Irreversible inhibition of Na,K-ATPase and K⁺-dependent p-nitrophenylphosphatase activities was produced by incubation of purified Na,K-ATPase enzyme with 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EPC). Inhibition was time and [EPC] dependent and displayed first order kinetics with respect to time. The [EPC] to reduce the enzyme velocity by 50% for Na,K-ATPase and phosphatase activities was 1.6 and 2.2 mM, respectively. Analysis of the kinetics of inhibition by EPC indicated that reaction at one site was sufficient to produce inhibition. Inhibition was greatly reduced by the presence of Mg²⁺, Na⁺, K⁺, choline, or Tris (decreasing order of effectiveness); ATP was without effect. This suggests that cation-bound enzyme forms were less reactive with the carbodiimide than free enzyme; ATP-bound enzyme was as reactive. Apparently the cations Na⁺, Mg²⁺, Tris, and choline stabilize E₁ forms of the enzyme which are different from the E₀ form stabilized by ATP.

Addition of [¹⁴C]glycine ethyl ester (Gly-OEt) resulted in incorporation of radioactivity into both α and β subunits that was dependent upon the presence of EPC, and the incorporation was reduced by the cations which reduced the inhibition due to EPC. Simultaneous addition of Gly-OEt with EPC prevented inhibition, although [¹⁴C] incorporation still took place. If Gly-OEt addition was delayed the initial inactivation was not inhibited, but little subsequent inactivation occurred. The protection against inactivation by EPC occurs on the addition of other exogenous nucleophiles, such as aminothane or ethylenediamine. Dicyclohexylcarbodiimide, a more potent hydrophobic carbodiimide inhibitor, shows similar effects; the inhibition due to dicyclohexylcarbodiimide is also prevented by the simultaneous presence of a nucleophile. After treatment with a carbodiimide and exogenous nucleophile the Na,K-ATPase has modified carboxyl residues but is not inhibited. Thus, modification of the cation-protectable carboxyl groups does not by itself cause inhibition. It seems likely that the inhibition of activity due to carbodiimide alone is not due to the modification of a carboxyl group per se but to the formation of an intramolecular bond between the carbodiimide-activated carboxylic acid and an endogenous nucleophile. The formation of such bonds suggests the close juxtaposition of amine and carboxyl groups in the secondary structure of the enzyme.

The Na,K-ATPase (EC 3.6.1.3) is the plasma membrane enzyme which carries out the transmembrane-coupled active transport of Na⁺ and K⁺ ions (1–3). Substantial progress has been made in recent years in establishing the enzyme subunit composition and structure (4, 5) and in relating the biochemical activities catalyzed by the enzyme to the associated transport reactions (6, 7). The use of a variety of group-specific chemical modifying agents has enabled the identification of a number of amino acid residues likely to be present at binding sites for the sodium pump ligands or of importance in the conformational transitions involved in the catalytic or transport cycles. These include butane dine for arginine residues at the ATP site (8), sulphydryl-reactive ATP analogs for —SH groups at the ATP site (9), N-CbdCl (7-chloro-4-nitrobenzo-2-oxa-1,3 diazole) reacting with a tyrosine residue at the ATP site (10), fluorescein isothiocyanate reacting with a lysine residue at the ATP site (11, 12), [³H]NaBH₄ reduction (13) confirming an aspartyl carboxyl residue as the phosphorylation site (14) etc. However, little information is currently available about the nature of the monovalent cation-binding sites on the enzyme. Previous studies have utilized dicyclohexylcarbodiimide (DCCD) as a —CO₂H selective reagent and observed inhibition that was reduced by the presence of Na⁺ or K⁺ ions, concluding that carboxyl groups were involved at the sites of cation binding (15–17).

