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# Solubilization and Reconstitution of an Anion-Sensitive $H^+$-ATPase from Corn Roots

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**Summary.** The distribution of corn root microsomal ATPase activity on continuous sucrose gradients was nearly identical to that of membrane vesicles capable of ATP-dependent $H^+$ transport when assayed in the presence of 20 μM vanadate, 3 mM azide, 5 mM $MgSO_4$ and 50 mM LiCl. These assay conditions were used to monitor solubilization of an anion-sensitive $H^+$-ATPase from a purified membrane fraction (20–30% (wt/wt) sucrose interface) believed to be derived from tonoplast membranes.

The detergents N-tetradecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate (Zwittergent® 3-14) and deoxycholate were used to solubilize the $H^+$-ATPase. In the presence of 40% glycerol, the $H^+$-ATPase was solubilized in active form by both detergents. Glycerol was essential for the preservation of activity during solubilization and added phospholipid was required for maximal activity of the deoxycholate-solubilized, but not the Zwittergent® 3-14-solubilized, $H^+$-ATPase. Phospholipid reactivation of the deoxycholate-solubilized $H^+$-ATPase was maximal with mixed phospholipids, although several purified phospholipids gave substantial reactivation, indicating a relatively nonspecific interaction of the $H^+$-ATPase with the hydrophobic lipid environment.

Reconstitution of the deoxycholate-solubilized, anion-sensitive $H^+$-ATPase was accomplished by removal of deoxycholate by gel filtration through Sephadex G-200 in the presence of added phospholipid. ATP-dependent $H^+$ transport in reconstituted vesicles was similar to that in native vesicles with respect to substrate specificity, salt stimulation, and inhibitor sensitivity.

**Key Words** $H^+$-ATPase · solubilization · reconstitution · lipid activation · corn roots

## Introduction

Several groups have recently reported the existence of an electrogenic $H^+$-ATPase in a microsomal membrane fraction from plant cells (Sze & Churchill, 1981; DuPont, Bennett & Spanswick, 1982*a*; Mettler, Mandala & Taiz, 1982; Stout & Cleland, 1982). This $H^+$-ATPase is primarily sensitive to anions (Hager & Helmle, 1981; DuPont, Giorgi & Spanswick, 1982*c*; Stout & Cleland, 1982), and based on a kinetic analysis of $Cl^-$-stimulation of $H^+$ influx and ATPase activity, we have proposed that the $H^+$-ATPase is closely associated with an anion channel (Bennett & Spanswick, 1983). The subcellular localization of this $H^+$-ATPase remains controversial, but we, and others, have presented evidence that it is associated with tonoplast membrane vesicles (DuPont, Bennett & Spanswick, 1982*b*; Mettler et al., 1982).

Membrane fractions collected from discontinuous dextran gradients have been extremely useful in characterizing transport activity in sealed membrane vesicles from a variety of plant tissues (Sze, 1980; Sze & Churchill, 1981; DuPont et al., 1982*a*, Stout & Cleland, 1982). This fraction is, however, a collection of vesicles derived from many subcellular membranes (DuPont et al., 1982*b*) and has contributed to some confusion in identifying the particular ATPase activity responsible for the observed ATP-dependent $H^+$ transport in a mixed milieu of ATP hydrolyzing activities. In this report we identify a membrane fraction enriched specifically in an anion-sensitive $H^+$-ATPase.

In order to gain additional insights into the structural and catalytic properties of the anion-sensitive $H^+$-ATPase, it is necessary to solubilize and purify the enzyme, preferably in a reconstitutively active form. We report here solubilization of the anion-sensitive $H^+$-ATPase in a catalytically and reconstitutively active form. This represents the first step in purification of a reconstitutively active $H^+$-ATPase.

