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Amino Group Modification of $(Na^+ + K^+)$-ATPase[1]

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Abstract

The effects of three amino group reagents on the activity of $(Na^+ + K^+)$-ATPase[3] and its component K^+-stimulated *p*-nitrophenylphosphatase activity from rabbit kidney outer medulla have been studied. All three reagents cause inactivation of the enzyme. Modification of amino groups with trinitrobenzene sulfonic acid yields kinetics of inactivation of both activities, which depend on the type and concentration of the ligands present. In the absence of added ligands, or with either Na^+ of Mg^{2+} present, the enzyme inactivation process follows complicated kinetics. In the presence of K^+, Rb^+, or Tl^+, protection occurs due to a change of the kinetics of inactivation toward a first-order process. ATP protects against inactivation at a much lower concentration in the absence than in the presence of Mg^{2+} (P_{50} 6 μM vs. 1.2 mM). Under certain conditions (100 μM reagent, 0.2 M triethanolamine buffer, pH 8.5) modification of only 2% of the amino groups is sufficient to obtain 50% inhibition of the ATPase activity. Modification of amino groups with ethylacetimidate causes a nonspecific type of inactivation of $(Na^+ + K^+)$-ATPase. Mg^{2+} and K^+ have no effects, and ATP only a minor effect, on the degree of modification. The K^+-stimulated *p*-nitrophenylphosphatase activity is less inhibited than the $(Na^+ + K^+)$-ATPase activity. Half-inhibition of the $(Na^+ + K^+)$-ATPase is obtained only after 25% modification of the amino groups. Modification of amino groups with acetic anhydride also causes nonspecific inactivation of $(Na^+ + K^+)$-ATPase. Mg^{2+} has no effect, and ATP has only a slight protecting effect. The K^+-stimulated *p*-nitrophenylphosphatase activity is inhibited in parallel with the $(Na^+ + K^+)$-ATPase activity. Half-inactivation of the $(Na^+ + K^+)$-ATPase activity is obtained after 20% modification of the amino groups.

Key Words: $(Na^+ + K^+)$-ATPase; amino groups; trinitrobenzene sulfonic acid; ethylacetimidate; acetic anhydride; ion-induced conformational changes.

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Introduction

The enzyme ($Na^+ + K^+$)-ATPase[3] (ATP phosphohydrolase EC: 3.6.1.3) is involved in the active transport of Na^+ and K^+ ions across mammalian plasma membranes (Bonting, 1970; Glynn and Karlish, 1975; Robinson and Flashner, 1979b; Schuurmans Stekhoven and Bonting, 1981; Jørgensen, 1982). Relatively little is known about the relation between its structure and its function, although the enzyme has been available in virtually pure form for a number of years (Jørgensen, 1975). One approach to obtain some insight into this relation is by group-specific chemical modification. Modification of arginine (De Pont *et al.*, 1977), cysteine (Patzelt-Wenczler *et al.*, 1975; Schoot *et al.*, 1977, 1978), and tyrosine residues (Cantley *et al.*, 1978) has yielded valuable information on the ATP binding center (Grisham, 1980). Effects of Na^+ and K^+ on the extent of enzyme inactivation by these reagents suggest that these activating cations affect the conformation of the enzyme (De Pont *et al.*, 1977; Cantley *et al.*, 1978; Schoot *et al.*, 1980). Comparison of the effects of these reagents on the overall activity and the so-called partial reactions (e.g., K^+-stimulated *p*-nitrophenylphosphatase activity) of the enzyme has yielded additional information about the reaction mechanism (De Pont *et al.*, 1977; Schoot *et al.*, 1977, 1978; Cantley *et al.*, 1978).

Free amino groups are present in and around the enzyme molecule in the form of lysine residues in the enzyme protein (Peters *et al.*, 1981a) and as the amino groups of the accompanying phospholipids phosphatidylethanolamine and phosphatidylserine (De Pont *et al.*, 1978). Many of the fluorescent probes, which have been used to study the conformational changes in the enzyme, such as fluorescein isothiocyanate (Karlish, 1980; Sen *et al.*, 1981; Carilli *et al.*, 1982) and pyridoxal 5-phosphate (Skou, 1982) probably react with amino groups. The same is true for the bifunctional imidates (Kyte, 1972; Sweadner, 1977; De Pont, 1979) which have been used to cross-link the subunits of the enzyme.

However, a reliable picture on the quantity and the role of the amino groups is still lacking. Hence, we have studied these groups by means of three different group-specific reagents: trinitrobenzene sulfonate, acetic anhydride, and ethylacetimidate.

[3]Abbreviations: ($Na^+ + K^+$)-ATPase: sodium plus potassium-activated adenosine triphosphatase; CDTA: *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*',*N*'-tetraacetic acid; TNBS: trinitrobenzene sulfonic acid.

Materials and Methods

Enzyme Preparation

Purified $(Na^+ + K^+)$-ATPase is obtained from rabbit kidney outer medulla microsomes by extraction with sodium dodecyl sulfate and continuous sucrose gradient centrifugation, as described by Jørgensen (1974). The preparation is freed from ATP and washed in 2 mM CDTA and 25 mM imidazole-HCl (pH 7.5) as previously described (Schoot *et al.*, 1978). The enzyme is stored at −20°C in this buffer with 0.25M sucrose added. When the enzyme is to be treated in triethanolamine buffer, it is stored at −20°C in 0.2 M triethanolamine and 0.25 M sucrose (pH 8.5). The specific $(Na^+ + K^+)$-ATPase activity of the preparations amounts to 2000–2700 μmol ATP hydrolysed · mg^{-1} protein · h^{-1}. Ouabain-insensitive activity is not detectable, and after sodium dodecyl sulfate gel electrophoresis only two bands (M_r 100 and 50 kDa) are observed.

Chemical Modification

Reaction with trinitrobenzene sulfonic acid (TNBS) is carried out either in 0.2 M triethanolamine · HCl or in 120 mM $NaHCO_3$/40 mM NaCl (pH 8.5) with 100–200 μg protein · ml^{-1} and 50–200 μM TNBS for varying times (0–60 min) at 20°C. After incubation the reaction is stopped by filtration over a column (5 × 100 mm) of Sephadex G25 coarse, using the same buffer as eluent.

Reaction with ethylacetimidate is carried out by mixing equal volumes of $(Na^+ + K^+)$-ATPase (2 mg · ml^{-1} protein) and a freshly prepared ethylacetimidate solution (variable concentration), both in 0.2 M triethanolamine · HCl (pH 8.5). The mixture is incubated for 30 min at 25°C. The reaction is stopped either by 25-fold dilution with the same buffer or by gel filtration as described above.