In the present study we have examined the effects of a water-soluble carbodiimide (1-ethyl-3(3-dimethylaminopropyl)carbodiimide) on the properties of the Na,K-ATPase enzyme. The expectation was that such a positively charged reagent for carboxyl residues might target the modification in a more selective way toward those carboxyl groups on the enzyme which were involved in cation binding. The studies reported here demonstrate cation protection against the inhibition caused by modification of the enzyme with the carbodiimide, in agreement with similar results previously obtained using dicyclohexylcarbodiimide (15–17). However, our results indicate, contrary to the conclusions of other workers (16, 17), that carboxyl modification by carbodiimide per se does not inhibit the Na,K-ATPase. The most likely cause of such inhibition is via the reaction of a carbodiimide-activated carboxyl residue with an endogenous nucleophile (a side-chain amine for example) leading to the formation of an intramolecular cross-link. Modification of cation-protectable carboxyl groups does not itself lead to inhibition, so that it is unlikely that the free carboxyl groups (which react with the carbodiimide) prior to modification are essential for competent cation

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The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; EPC, 1-ethyl-3(3-dimethylaminopropyl)carbodiimide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Gly-OEt, glycine ethyl ester; HEPES, 4-[2-hydroxyethyl]-1-piperazineneethanesulfonic acid.
binding. A preliminary report of some of these findings has been presented (18).

MATERIALS AND METHODS

Partially purified Na,K-ATPase was obtained from dog kidneys according to Jorgensen (19) with some modifications (20). The standard assay medium for Na,K-ATPase activity had the following composition (mM): EGTA, 0.5; NaCl, 130; KCl, 20; MgCl2, 5; ATP, 3; imidazole, 50; pH 7.2, 20 'C. In all cases the ATPase assay medium contained 0.3 mg/ml bovine serum albumin.

When the concentration of Na+ or K+ was varied they were replaced by choline chloride. The Na,K-ATPase activity was determined by the method of Brotherus et al. (21). The activity of the Na,K-ATPase was calculated from the difference between the ATP hydrolysis measured in the absence and presence of 6 x 10-4 M ouabain. Na,K-ATPase used in these studies had a specific activity of between 10 and 15 pmol of Pi liberated mg-1 min-1. Protein was assayed by the method of Lowry et al. (22) using bovine serum albumin as standard.

Phosphatase Activity—This was measured continuously at 20 'C following the release of p-nitrophenol from p-nitrophenyl phosphate in a spectrophotometer at 410 nm. For the activity of the enzyme to be a linear rate of 1.7 x 104 M-1 s-1 was used. It was also measured at 37 'C when the reaction was stopped with 1 volume of 0.2 M NaOH, 2.5% SDS, 4 mM EDTA, and the production of p-nitrophenol was estimated at 410 nm (24). The phosphatase assay medium contained (mM): EGTA, 0.5; MgCl2, 5; p-nitrophenyl phosphate, 5; Tris, 50; KCl, 20, pH 7.2.

Reaction of the Enzyme with the Carbodiimides—The enzyme was incubated at pH 7.2 with the carbodiimide at 37 'C before measuring activity. The 20-100-fold dilution of the protein samples in the assay medium completely quenched the modification reaction. In the protection experiments the ligands were added to the enzyme suspension before the carbodiimide. Control experiments showed that the "preincubation" of the enzyme in the presence or absence of any other ligand had no significant effect on the ATPase activity. The composition of the solutions used for inactivation of the enzyme varied in different experimental conditions and are detailed in figure and table legends.

Labeling of the Protein with [14C]Gly-OEt—Aliquots of the enzyme were incubated in a medium (0.1 ml) containing (mm): [14C]Gly-OEt, 6; HEPES, 10; EDTA, 0.1, pH 7.2, 20 'C, and 1 mg/ml enzyme protein. At different times 1 mM EPC was added. Samples (5 μl) were withdrawn to determine ATPase activity. To the remainder, 100 μl of 10% trichloroacetic acid was added and filtered through Millipore filters (pore size, 0.45 μm). The filters were washed with 150 μl (5-ml aliquots) of 25 mM imidazole, 10 mM Gly-OEt, 5% trichloroacetic acid, and counted in a scintillation counter.

SDS-Gel Electrophoresis—The enzyme (2.5 mg/ml) was incubated with [14C]Gly-OEt and EPC in the presence of 3 mM HEPES, 0.05 mM EDTA, and ligands as shown in the figures, collected by centrifugation, and resuspended in a medium containing 0.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS and 5% mercaptoethanol. Electrophoresis was carried out by the method of Laemmli (25) on a 7.5% gel.

