mM) calculated previously (27) for the enhancement of fluorescence of dansylphosphatidylethanolamine which had been incorporated into the lipids associated with this enzyme preparation. This suggests that the observed magnesium-induced reaction of a subunit sulphydryl groups with Mal-ANS (12) and with Mal-pyrene (Fig. 4) is due to the effect of magnesium on the fluidity of the membrane lipids, and is not due to a direct or specific magnesium-induced conformational change in the α subunit. In this regard, it is interesting to note that it is the binding of Mg2+ to a site on the (Na,K)-ATPase preparation (Kd approximately 0.2 mM) that results in a 150-fold increase in the enzyme’s affinity for ouabain (28). This magnesium-induced change in affinity for ouabain, which may also be mediated via the lipids, is greater than the changes in affinity caused by the addition of either inorganic phosphate or ATP plus sodium. From measurements of fluorescence polarization, it appears that the 3 additional sulphydryl groups α that react with Mal-pyrene in the presence of 5 mM MgCl2 are located in a more fluid environment than those which react in the absence of magnesium. This fluid environment could be near that polar head group region of the lipids on the enzyme surface.

It is possible that at least some of the sulphydryl groups which are essential for ouabain binding may be located within an ATP binding site on the (Na,K)-ATPase. The observation that alklyation by Mal-ANS prevents the phosphorylation of (Na,K)-ATPase by ATP, but does not prevent ATP-supported ouabain binding, could have several interpretations but might suggest that the ATP and sodium sites which regulate ouabain binding are different from the ATP and sodium sites which are involved in the phosphorylation of the (Na,K)-ATPase. If this were the case, then the reaction of Mal-ANS with sulphydryl groups at the ATP site which leads to phosphorylation could result in the observed inhibition of phosphorylation, without inhibiting inorganic phosphate or ATP plus sodium-stimulated ouabain binding. Since the ATP site which regulates ouabain binding is a high affinity site, this would require the existence of two distinct high affinity ATP sites/molecule of enzyme; one leading to phosphorylation, and another at which ATP binds, but does not form an acid-stable phosphointermediate, to increase the affinity of the (Na,K)-ATPase for ouabain.

An alternative possibility is that a single ATP site on the enzyme is involved in both phosphorylation and ouabain binding, but that the observed stimulation of ouabain binding by ATP plus sodium is not mediated via phosphorylation of the (Na,K)-ATPase. If this second hypothesis were correct, then alklyation of sulphydryls by Mal-ANS would not prevent ATP binding to the site, but would prevent a subsequent step (possibly sodium binding) that is required for phosphorylation. The increased rate of ouabain binding caused by the addition of sodium would be mediated via the binding of sodium at a regulation site for ouabain binding that’s distinct from the sodium site that promotes phosphorylation. Mal-pyrene, by reacting with additional sulphydryls within the same ATP site which are not accessible to Mal-ANS, would then prevent the binding of ATP and inorganic phosphate, thereby inhibiting both inorganic phosphate- and ATP-stimulated ouabain binding.

Although Askari and Huang (29) recently reported evidence for the simultaneous existence of both a high and a low affinity ATP site on the enzyme, there is no evidence at present to support the existence of two distinct high affinity ATP sites on the enzyme. There is, however, evidence to suggest that the sodium site which modulates ouabain binding to the (Na,K)-ATPase is different from the site which regulates phosphorylation. Anner et al. (30) reported that in the absence of potassium the Kd for sodium activation of ouabain binding is as high as 20 mM, while the Kd for sodium activation of phosphoenzyme formation is as low as 0.5 mM, and that sodium still modulates the rate of [3H]ouabain binding even when the (Na,K)-ATPase is completely phosphorylated.

Our present results do not permit us to determine which, if either, of these alternatives is correct. These results do demonstrate that several sulphydryl groups are essential for the various functional parameters of the (Na,K)-ATPase, and that these sulphydryl groups are differentially modified by Mal-ANS and Mal-pyrene. These results also support the previous suggestion by Mandel et al. (28) and Anner et al. (30) that phosphorylation of the (Na,K)-ATPase per se is not required for high affinity ouabain binding, despite the fact that the same ligand conditions which promote phosphoenzyme formation also promote ouabain binding.

Acknowledgments—We are grateful to Drs. Earl T. Wallick and Arnold Schwartz for their helpful suggestions and encouragement, M. Rowe and E. Lavash for their expert technical assistance, G. Kraft for the illustrations, and the Kleuner Packing Company of Cincinnati, Ohio, for providing the frozen lamb kidneys.