Reaction with acetic anhydride is carried out by mixing 20 μl enzyme (2 mg · ml^{-1} protein) with 1 ml half-saturated sodium acetate (pH 8.5) to which 100 μl 10 mM acetic anhydride is added. After incubation for 60 min at 25°C the reaction is stopped by 25-fold dilution with demineralized water, followed by centrifugation for 30 min at 200,000 *g*.

Various Assays

Immediately after stopping the reaction, aliquots are taken for determination of enzyme activities and numbers of free and modified amino groups.

Occasionally extraction of lipids is carried out according to Folch *et al.* (1957).

Assays of $(Na^+ + K^+)$-ATPase activity and K^+-stimulated *p*-nitrophenylphosphatase activity are carried out as previously described (Schoot *et al.*, 1978). Protein is determined by the method of Lowry *et al.* (1951), after precipitation with trichloroacetic acid as described by Jørgensen (1974), using bovine serum albumin as a standard. Since the Lowry method gives 35% too high protein values for $(Na^+ + K^+)$-ATPase, as compared with amino acid analysis which can be used as an absolute method, we have multiplied all parameters based on the Lowry method by a factor of 1.35 (Peters *et al.*, 1981b).

The number of free amino groups in enzyme preparations or in lipid extracts is determined fluorimetrically (Böhlen *et al.*, 1973). To each sample 1% sodium dodecyl sulfate is added immediately after reaction with the modifying reagents. Leucine is used as a standard.

After modification with TNBS the number of modified amino groups is determined in some experiments by direct spectroscopic assay of the trinitrophenylated protein and lipids. To 500 µl of the incubation mixture 175 µl 1 M HCl is added followed by 75 µl 10% (w/v) sodium dodecyl sulfate. The

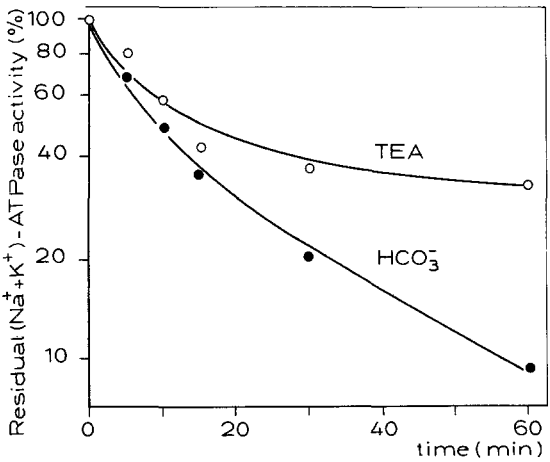

Fig. 1. Time dependence of $(Na^+ + K^+)$-ATPase inactivation by TNBS in two media. The enzyme (0.2 mg/ml) is incubated with 100 µM TNBS in either 0.2 M triethanolamine · HCl (○) or in 120 mM $NaHCO_3$/40 mM NaCl (●) (pH 8.5). The reaction is stopped by gel filtration, and $(Na^+ + K^+)$-ATPase activity is determined as described under Materials and Methods. Representatives for 3–7 experiments.

absorbance at 340 nm is measured against a blank in which TNBS is added after the addition of detergent. The number of modified lipid amino groups is determined by diluting 500 μl incubation mixture with 8 ml 0.2 M triethanolamine · HCl (pH 8.5), sedimenting the enzyme by centrifugation for 30 min at 200,000 *g* and taking up the pellet in 125 μl triethanolamine and 600 μl chloroform/methanol 2:1 (v/v). This mixture is centrifuged for 10 min at 10.000 *g* and the absorbance at 340 nm is measured against a blank without TNBS. The method is standardized by treating lysine solutions of different concentrations with 1 mM TNBS in the same way as the samples. The extinction coefficient in the HCl/sodium dodecyl sulfate solution (total amino groups) is 6200 $M^{-1} \cdot cm^{-1}$ and in the triethanolamine/chloroform/methanol solution (lipid amino groups) 8600 $M^{-1} \cdot cm^{-1}$.

Materials

Trinitrobenzene sulfonic acid (TNBS) has been obtained from BDH chemical Ltd. (Poole, England), ethylacetimidate from Eastman Kodak Co. (Rochester, New York), fluorescamine (Fluram) from Hoffman-La Roche (Nutley, New Jersey), ATP from Boehringer (Mannheim, FRG), and *p*-nitrophenyl phosphate, ouabain, CDTA, and acetic anhydride from Merck (Darmstadt, FRG). All other reagents are of analytical grade.

Results

Number of Amino Groups

The total number of amino groups in the $(Na^+ + K^+)$-ATPase preparations, determined by means of the fluorescamine assay in the presence of sodium dodecyl sulfate, is 1193 (SE 51; $n = 30$) leucine μeq. · g^{-1} protein. The phospholipid amino group content as determined previously by phospholipid analysis (De Pont *et al.*, 1978) is 591 μmol · g^{-1} protein (SE 38; $n = 15$). Amino acid analysis of the enzyme has shown that it contains 98 lysine residues per $\alpha\beta$-dimer (Peters et al., 1981a), which at a molecular weight of 163,400 protein is equivalent to 599 μmol · g^{-1} protein. Thus the sum of lysine residues and lipid amino groups amounts to 1190 μmol · g^{-1} protein, which is in excellent agreement with the value of 1193 μmol · g^{-1} protein determined by the fluorescamine assay. In a lipid extract of the enzyme preparation we find with the fluorescamine assay 675 (SE 38; $n = 20$) leucine μeq. · g^{-1} protein, which is not significantly different from 591 μmol · g^{-1} protein (SE 38; $n = 15$) amino groups of phosphatidylethanolamine and phosphatidylserine. The glucosamine and galactosamine residues, which amount to 371 μmol · g^{-1} protein (Peters *et al.*, 1981), are assumed to be acetylated.

Notwithstanding the different fluorescence of various amino acid residues (Böhlen *et al.*, 1973), the results of the two approaches (fluorescamine assay and analysis of amino acids and phospholipids) agree rather well, indicating that the fluorescamine method is a valid technique for the determination of the number of free amino groups.