The gels were stained using Coomassie Blue (26). After destaining, the gel was cut in 5-mm slices and placed in vials with 5% trichloroacetic acid and counted in a scintillation counter (Beckman 7500 LSC).

Trypsin inactivation of Na,K-ATPase was performed as described by Jorgensen (27).

RESULTS

Inactivation of Enzymatic Activities—Treatment of the partially purified enzyme with EPC resulted in parallel inhibition of Na,K-ATPase and p-nitrophenylphosphatase activities. The extent of inhibition of these activities was increased as the concentration of EPC was raised (Fig. 1); the concentration of EPC necessary to reduce these activities to 50% of their initial values was 1.6 and 2.2 mM for the Na,K-ATPase and p-nitrophenylphosphatase, respectively. The inhibition was irreversible, and the initial activity could not be recovered by either washing the enzyme or incubation with any of the agents which protected against inhibition if present during the treatment (see below). A logarithmic plot of the remaining ATPase activity versus time of treatment with EPC gave

![Fig. 1. Inactivation of Na,K-ATPase and p-nitrophenylphosphatase activity by EPC.](image)

![Fig. 2. Time course of inactivation in the presence of different EPC concentrations.](image)
straight lines at all the carbodiimide concentrations used (Fig. 2) indicating that the inhibition reaction approximates first order kinetics with respect to time.

If we suppose that the carbodiimide reacts with a side-chain carboxylate group on the enzyme to give a complex which then rearranges or reacts to give a stable product (28-30), the reaction can be described by

\[
E + I \rightarrow EI \rightarrow E_i \quad (A)
\]

where \( I \) is the carbodiimide which reversibly combines with the free enzyme (\( E \)) with a dissociation constant \( K_i \). The complex (\( EI \)) is converted to the dissociated form (\( E_i \)) at a rate \( k_i \).

The formation rate of the inactive enzyme (\( E_i \)) from free enzyme (\( E \)) is

\[
\frac{dE_i}{dt} = \frac{dE}{dt} = k_i[E] = \frac{k_i}{K_i}E = k' E
\]

and integrating gives \( "E" \) the amount of free enzyme at time \( t \).

\[
E = E_0 e^{-k't}
\]

Thus, the enzyme velocity before and after inactivation is, respectively,

\[
V_e = \frac{k_i E_0[I]}{K_i + [I]} \quad \text{and} \quad V_i = \frac{k_i E_0[I]}{K_i + [I]}
\]

Then

\[
\log \frac{V_i}{V_e} = \frac{k't}{2.3} = \frac{k_i[I]}{K_i 2.3}
\]

and

\[
k' = \frac{k_i[I]}{K_i} = 0.693
\]

where \( t_{1/2} \) is the half-time of inactivation (31). From this equation it is possible to calculate the value of \( k_i/K_i \) and (as shown by Levy et al. (32)) the average order of reaction ("n") with respect to [EPC].

If it is assumed that \( n \) molecules of EPC bind to the enzyme then

\[
k' = \frac{k_i}{K_i} n^*
\]

and

\[
\log k' = \log \frac{k_i}{K_i} + n \log[I].
\]

The data from Fig. 2 have been plotted in the inset for Fig. 2 and show a linear relationship between \( \log k' \) and \( \log [I] \). The slope of unity indicates that 1 molecule of EPC binding per molecule of enzyme is sufficient to produce the inactivation. The intercept of this plot yields a value of 40 min\(^{-1}\) M\(^{-1}\) for \( k_i/K_i \).

Protection by Ligands—If Na\(^+\), K\(^+\), or Mg\(^{2+}\) ions were present during the incubation of the enzyme with EPC a protective effect was observed (Fig. 3). The effectiveness of these cations in protecting against inactivation by EPC followed the order \( \text{Mg}^{2+} > \text{Na}^+ > \text{K}^+ \) at all concentrations used (Fig. 4). Tris\(^+\) and choline which have been shown to have a weak Na\(^+\)-like effect on the enzyme (19, 37, 38) also protected, although higher concentrations were needed. The observation that 20 mM MgCl\(_2\) protected the enzyme against EPC inactivation to a greater extent than did 200 mM NaCl or 400 mM KCl showed that the protective effects were not due simply to an increase in ionic strength.