## Materials and Methods

### *Membrane Preparation*

Root tips (1.5-cm apical segments) were excised from 3 to 4 day old corn seedlings (Crow Single Cross Hybrid WF9 X Mo17, Crow Hybrid Seed Co., Milford, IL) into aerated 0.1 mM

$CaCl_2$ at room temperature. Approximately 50 grams of roots were ground with a chilled mortar and pestle in 0.25 M sucrose, 2 mM EGTA, 2 mM dithiothreitol (DTT), 0.1% bovine serum albumin (BSA), and 25 mM Bis-tris-propane/Mes (pH 7.4). The homogenate was centrifuged at $10,000 \times g$ for 10 min and the pellet discarded. For experiments with continuous sucrose gradients this low speed spin was performed once. For routine membrane preparation with discontinuous sucrose gradients (described below) the $10,000 \times g$ centrifugation was repeated, and both pellets were discarded. The resulting supernatant was centrifuged at $80,000 \times g$ for 30 min. The $80,000 \times g$ pellet was resuspended in 0.25 M sucrose, 1 mM DTT, and 5 mM Bis-tris-propane/Mes (pH 6.75) and layered onto either 15–45% (wt/wt) continuous sucrose gradients or 20, 30, 38% (wt/wt) discontinuous sucrose gradients and centrifuged at $80,000 \times g$ for 2 or 3 hr. All gradient solutions, in addition to sucrose, contained 1 mM DTT, 1 mM EDTA and 5 mM Bis-tris-propane/Mes (pH 6.75). Membranes at the interfaces of discontinuous gradients were collected with a bent Pasteur pipette, diluted, and pelleted prior to use. For solubilization, pellets were resuspended in 40% glycerol (or as indicated in text), 5 mM buffer (either Bis-tris-propane/Mes or Tris/Mes) (pH 6.75) and 1 mM DTT at a final concentration of 2 mg membrane protein/ml. Continuous gradient fractions were collected in 2-ml aliquots and assayed without pelleting.

### *Solubilization*

Deoxycholate and N-tetradecyl-N,N-dimethyl-3-ammonio-1-propane-sulfonate (Zwittergent® 3-14) were kept as 10% aqueous stocks at room temperature. Prior to membrane solubilization, the 10% stock was diluted with appropriate buffer to a concentration twice that desired for solubilization. Solubilization was initiated by adding a volume of detergent dropwise to an equal volume of membrane suspension with constant stirring. The resultant detergent-membrane sample was incubated 10 min on ice and centrifuged at $160,000 \times g$ for 45 min. The supernatant and pellet were collected separately. Activities released to the supernatant by this treatment were considered to be solubilized. Deoxycholate was purified by recrystallization from ethanol after filtration through activated charcoal (MacLennan, 1970) before use.

### *Reconstitution*

Deoxycholate was removed after membrane solubilization by gel filtration through Sephadex G-200 (Pharmacia). A $1.6 \times 26$ cm column of Sephadex G-200 was equilibrated with 2.5 mM Bis-tris-propane/Mes (pH 6.75) and 1 mM DTT (elution buffer) and maintained at 20 °C with a water jacket. A 0.5-ml aliquot of 5 mg/ml sonicated phospholipid in elution buffer was layered on and allowed to enter the column. Subsequently a 1–2 ml aliquot of deoxycholate-solubilized $H^+$-ATPase with sonicated phospholipid added to a final concentration of 10 mg/ml was layered on and eluted at a flow rate of 20–30 ml/hr. The cloudy void volume was collected and either pelleted at $160,000 \times g$ for 45 min and resuspended or assayed for ATP-dependent $H^+$ transport directly from the column.

### *Fluorescence Assays*

Formation of acid interior pH gradients were measured as quenching of fluorescence of the permeant amine dyes, quinacrine or acridine orange. Vesicles, appropriate salts, and either quinacrine or acridine orange were added to an assay buffer of 25 mM Bis-tris-propane/Mes (pH 6.75), with or without 0.25 M sucrose for native or reconstituted vesicles, respectively, to a final volume of 1.5 ml. Dye concentrations were 10 and 5 μM for quinacrine and acridine orange, respectively. Fluorescence was measured at 25 °C with a Perkin-Elmer 650-10S spectrofluorometer at excitation and emission wavelengths (exc.> em.) of 423 nm>500 nm for quinacrine and 472 nm>525 nm for acridine orange.

### *Assays*

ATPase activity was assayed at 25 °C as described previously (Bennett & Spanswick, 1983). Glucan-synthetase II, NADPH-cytochrome C reductase, and cytochrome C oxidase were assayed as described previously (DuPont et al., 1982*b*).

Protein was determined by either the method of Peterson (1977) or Bradford (1976) with BSA as standard or by the method of Bradford with bovine gamma globulin (BGG) as standard. Since each method gave a different estimate of protein for a given sample, all values were normalized to that obtained with BGG standards and the Bradford assay, which was an intermediate estimate as compared to the other two methods.