Effects of TNBS on (Na^+ + K^+)-ATPase Activity

Incubation of the (Na^+ + K^+)-ATPase preparation with trinitrobenzene sulfonate (TNBS) results in a time-dependent decrease in enzyme activity. Under most conditions a curved inactivation plot is obtained, suggesting that inactivation does not follow a single first-order rate process (Fig. 1), but rather a biphasic course. A more complex inactivation process cannot be excluded, particularly since the residual activity is relatively high.

The rate of inactivation depends on the buffer used. In a mixture of 120 mM $NaHCO_3$ and 40 mM NaCl (pH 8.5) the rate of inactivation is larger than in 0.2 M triethanolamine · HCl (pH 8.5). The larger rate of inactivation in the former buffer does not seem to be due to the presence of Na^+ in the $NaHCO_3$/NaCl medium, since addition of Na^+ to the inactivation medium with triethanolamine · HCl only slightly affects the level of inactivation (Fig. 2) or the rate of inactivation (not shown) with 100 μM TNBS at 20°C.

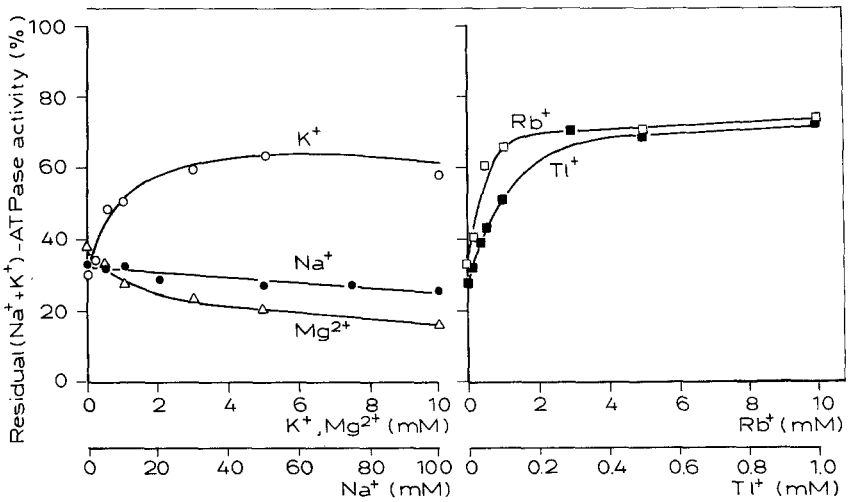

Fig. 2. Effect of cations on the inactivation of (Na^+ + K^+)-ATPase activity by TNBS. The enzyme (0.2 mg/ml) is incubated for 30 min at 20°C with 100 μM TNBS in 0.2 M triethanolamine · HCl (pH 8.5) to which KCl (○), NaCl (●), $MgCl_2$ (△), RbCl (□), or TlCl (■) is added in the concentrations indicated. Assays as in the legend to Fig. 1. Average values from 3–5 experiments.

In contrast, K^+ ions markedly decrease the extent of inactivation by TNBS, maximally at 3 mM K^+, and half-maximally at ca. 0.6 mM. This protecting effect is also found with Rb^+ and Tl^+, with Rb requiring the same concentration for K^+, whereas with Tl^+ one-tenth of this concentration is sufficient (Fig. 2).

Kinetic analysis indicates that the protecting effect of the monovalent cations K^+, Rb^+, and Tl^+ is correlated with a change in the inactivation process. In the presence of maximally protecting concentrations of these ions, the inactivation reaction seems to obey first-order rate kinetics with a $t_{1/2}$ of 35–42 min (Fig. 3). The inactivation plot also becomes linear over the time range studied, when Na^+ is replaced by K^+ in the HCO_3^-/Cl^- medium (not shown).

When both Na^+ and K^+ (total concentration 50 mM) are present in the triethanolamine · HCl medium, the protecting effect of K^+ occurs only at relatively high K^+ concentrations (Fig. 4). The half-maximally protecting effect is found at 37 mM K^+ (K/Na^+ ratio = 3), while in the absence of Na^+

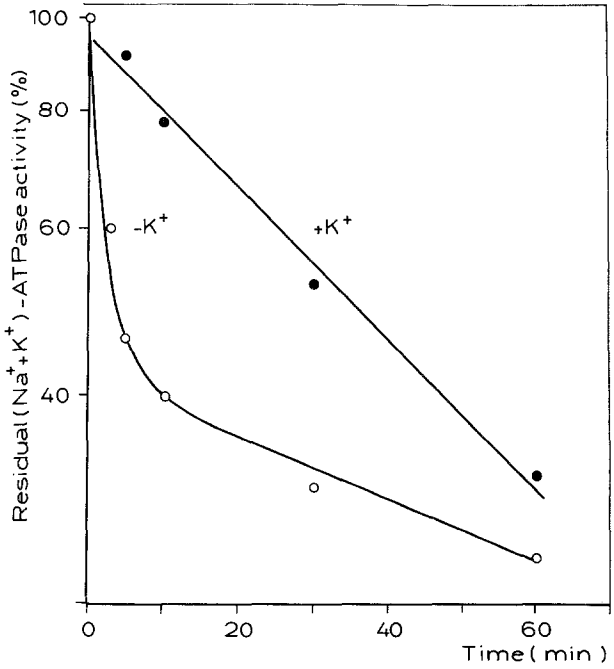

Fig. 3. Effect of K^+ on time dependence of $(Na^+ + K^+)$-ATPase inactivation by TNBS. The enzyme (0.2 mg/ml) is incubated at 20°C with 200 μM TNBS in 0.2 M triethanolamine · HCl (pH 8.5) with (○) or without (●) 5 mM KCl. Assays as in the legend to Fig. 1. Representative for 3 experiments.

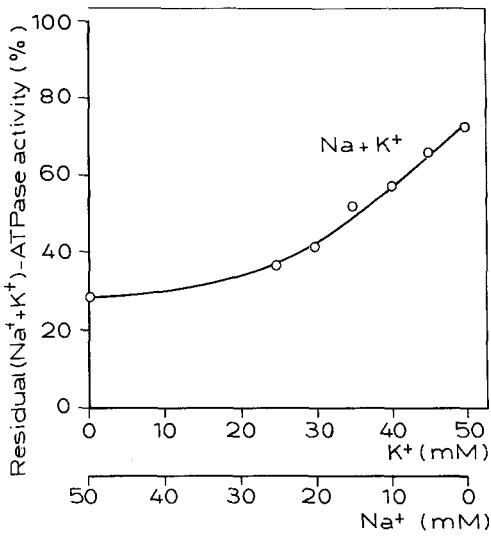

Fig. 4. Effect of Na^+ and K^+ on the inactivation of (Na^+ + K^+)-ATPase activity by TNBS. The enzyme (0.3 mg/ml) is incubated for 30 min at 20°C with 100 μM TNBS in triethanolamine · HCl (0.2 M, pH 8.5) with NaCl and KCl added in the concentrations indicated. Assays as in the legend to Fig. 1. Representative for 3 experiments.

only 0.6 mM K^+ is necessary for half-maximal protection. This indicates that Na^+ strongly antagonizes the K^+ effect.