In order to analyze the effects of the cations, the simplest mechanism might be

\[
EC \xleftarrow{K_c} E + I \xrightarrow{k_i} E_i \quad (B)
\]

where the cation (C) competes with EPC (I) for the free enzyme (\( E \)). The pseudo first-order constant of inactivation (see Equation 1 above) becomes

\[
k^* = \frac{k_i[I]}{K_i \left( 1 + \frac{C^n}{K_c} \right)}
\]

where \( n \) is the number of cations that bind to the enzyme to prevent the inactivation. The ratio of the enzyme velocity in the presence of both the cation and inhibitor (\( V^* \)) to the velocity in the presence of inhibitor alone (\( V_i \)) is

\[
\log \frac{V^*}{V_i} = \frac{k_i[I]}{2.3K_i} C^n + K_c
\]

From Equations 4 and 9

\[
\log \frac{V^*}{V_i} = \frac{K_c}{C^n + K_c}
\]

and

\[
\log \frac{V^*}{V_i} = \log K_c - n \log C
\]

The linearity of the plots shown in Fig. 4B using Equation 11 shows that the data is at least consistent with this model. This plot enabled the calculation of the kinetic constants for each cation. Using the data from Fig. 4 the values for \( K_c \) and \( n \) are, respectively, 2.7 mM and 3.1 for K\(^+\), 2.7 mM and 1 for Mg\(^{2+}\), and 3.2 mM and 2.5 for Na\(^+\). While it would be inappropriate to make too much of the precise values of these kinetic constants it is interesting that (a) an appropriately small number of ions is involved and (b) the values are close to those described in the literature for activation of the various cation-dependent activities of the enzyme (1, 33, 34). None of the cations were able to fully protect against the inactivation by EPC, and their effects were not additive when more than one cation was simultaneously present (not shown). The essential cations of the p-nitrophenylphosphatase activity (K\(^+\)
and Mg$^{2+}$) showed a similar protective effect against the inactivation by EPC of this activity (see Fig. 3).

ATP, when present during the incubation of the enzyme with EPC showed no protective effect against inactivation (Fig. 3). The sequence of addition of EPC and ATP did not alter this. In the same way, when inactivation was carried out in the presence of a cation (Na$^+$, K$^+$, or Mg$^{2+}$) the addition of ATP led to no enhancement of the effect of the cation alone.

**Effects of Added Nucleophiles**—Carboxylic acids activated by carbodiimides bind nucleophiles such as glycine ethyl ester (28–30). Carbodiimides are also able to bind to other groups such as sulfhydrils, phenols, and amino groups, for example (28). However, only carboxylic acids produce an adduct favoring a nucleophilic attack and release of a urea with modification of the original residue. The addition of exogenous nucleophiles, such as glycine ethyl ester, has become a useful tool in elucidating the number and location of modified protein carboxyl residues.

A direct measurement of the binding of glycine ethyl ester (Gly-OEt) to the enzyme was performed using $[^{14}C]Gly$-OEt and EPC (Fig. 5). The correlation between the binding of Gly-OEt and inactivation of the enzyme suggested that incorporation of the nucleophile, and by extension, modification of carboxyl residues by the carbodiimide was responsible for the enzyme inactivation. Treatment with EPC and $[^{14}C]Gly$-OEt yielded a modified enzyme retaining 24% of the original activity which was analyzed by SDS-PAGE (Fig. 6). The profile of radioactivity showed that both subunits ($\alpha$ and $\beta$) of the enzyme were labeled. The pattern was not changed by the presence of ATP. However, when Na$^+$, K$^+$, or Mg$^{2+}$ was included in the incubation mixture, the binding of the nucleophile (i.e. $^{14}$C labeling) was significantly reduced. As with the inactivation experiments described above, Mg$^{2+}$ had the greatest protective effect against labeling, and in the presence of Mg$^{2+}$ ions almost all the $[^{14}C]Gly$-OEt binding was prevented. Under these conditions simultaneous measurements of binding and enzymatic activity showed that when binding was almost completely prevented the activity was only minimally inhibited.