REFERENCES
1. Wallick, E. T., Lane, L. K., and Schwartz, A. (1979) Annu. Rev. Physiol. 41, 307–411
2. Schoot, B. M., De Pont, J. J. H. M., and Bonting, S. L. (1978) Biochim. Biophys. Acta 483, 602–613
3. Harris, W. E., and Stahl, W. L. (1977) Biochim. Biophys. Acta 483, 203–214
4. Patzel-Wenzcher, H. P., Erdmann, E., and Schoner, W. (1975) Eur. J. Biochem. 53, 301–312
5. Fahn, S., Moira, R., Koval, G. J., and Albers, R. W. (1966) J. Biol. Chem. 241, 1890–1895
6. Post, R. L., Kunne, S., Tobin, T., Orcutt, R., and Sen, A. K. (1969) J. Gen. Physiol. 54, 3066–3266
7. Siegel, G. J., Koval, G. J., and Albers, R. W. (1969) J. Biol. Chem. 244, 3264–3269
8. Bannerjee, S. P., Wong, S. M. E., and Sen, A. K. (1972) Mol. Pharmacol. 8, 8–17
9. Skou, J. C., and Hilberg, C. (1969) Biochim. Biophys. Acta 185, 188–219
10. Wallick, E. T., Anner, B. M., Ray, M. V., and Schwartz, A. (1978) J. Biol. Chem. 253, 8778–8786
11. Schoot, B. M., Schoots, A. F. M., De Pont, J. J. H. M., Schuurmans-Stekhoven, F. M. A. H., and Bonting, S. L. (1977) Biochim. Biophys. Acta 483, 181–192
12. Gupta, S. S., and Lane, L. K. (1979) J. Biol. Chem. 254, 10362–10369
13. Lane, L. K., Potter, J. D., and Collins, J. H. (1979) Prep. Biochem. 9, 157–170
14. Schwartz, A., Allen, J. C., and Harigaya, S. (1969) J. Pharmacol. Exp. Ther. 188, 31–41
15. Lane, L. K., Anner, B. M., Wallick, E. T., Ray, M. V., and Schwartz, A. (1978) Biochem. Pharmacol. 27, 255–231
16. Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468
17. Lamsili, U. K. (1970) Nature (Lond.) 227, 680–685
18. Peters, W. H. M., Schwartz, H. G. P., De Pont, J. J. H. M., Schuurmans-Stekhoven, F. M. A. H., and Boning, B. S. (1981) Nature (Lond.) 290, 338–339
19. Moczylowski, E. G., and Fortes, P. A. G. (1981) J. Biol. Chem. 256, 2346–2356
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21. Craig, W. S., and Kyte, J. (1980) J. Biol. Chem. 255, 6262–6269

E. T. Wallick, personal communication.
Labeling of (Na,K)-ATPase Sulphydryl Groups

22. Kyte, J. (1981) *Nature (Lond.*)** 292, 201-204
23. Peterson, G. L., and Hokin, L. E. (1981) *J. Biol. Chem.* 256, 3751-3761
24. Lux, B., and Gérard, D. (1981) *J. Biol. Chem.* 256, 1767-1771
25. Watt, R. M., and Voss, E. W., Jr. (1979) *J. Biol. Chem.* 254, 1684-1690
26. Tu, S., Wu, C., and Hastings, J. W. (1978) *Biochemistry* 17, 987-993

27. Gupte, S. S., Lane, L. K., Johnson, J. D., Wallick, E. T., and Schwartz, A. (1979) *J. Biol. Chem.* 254, 5099-5103
28. Mandel, F., Wallick, E. T., and Schwartz, A. (1977) *Fed. Proc.* 36, 274
29. Askari, A., and Huang, W. (1982) *Biochem. Biophys. Res. Commun.* 104, 1447-1453
30. Anner, B. M., Wallick, E. T., Lane, L. K., and Schwartz, A. (1976) *Fed. Proc.* 35, 833
Reaction of (Na,K)-ATPase with fluorescent maleimide derivatives. Probes for studying ATP site(s) function.

S S Gupte and L K Lane

J. Biol. Chem. 1983, 258:5005-5012.

Access the most updated version of this article at http://www.jbc.org/content/258/8/5005

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/258/8/5005.full.html#ref-list-1