In the presence of Mg^{2+} the inactivating effect of TNBS is enhanced (Fig. 2) with a half-maximal effect at approximately 1 mM Mg^{2+}, but in the presence of this ion the inactivation plot is still curved (Fig. 5).

ATP protects against the inactivation by TNBS (Fig. 6). In the absence of Mg^{2+} (with 2 mM CDTA to complex any free Mg^{2+}) the half-maximally protecting concentration for ATP is 6 μM, whereas 100 μM ATP nearly completely protects against inactivation. In the presence of 5 mM Mg^{2+} half-maximal protection requires 1.2 mM ATP and 10 mM is needed for complete protection.

Hiratsuka and Uchida (1973) have shown that TNBS can react with ATP with the formation of trinitrophenyl-ATP, which is a competitive inhibitor of (Na^+ + K^+)-ATPase (Moczydlowski and Fortes, 1981). However, we believe that it is unlikely that this may have played a role for the following reasons: (1) the two substances are in contact for only 30 min at pH 8.5, while Hiratasuka and Uchida left them in contact for 4 days at pH 9.5; (2) the gel filtration procedure applied after modification and before the assay would remove the trinitrophenyl-ATP; (3) the reduction of the protecting

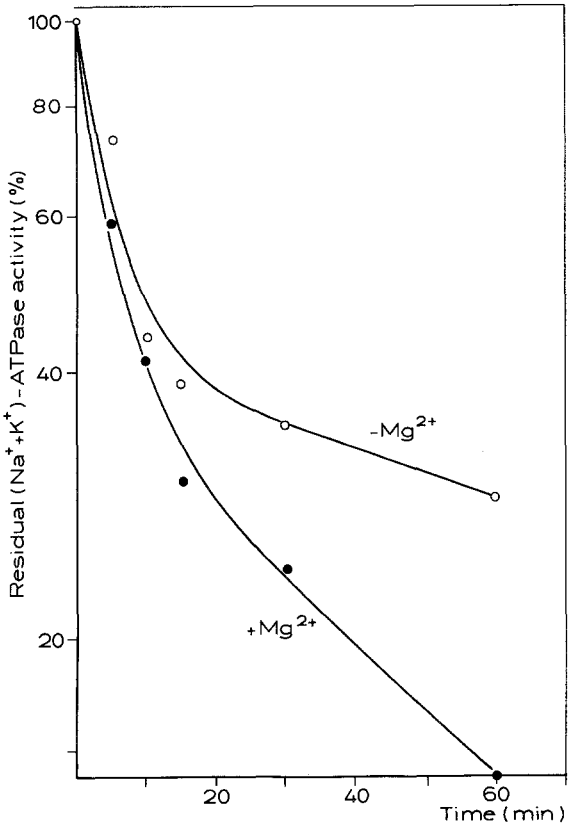

Fig. 5. Effect of Mg^{2+} on the time dependence of $(Na^+ + K^+)$-ATPase inactivation by TNBS. The enzyme (0.4 mg/ml) is incubated at 20°C with 100 μM TNBS in 0.2 M triethanolamine (pH 8.5) with (●) or without (○) 3 mM $MgCl_2$. Assays as in the legend to Fig. 1. Representative for 3 experiments.

effect of ATP by Mg^{2+} is also observed in the modification of arginine by butanedione (De Pont *et al.*, 1977) and of sulfhydryl groups by 5,5'-dithiobis-2-(nitrobenzoic acid) and *N*-ethylmaleimide (Schoot *et al.*, 1977, 1978).

Effects of TNBS on K^+-Activated p-Nitrophenylphosphatase Activity

The effect of TNBS treatment on the K^+-stimulated *p*-nitrophenylphosphatase activity has also been measured in the experiments described before. Qualitatively there is no difference between the effects on the K^+-stimulated phosphatase and the $(Na^+ + K^+)$-ATPase activities. The nonlinear inactiva-

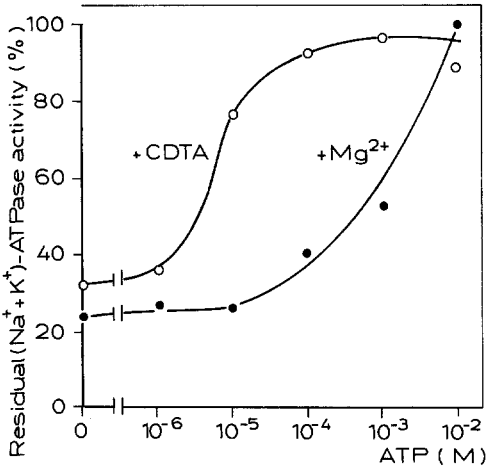

Fig. 6. Effect of ATP on the inactivation of $(Na^+ + K^+)$-ATPase by TNBS. The enzyme (0.2 mg/ml) is incubated for 30 min at 20°C with 100 μM TNBS in 0.2 M triethanolamine · HCl (0.2 M, pH 8.5). ATP is added in the concentrations indicated. Additionally 2 mM CDTA (○) or 5 mM $MgCl_2$ (●) is added to the incubation medium. Assays as in the legend to Fig. 1. Representative for 4 experiments.

tion pattern in the absence of ions and in the presence of Na^+ or Mg^{2+} is also observed for the K^+-stimulated ATPase activity. The nature of the buffer also affects the activities in similar fashion. The protection by ATP shows the same dependence on Mg^{2+} with approximately the same half-maximally protecting concentrations. In the presence of K^+, Rb^+, or Tl^+ the inactivation curve again shows first-order rate kinetics, but the $t_{1/2}$ values are 1.5 times as high as those for the inactivation of the $(Na^+ + K^+)$-ATPase activity.