The labeling of the protein by $[^{14}C]Gly$-OEt and the correlation between time-dependent binding of Gly-OEt and enzyme inactivation fit well with the idea that the inhibition is produced by the modification of carboxyl groups. However, when the inactivation of the enzyme was investigated at increasing concentrations of Gly-OEt a protective effect of

![Fig. 4](image-url)  
**Fig. 4.** Protective effect of cations on the inactivation of Na,K-ATPase. A, the enzyme was incubated for 10 min at 37 °C in the presence of 5 mM EPC and different concentrations of NaCl (A), KCl (O), and MgCl$_2$ (0) before assaying the Na,K-ATPase as described under "Materials and Methods." The lines through the points were drawn by eye. B, replot of the data using Equation 11 (see text).

![Fig. 5](image-url)  
**Fig. 5.** Correlation between $[^{14}C]Gly$-OEt binding and inhibition of the Na,K-ATPase. The labeling and assay of enzyme activity was performed as detailed under "Materials and Methods." The continuous line through the enzyme activity points (○) was drawn according to the equation, $v = v_O - k/K_1 + k/K_1 - k/K_2 + v_O$ (see text). The parameter values used were: 40 min$^{-1}$ $^{-1} M$ for $k/K_1$; 2.9 $\times 10^{-3}$ min$^{-1}$ $^{-1} M$ for $k/K_2$; 5 $\times 10^{-5}$ $^{-1} M$ for $N_i$; and 1.1$^{-5}$ $^{-1} M$ for $I$. The line through the labeling points (△) was drawn symmetrically to that of the enzyme inactivation.

![Fig. 6](image-url)  
**Fig. 6.** Polyacrylamide gel electrophoresis of enzyme labeled with $[^{14}C]Gly$-OEt in the presence of EPC. The enzyme (30–50 µg of protein/well) was preincubated with EPC (2.9 mM) and $[^{14}C]Gly$-OEt (5 mM) and then subjected to SDS-gel electrophoresis as indicated under "Materials and Methods." The positions of $\alpha$- and $\beta$-subunits are indicated. The background labeling obtained in the absence of EPC was subtracted. The remaining enzyme activities were (in %) 24, 20, 70, 52, and 59 for enzyme treated with EPC alone, plus ATP, Mg$^{2+}$, K$^+$ and Na$^+$, respectively.
the added nucleophile was observed (Fig. 7). The inhibition due to EPC was reduced at elevated [Gly-OEt], and with [Gly-OEt] at 120 mM almost complete activity was retained. The protective effect of the nucleophile was not restricted to Gly-OEt. Other nucleophilic reagents were tested; these included aminooethane and ethylenediamine; both of these agents were effective in protecting the enzyme against inhibition by the EPC (Fig. 7).

Gly-OEt alone had no effect on the enzyme activity in the absence of EPC (Fig. 8). The nucleophile reduced the inactivation if it was added to the enzyme suspension before or together with the carbodiimide. When addition of adequate Gly-OEt was made after the addition of EPC inactivation of the enzyme activity was observed (Fig. 8). This indicates that once the enzyme is inhibited the nucleophile does not restore activity but simply prevents further inactivation. In order to kinetically distinguish the inactivation in the presence of Gly-OEt and the protective action of Gly-OEt the time course of inactivation was measured in the absence and presence of Gly-OEt. The data in Fig. 9 shows that 10 mM Gly-OEt added at the same time as EPC drastically reduced the inactivation rate. When the same [Gly-OEt] was added 6 min later than EPC, the nucleophile reduced the inactivation rate to the value seen with simultaneous addition, but the enzyme activity already lost was not recovered. The protective effect of Gly-OEt is different from that shown by cations in that the nucleophile requires the prior binding of the carbodiimide to the enzyme to produce its effect. From the characteristics of

The protection against inhibition provided by Gly-OEt we can assume that the nucleophile (N), Gly-OEt, binds to Ei (see Reaction A) to produce an active form of the enzyme EN, with a rate constant $k_b$ with the associated loss of a dialkylurea (29, 30). Under these conditions Equation 1 becomes

$$\frac{dE_i}{dt} = \frac{k_b}{K_i} - \frac{k_p}{K_p}N_i.$$ 

The pseudo first-order rate constants can be calculated from Fig. 9. With the concentration of Gly-OEt and EPC used in this experiment a value of $3 \times 10^{-4} \text{ min}^{-1} \text{ M}^{-1}$ can be calculated for $k_b/K_i$. Thus, the ratio $k_b/k_p$ (15 mM) is the concentration of Gly-OEt at which the pseudo first-order reactions in the presence and absence of the nucleophile are equal. This means that after binding of EPC the inactivation reaction and the protective reaction with the nucleophile would occur with equal probability. Considering the error in the determinations this value is close to the concentration of Gly-OEt necessary for half-maximal protection (see Fig. 7). Enzyme which has been protected by the addition of nucleophile and which has Gly-OEt bound to its carboxyl groups behaves like normal enzyme with respect to the binding and activation by Na$^+$ and K$^+$. The activation curves of Na,K-ATPase and $\mu$-nitrophosphophatase by these cations are exactly the same in control and Gly-OEt-protected enzyme (data not shown). In the same way, the enzymes showed similar SDS-PAGE patterns of degradation by trypsin in the presence of Na$^+$ or K$^+$ (data not shown).

Inactivation by DCCD—It has been reported that EPC (0.5 mM) did not inhibit the sarcoplasmic reticulum Ca$^+$-ATPase while 100 times lower concentrations of DCCD were inhibitory (35). As this might suggest that EPC reacted in a different way with ATPases than the more commonly employed DCCD we carried out several experiments using DCCD with the renal Na,K-ATPase. The more hydrophobic and neutral carbodiimide, DCCD, inactivated the Na,K-ATPase with an apparent affinity some 50 times higher than that of EPC (Fig. 10). A pseudo first-order rate constant of 0.179 min$^{-1}$ was obtained with 0.1 mM DCCD, compared with 5 mM EPC necessary to obtain the same inactivation rate constant (Fig. 2). In the presence of 50 mM Gly-OEt, the pseudo first-order rate constant was reduced 7-fold. When the inactivation of the enzyme was measured at various [Gly-OEt], full activity of the enzyme was maintained when the [Gly-OEt] was 250 mM and [DCCD] was 0.25 mM (Fig. 11). These experiments suggest that the mechanism of inactivation of the Na,K-ATPase was similar for both carbodiimides. A lower $K_i$ could
the points with the same equation used in Fig. 4. The values used were 2.1 mM·min⁻¹ and 0.036 min⁻¹ mM⁻¹ for k1/Ki and k2/Ki, respectively.

The experiment is similar to that described in Fig. 7 for EPC. The line was drawn through the points with the same equation used in Fig. 6. The parameter values used were 2.1 mM·min⁻¹ and 0.036 min⁻¹ mM⁻¹ for k1/Ki and k2/Ki, respectively.

account for the greater effectiveness of DCCD as an inhibitor, but since higher [Gly-OEt] was required with DCCD than with EPC to prevent inactivation it suggests that the inactivation rate constant (k1) was also increased by DCCD. The ratio k1/k2 was about 58 mM for DCCD (see Fig. 10). This value is 4.5-fold higher than that obtained for EPC and is very close to the Gly-OEt concentration necessary for half-maximal protection against DCCD (Fig. 11).

FIG. 10. Inactivation of Na,K-ATPase by DCCD. The enzyme (0.024 mg/ml) was incubated at (37 °C in the presence of 0.1 mM DCCD for different times (A). Gly-OEt (50 mM) was also added to the inactivation medium (C). The continuous lines were drawn through the points with the same equation used in Fig. 6. The parameter values used were 2.1 mM·min⁻¹ and 0.036 min⁻¹ mM⁻¹ for k1/Ki and k2/Ki, respectively.

FIG. 11. Protection by Gly-OEt against the inactivation of Na,K-ATPase by 0.2 mM DCCD. The experiment is similar to that described in Fig. 7 for EPC. The line was drawn by eye. Note: the Gly-OEt concentration necessary for half-maximal protection is 66 mM.