Number of Amino Groups Modified by TNBS

Analysis of the number of residual free amino groups by the fluorescamine method indicates that after treatment of $(Na^+ + K^+)$-ATPase with TNBS (100 μM, 15 min, $NaHCO_3$/NaCl medium) relatively few amino groups are modified. In 9 experiments, in which the $(Na^+ + K^+)$-ATPase activity is inhibited by 62% (SE 2.8), only 10% (SE 3.0) of the amino groups are modified by TNBS; Measurement of the difference between the total number of amino groups before and after TNBS treatment shows a considerable amount of variation and is therefore not accurate enough to detect even smaller levels of chemical modification.

The number of modified amino groups after TNBS treatment can also be

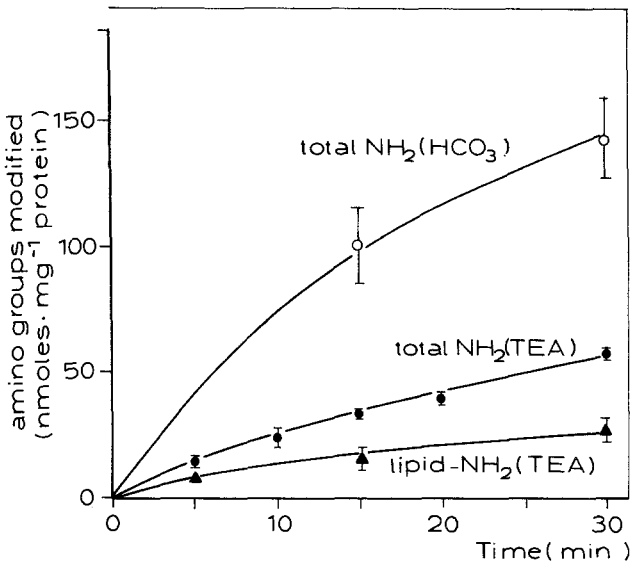

Fig. 7. Time dependence of amino group modification upon treatment of $(Na^+ + K^+)$-ATPase with TNBS. The enzyme (0.2 mg/ml) is incubated at 20°C with 100 μM TNBS in 0.2 M triethanolamine · HCl (●, ▲) or in 120 mM $NaHCO_3$/40 mM NaCl (○), both media at pH 8.5. After stopping the reaction the total number of modified amino groups (○, ●) and the number of modified lipid amino groups (▲) are determined as described under Materials and Methods. Mean with SEM from 3 experiments.

measured directly from the absorbance at 340 nm of the incorporated trinitrobenzene group. The number of modified amino groups thus determined increases with time (Fig. 7) and TNBS concentration (Table I). In the $NaHCO_3$/NaCl medium the incorporation is much larger than in the triethanolamine medium: 11% (SE 1.7; $n = 3$) modification after 15 min in $NaHCO_3$/NaCl medium, but only 3.6% (SE 0.24; $n = 3$) in triethanolamine medium under the same circumstances. An even larger difference is found when the number of modified amino groups necessary for 50% inhibition of the $(Na^+ + K^+)$-ATPase activity is measured: 122 (SE 7.6; $n = 5$) μmol · g^{-1} protein in the $NaHCO_3$/NaCl medium vs. only 24 (SE 1.8; $n = 8$) in the triethanolamine medium. The number of modified amino groups after 5 min incubation in the presence of K^+ is 44% (SE 8.0; $n = 4$) lower than in the absence of K^+ (Table I), which is in agreement with the protecting effect of K^+ on the enzyme activity. However, after 60 min incubation the number of amino groups modified by TNBS in the presence of K^+ is significantly higher (13%, SE 2.6, $n = 5$) than in the absence of K^+.

Table I. Effects of TNBS on Modification of Total and Lipid Amino Groups and on $(Na^+ + K^+)$-ATPase Activity[f]

Time	TNBS (μM)	Buffer	K^+ (5 mM)	$(Na^+ + K^+)$-ATPase activity (%)	Total amino groups modified		Lipid amino groups modified	
					μmol · g^{-1} protein	Percent[a]	μmol · g^{-1} protein	Percent[b]
5	100	TEA	–	60 ± 9.5 (3)	18 ± 1.8 (3)	1.5	11.9 ± 1.1 (3)	4.4
5	200	TEA	–	58 ± 4.8 (8)	43 ± 4.7 (4)	3.6	n.d.[e]	n.d.
5	200	TEA	+	88 ± 3.8 (3)	26 ± 6.7 (4)[c]	2.1	n.d.	n.d.
15	100	TEA	–	44 ± 4.6 (3)	43 ± 2.8 (3)	3.6	20.2 ± 4.3 (3)	7.5
15	100	HCO_3^-/Cl^-	–	37 ± 6.1 (3)	130 ± 20 (3)	10.9	n.d.	n.d.
60	200	TEA	–	25 ± 3.0 (8)	312 ± 28 (5)	26	n.d.	n.d.
60	200	TEA	+	35 ± 4.1 (8)	356 ± 41 (5)[d]	30	n.d.	n.d.

[a] Percent of total amino groups (1193 μmol · g^{-1} protein).
[b] Percent of lipid amino groups (675 mol · g^{-1} protein).
[c] Ratio modification with vs. without K^+ in paired experiments 0.56 ± 0.08 (4).
[d] The same, 1.13 ± 0.0026 (5).
[e] n.d. = not determined.
[f] $(Na^+ + K^+)$-ATPase (0.2 mg/ml) is incubated at 20°C with TNBS (concentration indicated) in either 0.2 M triethanolamine · HCl (TEA, pH 8.5) or 120 mM $NaHCO_3$/40 mM NaCl (pH 8.5) with or without 5 mM KCl. The residual $(Na^+ + K^+)$-ATPase activity and the number of total and lipid amino groups are determined as described under Materials and Methods. Values represent means with SEM, and values in parentheses represent the number of experiments.

Effects of Ethylacetimidate

Ethylacetimidate reacts with amino groups without changing the charge of the residues. Imidination leads to inactivation of the $(Na^+ + K^+)$-ATPase activity, but there are a number of important differences as compared to the effects of TNBS. Addition of Na^+ (20 mM), K^+ (10 mM), or Mg^{2+} (5 mM) has no significant effect on the degree of inactivation by ethylacetimidate. Neither does addition of $Mg^{2+} + Na^+$ or $Mg^{2+} + K^+$ affect the degree of inactivation. ATP (5 mM) has a small but significant protecting effect on the degree of inactivation (residual activity 42 ± 4.4 ($n = 13$) vs. 29 ± 4.2% ($n = 13$) of unmodified enzyme; $P < 0.05$).

The K^+-stimulated *p*-nitrophenylphosphatase activity is inhibited considerably less than the $(Na^+ + K^+)$-ATPase activity (Fig. 8). The inhibition

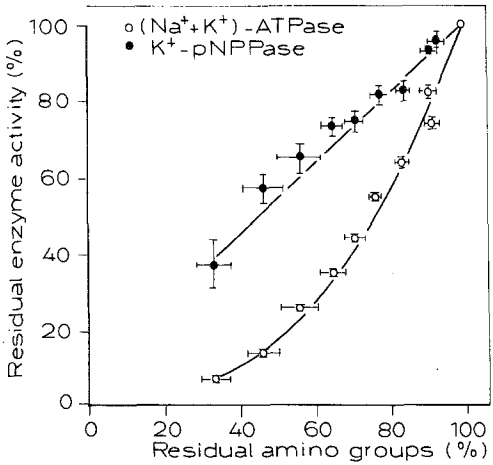

Fig. 8. $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities vs. number of amino groups modified by ethylacetimidate. The enzyme (1 mg/ml) is incubated in 0.2 M triethanolamine · HCl (pH 8.5) with ethylacetimidate (3 mg/ml) for various lengths of time. The reaction is stopped by 25-fold dilution or by gel filtration, and the residual $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities and the number of unmodified amino groups are determined. The experimental points with approximately equal residual $(Na^+ + K^+)$-ATPase activity are averaged, and these values are plotted against the number of residual amino groups (O). The values for the residual K^+-stimulated *p*-nitrophenylphosphatase activity from the same experiments are treated in the same way (●). Each point is the mean of 5–15 experimental points and is given with SEM.

of the K^+-stimulated *p*-nitrophenylphosphatase activity is not affected by Na^+, K^+, Mg^{2+}, or ATP.

The number of amino groups that must be modified in order to inhibit the ($Na^+ + K^+$)-ATPase activity is considerably larger with ethylacetimidate as modifying agent than with TNBS. Figure 8 shows that modification of 25% of the amino groups is necessary to obtain 50% inhibition of the enzyme activity. The inhibition of the K^+-stimulated *p*-nitrophenylphosphatase activity nearly parallels the degree of amino group modification (Fig. 8). The presence of Na^+, K^+, Mg^{2+}, or ATP has no clear effect on the number of amino groups modified by ethylacetimidate.

Modification with ethylacetimidate affects the lipid amino groups to the same extent as the protein amino groups. In 29 determinations in 8 experiments, in which from 20 to 70% of the amino groups were modified, the percentage of modified lipid amino groups is on the average 0.98 (SE 0.05) times that of the total modified amino groups.

Effects of Acetic Anhydride

Acetylation of amino groups by acetic anhydride alters the charge of the amino groups, but the incorporated groups are less bulky than in the case of modification by TNBS. Modification with acetic anhydride leads to inhibition of ($Na^+ + K^+$)-ATPase activity. The degree of inhibition is not affected by Mg^{2+}. ATP (5 mM) significantly protects against modification both in the presence and absence of Mg^{2+} (Table II), but this effect is much smaller than with TNBS as modifying agent.

The K^+-stimulated *p*-nitrophenylphosphatase activity is inhibited nearly in parallel with the ($Na^+ + K^+$)-ATPase activity. The effects of Mg^{2+} and ATP are the same as for the ($Na^+ + K^+$)-ATPase activity (Table II).

Half-inhibition of the ($Na^+ + K^+$)-ATPase activity upon acetylation

Table II. Influence of CDTA, Mg^{2+}, and ATP on the Effects of Acetic Anhydride on ($Na^+ + K^+$)-ATPase[a]

Additions	Residual ($Na^+ + K^+$)-ATPase activity	Residual K^+-stimulated *p*-NPPase activity (percent of control)	Amino groups modified
CDTA	19 ± 5.4 (6)	11 ± 2.5 (6)	39 ± 6.2 (4)
CDTA + ATP	34 ± 4.8 (6)	25 ± 4.1 (6)	32 ± 4.9 (4)
Mg^{2+}	14 ± 2.5 (6)	9 ± 2.9 (6)	36 ± 9.5 (4)
Mg^{2+} + ATP	24 ± 3.7 (6)	26 ± 4.3 (6)	39 ± 6.2 (4)

[a] ($Na^+ + K^+$)-ATPase (40 μg/ml) is preincubated for 60 min at 25°C with 10 mM acetic anhydride in half-saturated Na acetate (pH 8.5) with additions (5 mM each) as indicated. Values (with SE and number of experiments) are expressed as percent of controls incubated without acetic acid anhydride.

with acetic anhydride is obtained after modification of 20% of the amino groups. The acetic anhydride inactivation vs. residual amino group profile is virtually identical to that observed with ethylacetimidate (Fig. 8). The presence of Mg^{2+} or ATP does not significantly affect the number of amino groups modified.

The lipid amino groups are slightly more susceptible to acetylation than the protein amino groups. In 23 determinations in 6 experiments with modifications varying from 20 to 75%, the percentage of modified lipid amino groups is on the average 1.10 (SE 0.032) times that of the total modified amino groups.

Discussion

Amino Groups

The free amino groups in a highly purified preparation of $(Na^+ + K^+)$-ATPase from rabbit kidney represent lysine residues in the enzyme protein and phosphatidylethanolamine and phosphatidylserine in the lipid matrix. The total number of amino groups is 1190 μmol $\cdot$ g^{-1} protein, which is equivalent to 195 amino groups per $\alpha\beta$ unit. About half of these (96–115) are lipid amino groups and the other half (85–99) are lysine residues.

The amino group modifying agents used in this study are ethylacetimidate, acetic anhydride, and TNBS. Amidination, occurring with the first reagent, is a very specific modification reaction, which does not alter the charge of the amino group (Hunter and Ludwig, 1962). Acetylation is less specific and changes the charge of the amino group, but this type of modification has the advantage that the incorporated group is small. Modification of tyrosine residues in particular cannot be excluded, although the use of a half-saturated acetate medium favors acetylation of amino groups (Fraenkel-Conrat, 1957). TNBS is a relatively large and rather hydrophobic reagent, which at the chosen pH (8.5) specifically modifies amino groups, the thiol derivative being unstable at this pH (Glazer *et al.*, 1975).

Effects of Ethylacetimidate and Acetic Anhydride

Modification with the reagents used in this study is unlikely to be restricted to amino groups essential to $(Na^+ + K^+)$-ATPase activity. This is particularly true for ethylacetimidate and acetic anhydride, where modification of 40–50 amino groups per $\alpha\beta$ unit (20–25% of the total number of amino groups) is required in order to obtain 50% inactivation of the enzyme. Moreover, neither mono- nor divalent cations affect the degree of enzyme inactivation and the number of amino groups modified by these two reagents.