DISCUSSION

Incubation of renal Na,K-ATPase with EPC resulted in inhibition of enzymatic activity. The presence of cations, which are known to bind to the Na,K-ATPase, during the reaction of the protein with EPC reduced the rate of inactivation (see Fig. 4). This kind of effect has previously been observed in studies of the inactivation of the enzyme by DCCD (15–17). The kinetic analysis of this protection suggested that the cations reduced the inactivation rate by competing with EPC for the free enzyme. However, this does not show that the carbodiimide and the cations compete for the same site on the enzyme as has been suggested previously (15–17). In the present work we have shown that the carboxylic residues which reacted with EPC could be modified by coupling them with Gly-OEt or other nucleophiles. The resulting enzyme showed full activity and unmodified affinities for Na and K ions. Thus, the protective effect of the cations could be due to either a "nonspecific" binding to the carboxyl groups or an allosteric effect produced by the cations binding to their true sites. The apparent affinities calculated for the effects of the cations in protecting against inactivation by EPC appear to favor the second of these two possibilities. Whichever of these (or other) mechanisms is correct (although protection against inhibition is produced by cation binding to the enzyme) the inhibition itself is not a consequence of modifying cation-protectable sites. This suggests that either there are not carboxylic acid groups at the cation-binding sites or they are not accessible for reaction with the carbodiimides under our experimental conditions. Another specific carboxylic acid group which is a candidate for carbodiimide modification leading to enzyme inhibition is the aspartyl residue which is phosphorylated by ATP during the enzyme cycle yielding an aspartylphosphate (13, 14). Since modification of the enzyme with either EPC or DCCD followed by the subsequent coupling of a nucleophile to the modified carbonyl yields a fully active enzyme it is evident that the carboxyl site of modification is not the phosphate acceptor site in the ATPase cycle.

Although the rate of inactivation of the enzyme was slowed by the presence of cations, ATP showed no such protective effect. This implies that cation-bound forms of the enzyme react less readily with EPC than free enzyme and that ATP-bound forms of the enzyme react as readily as ligand-free enzyme.

It is known that Mg²⁺, Na⁺, Tris, and choline all produce k1 forms of the enzyme, and K⁺ is known to produce an E2 form (19, 36–38). These two major conformations of the dephosphoenzyme (E1 and E2) have been characterized from fluorescence (39) and tryptic digestion experiments (27, 40). Earlier studies on the differing rates of phosphorylation of the enzyme that were a consequence of the different sequence of addition of the various ligands (Na⁺, Mg²⁺, ATP) have provided kinetic evidence for subconformers of E1 states of the dephosphoenzyme (41); the present work provides evidence for structural differences between such subconformers. The results of our studies show that the E1 Na⁺ and the E1 ATP forms of the enzyme are substantially different as manifested in their differing reactivity toward carbodiimide modification.

The present studies were undertaken to see whether or not a positively charged carbodiimide would react with and modify carboxyl residues on the Na,K-ATPase which were associated with cation-binding and cation-activating functions of the enzyme. In Fig. 12 the various possible reaction products resulting from the modification of a protein carboxyl residue with a carbodiimide and addition of an exogenous nucleophile or reaction with an endogenous nucleophile are shown. The initial product of reaction between a carboxyl residue on the protein and the reagent (I in Fig 12) results in the formation of an unstable α-acylisourea (I) which can either rearrange to give the stable N-acylurea (III) or react with a nucleophile producing an amide bond and releasing a urea to the medium (II). In the absence of added nucleophile the reaction with endogenous nucleophile can result in the formation of an intramolecular amide which internally cross-links the protein (IV).

Carbodiimides are able to bind to various chemical groups, and the reaction will depend on the particular carbodiimide, the accessibility of these chemical groups, their reactivity, and the particular experimental conditions (23, 28). In the
present study it is not possible to rule out the occurrence of EPC and/or DCCD binding and modification of different kinds of chemical groups on the Na,K-ATPase (hydroxyl, amines, carboxyl, sulphydryl groups). However, it is possible to be certain that the reaction of these carbodiimides with any group other than carboxyls had no effect on the enzyme activity since nucleophiles will only bind to carbodiimide-activated carboxyl groups.