The protecting effect of ATP is rather small and requires a relatively high ATP concentration (5 mM). Thus, these findings do not prove involvement of essential amino groups in the high-affinity substrate binding center.

Ethylacetimidate is known to cause cross-linking between the α and β subunits (Sweadner, 1977), but this does not seem to explain the inactivation of the enzyme by this reagent. Estimation of the Coomassie Blue absorbance obtained after sodium dodecyl sulfate gel electrophoresis of ethylacetimidate-treated $(Na^+ + K^+)$-ATPase indicates that only 25% of the protein is in a cross-linked form, while the $(Na^+ + K^+)$-ATPase activity is completely lost (de Pont, unpublished observations). This indicates that the inactivation of the $(Na^+ + K^+)$-ATPase activity must by mainly due to amino group modification as such.

Our results with acetic anhydride disagree with the findings of Robinson and Flashner (1979a). They found a stimulating effect of Mg^{2+} on and marked protection by K^+ and ATP against modification with both acetic anhydride and TNBS, while we find this only for modification by TNBS. Perhaps differences in incubation conditions (lower pH and temperature, no Na^+ present in their experiments) are responsible for the difference between their results and ours.

Effects of TNBS

The results with TNBS seem to be more specific than with the other two reagents. Modification of about eight amino groups leads to a 50% inactivation. The kinetics of the inactivation is markedly influenced by cations, and ATP in micromolar concentration protects against inactivation. K^+ ions, and also Rb^+ and Tl^+, have a marked protecting effect on the inactivation by TNBS. These cations change the nonlinear inactivation process to a linear one. The concentrations of K^+, Rb^+, and Tl^+ that are needed to obtain half-maximal protection against inactivation by TNBS are about the same as for the activation of the $(Na^+ + K^+)$-ATPase activity (Bonting *et al.*, 1963) and for the K^+-stimulated *p*-nitrophenylphosphatase activity (Britten and Blank, 1968; Inturrisi, 1969). However, the stimulating effect of these cations on the activation of the $(Na^+ + K^+)$-ATPase activity is observed in the presence of Na^+, whereas their effect on the inactivation of the enzyme is measured in its absence. In the presence of Na^+ much higher K^+ concentrations are necessary to obtain protection by K^+ (Fig. 5). This suggests that the K^+-induced conformational change is antagonized by Na^+ and that the affinity for a Na^+ site is higher than that for the K^+-site involved in this conformational change.

These findings resemble in part the effects of Na^+ and K^+ on the rate of inactivation by tryptic digestion observed by Jørgensen and Petersen (1975, 1977). In that case a biphasic inactivation pattern exists in the presence of

Na^+, the rate of the second phase being only 5% of that of the first phase. In the presence of K^+ the inactivation curve becomes linear. There are also some differences: the Na^+/K^+ ratio for the half-maximal effect was 0.33 in their case and 3 in our TNBS experiments; K^+ protects against TNBS modification, but it enhances tryptic digestion; K^+ linearizes only the inactivation curve of the (Na^+ + K^+)-ATPase activity, but not that of the K^+-stimulated phosphatase. Antagonistic effects of Na^+ and K^+ have also been found in modification studies with butanedione (De Pont *et al.*, 1977), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (Cantley *et al.*, 1978), and Cu^{2+}-*o*-phenanthroline (Askari *et al.*, 1980), and in fluorescence measurements with intrinsic (Karlish and Yates, 1978, Jørgensen and Karlish, 1980) and extrinsic (Karlish *et al.*, 1978; Karlish, 1980) probes.

Magnesium ion increases the extent of inactivation by TNBS, as it does also in modification by *N*-ethylmaleimide and 5,5′-dithiobis-2-(nitrobenzoic acid) (Schoot *et al.*, 1980). The effect of Mg^{2+} is half-maximal at approximately 1 mM, at which concentration it also induces a low-affinity nucleotide binding site on the α-subunit (Schuurmans Stekhoven *et al.*, 1981, 1983). The kinetics of the inactivation process in the presence of Mg^{2+} resemble those in the presence of Na^+, but not those in the presence of K^+. This indicates that Mg^{2+} induces the E_1 conformation of the enzyme rather than the E_2 conformation.

The protecting effect of ATP is also markedly influenced by the presence of Mg^{2+}. In the absence of Mg^{2+} (CDTA present) the half-maximally protecting ATP concentration is 6 μM, implying the involvement of the high-affinity substrate binding site. In the presence of Mg^{2+}, phosphorylation occurs, leading to a change in the high-affinity binding region; the half-maximal ATP concentration of 1.2 mM in that case suggests involvement of the low-affinity nucleotide binding site (Schuurmans Stekhoven *et al.*, 1981). The same phenomenon is found upon modification of arginine with butanedione (de Pont *et al.*, 1977) and of sulfhydryl groups with *N*-ethylmaleimide (Schoot *et al.*, 1977) or 5,5′-dithiobis-2-(nitrobenzoic acid) (Schoot *et al.*, 1978). Upon modification of tyrosine residues with 7-chloro-4-nitrobenzo-2-oxo-1,3-diazole, however, the protecting effect of ATP is the same in the presence and the absence of Mg^{2+}. Protection of the inactivation reaction by ATP does not necessarily mean that the reactive groups are present in the ATP binding center. Conformational changes, induced by ATP binding and leading to a burying of essential groups elsewhere, could also explain protection.

TNBS in triethanolamine buffer modifies relatively few amino groups. Modification of only 2% of the amino groups, or about 8 amino groups per enzyme molecule, leads to 50% inactivation. Part of these amino groups belongs to phospholipids. However, there is some evidence suggesting that

modification of protein amino groups is mainly responsible. Robinson and Flashner (1979a) have delipidated a $(Na^+ + K^+)$-ATPase preparation, which was partially inactivated by treatment with acetic acid anhydride. Upon addition of exogenous lipids the same relative reactivation was obtained as for the unmodified control preparation, indicating that lipid modification does not play a considerable role. Furthermore, there does not seem to be a specific requirement of particular phospholipid polar head groups for the activity of $(Na^+ + K^+)$-ATPase as shown by studies involving enzymatic conversion of phospholipids (De Pont *et al.*, 1978).