Since carbodiimides activate carboxyls for the formation of peptide bonds (42) it is natural to expect carbodiimide modification of proteins to tend to produce cross-links. We have observed that if the EPC concentration is increased above 10 mM there is a concentration-dependent disappearance of the enzyme subunits from SDS gels. Since no other bands appear at high molecular weights we believe that an oligomeric cross-linked structure is formed between several enzyme molecules which does not enter the gels. At the low EPC concentration used in the majority of this work (1-5 mM) no change was seen in the Coomassie Blue-stained band at 95,000 daltons in SDS-PAGE. Thus, any inactivation or other effects due to carbodiimide treatment are not due to extensive interchain cross-linking between subunits of the Na,K-ATPase. In the experiment of Fig. 6 some binding of [14C]Gly-OEt to protein of molecular weight higher than that of the subunit of the enzyme was observed. This might suggest that intermolecular cross-linking has been produced. However, while this cannot be conclusively eliminated the small quantity of binding and the fact that ATP reduced this labeling while it had no effect on the inhibition are consistent with the interpretation that inhibition is produced by "intramolecular" not "intermolecular" cross-linking.

Korner et al. (43) and Lacombe et al. (44) showed that nucleophiles increased the inactivation of the ATPase of the cardiac myosin subfragment I by a water-soluble carbodiimide. This effect clearly demonstrates the necessity of the integrity of the carboxyl groups for that enzymatic activity. In the present work, at low concentrations of glycine ethyl ester in the presence of EPC, time-dependent binding of [14C] Gly-OEt and simultaneous inactivation of the enzyme could be measured. If the concentration of glycine ethyl ester was increased, the inactivation caused by EPC was completely prevented (Fig. 8). The experiments with EPC alone would suggest that cation-protectable carboxyl group modification by itself was producing the enzyme inactivation. However, the studies with added nucleophiles, aminoethane, ethylenediamine, and especially glycine ethyl ester show this is not the case. Carboxyl side chains are the only sites able to accept nucleophilic attack after the binding of a carbodiimide (29, 30). It is evident that carboxyl groups have been modified by formation of an amide bond (via activation by a carbodiimide) and the enzyme retains full activity. The observation of Yamaguchi et al. (17) that [14C]DCCD binding to the Na,K-ATPase enzyme was unstable shows that after binding the radioactivity is lost, probably via release of the label and the formation of cross-links between the activated carboxyl residue and an amino side chain. Similar observations have recently been reported using DCCD with the sarcoplasmic reticulum Ca-ATPase (45) and there the suggestion of formation of an intramolecular cross-link between activated carboxyl groups and other residues has also been made.

The conclusion that inhibition is caused by the formation of internal cross-links between carboxyl groups and endogenous nucleophiles (amino side chain) suggests that either the inhibition is a consequence of cross-linking producing a rigid nonflexible protein or alternatively the formation of the cross-links modifies an amino group necessary for activity. The present studies do not distinguish between these two possibilities. Whichever is the cause of inactivation the data strongly suggest that there is a close juxtaposition of carboxyl and amino residues in the secondary structure of the Na,K-ATPase protein. This would account for the relatively high concentration of exogenous nucleophiles necessary to protect against inactivation. DCCD is significantly more reactive against inactivation. DCCD is significantly more reactive toward the enzyme than EPC but its mode of inhibition seems to be the same as EPC. This implies that the close juxtaposition of carboxyl and amino groups (perhaps via salt linkages) occurs in relatively hydrophobic environments. The importance of such linkages has recently been emphasized for another transport system, the ion exchange system of red blood cells (46).

Addendum—After acceptance of this paper for publication a report appeared by Skou (47) describing some of the effects of modification of the Na,K-ATPase by EPC. The modification causes a decrease in the apparent affinity for K+. It was assumed that modification of carboxyl groups was responsible; the question of whether or not cross-links were formed was left open.

REFERENCES

Carbodiimide Inactivation of Na,K-ATPase