Conclusion

This study shows that amino groups are important for the $(Na^+ + K^+)$-ATPase and K^+-stimulated *p*-nitrophenylphosphatase activities. However, the effects of amino group modification strongly depend on the choice of the modifying reagent. In our hands, TNBS gives the most specific modification, the rate and extent of which are influenced by ligands which also influence the ATPase activity. An explanation for this different behavior of the modifying reagents can only be tentative. The crucial amino groups may be located in a rather hydrophobic environment since TNBS is more hydrophobic than the two other reagents, or they may have a pK value which makes them more reactive towards TNBS. A more definitive conclusion requires knowledge of the primary structure of the enzyme.

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References

Askari, A., Huang, W. H., and Antieau, J. M. (1980). *Biochemistry* **9,** 1132–1140.

Böhlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973). *Arch. Biochem. Biophys.* **155,** 213–220.

Bonting, S. L. (1970). In *Membranes and Ion Transport* (Bittar, E. E., ed), Vol. 1, Wiley-Interscience, London, pp. 257–363.

Bonting, S. L., Caravaggio, L. L., and Hawkins, N. M. (1963). *Arch. Biochem. Biophys.* **101,** 47–55.

Britten, J. S., and Blank, M. (1968). *Biochim. Biophys. Acta* **159,** 160–166.

Cantley, L. C., Gelles, J., and Josephson, L. (1978). *Biochemistry,* **17,** 418–425.

Carilli, C. T., Farley, R. A., Perlman, D. M., and Cantley, L. C. (1982). *J. Biol. Chem.* **257,** 5601–5606.

De Pont, J. J. H. H. M. (1979). *Biochim. Biophys. Acta* **567,** 247–256.

De Pont, J. J. H. H. M., Schoot, B. M., Van Prooijen-Van Eeden, A., and Bonting, S. L. (1977). *Biochim. Biophys. Acta* **482,** 213–227.
De Pont, J. J. H. H. M., Van Prooijen-Van Eeden, A., and Bonting, S. L. (1978). *Biochim. Biophys. Acta* **508,** 464–474.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957). *J. Biol. Chem.* **226,** 497–509.
Fraenkel-Conrat, H. (1957). *Adv. Enzymol.* **4,** 247–269.
Glazer, A. N., De Lange, R. J., and Sigman, D. S. (1975). *Chemical Modification of Proteins,* North-Holland, Amsterdam p. 76.
Glynn, I. M., and Karlish, S. J. D. (1975). *Annu. Rev. Biophys.* **37,** 13–55.
Grisham, C. M. (1980). *J. Biochem. Biophys. Methods* **3,** 39–59.
Hiratsuka, T., and Uchida, K. (1973). *Biochim. Biophys. Acta* **320,** 635–647.
Hunter, M. J., and Ludwig, M. L. (1962). *J. Am. Chem. Soc.* **84,** 3491–3504.
Inturrisi, C. E. (1969). *Biochim. Biophys. Acta* **173,** 567–569.
Jørgensen, P. L. (1974). *Biochim. Biophys. Acta* **356,** 36–52.
Jørgensen, P. L. (1975). *Q. Rev. Biophys.* **7,** 239–274.
Jørgensen, P. L. (1982). *Biochim. Biophys. Acta* **694,** 27–68.
Jørgensen, P. L., and Petersen, J. (1975). *Biochim. Biophys. Acta* **401,** 399–415.
Jørgensen, P. L., and Petersen, J. (1977). *Biochim. Biophys. Acta* **466,** 97–108.
Jørgensen, P. L., and Karlish, S. J. D. (1980), *Biochim. Biophys. Acta* **597,** 305–317.
Karlish, S. J. D. (1980). *J. Bioenerg. Biomembr.* **12,** 111–136.
Karlish, S.J. D., and Yates, D. W. (1978). *Biochim. Biophys. Acta* **527,** 115–130.
Karlish, S. J. D., Yates, D. W., and Glynn, I. M. (1978). *Biochim. Biophys. Acta* **525,** 252–264.
Kyte, J. (1972). *J. Biol. Chem.* **247,** 7642–7649.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265–275.
Moczydlowski, E. G., and Fortes, P. A. G. (1981). *J. Biol. Chem.* **256,** 2357–2366.
Patzelt-Wenczler, R., Pauls, H., Erdman, E., and Schoner, W. (1975). *Eur. J. Biochem.* **53,** 301–311.
Peters, W. H. M., De Pont, J. J. H. H. M., Koppers, A., and Bonting, S. L. (1981a). *Biochim. Biophys. Acta* **641,** 55–70.
Peters, W. H. M., Swarts, H. G. P., De Pont, J. J. H. H. M., Schuurmans Stekhoven, F. M. A. H., and Bonting, S. L. (1981b). *Nature (London)* **290,** 338–339.
Robinson, J. D., and Flashner, M. S. (1979a). *Arch. Biochem. Biophys.* **196,** 350–362.
Robinson, J. D., and Flashner, M. S. (1979b). *Biochim. Biophys. Acta* **549,** 145–176.
Schoot, B. M., Schoots, A. F. M., De Pont, J. J. H. H. M., Schuurmans Stekhoven, F. M. A. H., and Bonting, S. L. (1977). *Biochim. Biophys. Acta* **483,** 181–192.
Schoot, B. M., De Pont, J. J. H. H. M., and Bonting, S. L. (1978). *Biochim. Biophys. Acta* **522,** 602–613.
Schoot, B. M., Van Emst-De Vries, S. E., van Haard, P. M. M., De Pont, J. J. H. H. M., and Bonting, S. L. (1980). *Biochim. Biophys. Acta* **602,** 144–154.
Schuurmans Stekhoven, F., and Bonting, S. L. (1981). *Physiol. Rev.* **61,** 1–76.
Schuurmans Stekhoven, F. M. A. H., Swarts, H. G. P., De Pont, J. J. H. H. M., and Bonting, S. L. (1981). *Biochim. Biophys. Acta* **649,** 533–540.
Schuurmans Stekhoven, F. M. A. H., Swarts, H. G. P., De Pont, J. J. H. H. M., and Bonting, S. L. (1983). *Biochim. Biophys. Acta* **732,** 607–619.
Sen, P. C., Kapakos, J. G., and Steinberg, M. (1981). *Arch. Biochem. Biophys.* **211,** 652–661.
Skou, J. C. (1982). *Biochim. Biophys. Acta* **688,** 369–380.
Sweadner, K. J. (1977). *Biochem. Biophys. Res. Commun.* **78,** 962–969.