### *Lipids*

Phospholipid (PL) used in all experiments was mixed soybean phospholipid (L-α-phosphatidylcholine, type IV-S, Sigma) containing approximately 40% phosphatidylcholine. A 100-mg PL/ml stock was prepared daily in 2.5 mM Bis-tris-propane/Mes (pH 6.75) and 1 mM DTT and sonicated in a bath sonicator to near clarity. Less-pure grades of soybean phospholipid were apparently contaminated with phosphate and interfered with ATPase assays.

Purified phospholipids were purchased in chloroform, which was evaporated under a nitrogen stream prior to use. Phosphatidylcholine (PC, Sigma), phosphatidylglycerol (PG, Avanti Polar Lipids), and phosphatidylinositol (PI, Avanti Polar Lipids) were suspended in 2.5 mM Bis-tris-propane/Mes (pH 6.75) and 1 mM DTT and sonicated to clarity before use. Phosphatidylethanolamine (PE, Avanti Polar Lipids) and phosphatidic acid (PA, Calbiochem) did not disperse readily with sonication and were homogenized with a Teflon hand-held homogenizer and briefly sonicated to form a suspension prior to use. All purified phospholipids were derived from soybean except for PA which was from egg and PG which was synthesized with unsaturated fatty acids.

### *Chemicals*

ATP was obtained from Boehringer Mannhein (Indianapolis, IN) or Calbiochem (La Jolla, CA) as the disodium salt. Sodium was routinely removed by passage through Dowex 50 W and the pH adjusted to 6.75 with Bis-tris-propane. Sodium orthovanadate was from Fischer (Pittsburg, PA) and concentrations of stock solutions were verified spectrophotometrically using an extinction coefficient determined by Cantley et al. (1977). Oligomycin, gramicidin, and Zwittergent® 3-14 were obtained from Calbiochem (La Jolla, CA) and all other chemicals were from Sigma (St. Louis, MO).

## Results and Discussion

### *Membrane Fraction*

In order to begin solubilization of the anion-sensitive $H^+$-ATPase it was necessary to identify a membrane fraction enriched in this ATPase and

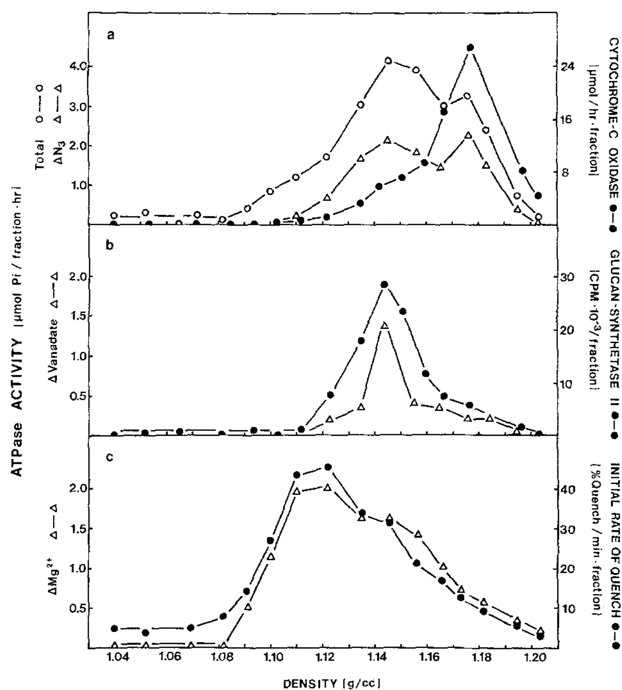


**Fig. 1.** Distribution of ATPase activity and subcellular markers on continuous gradients. Total ATPase (*a*) was assayed with 5 mM ATP, 5 mM $MgSO_4$, and 50 mM KCl. Vanadate or azide sensitive ATPase (*a* and *b*) was calculated as the difference in ATPase activity assayed with 5 mM ATP, 5 mM $MgSO_4$, 50 mM KCl and in the absence or presence of 20 μM vanadate or 3 mM azide. ATPase in *c* was assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 50 mM LiCl, 20 μM vanadate and 3 mM azide. All ATPase assays contained 2 μM gramicidin to ensure maximal activities

to define assay conditions appropriate to identify the ATPase in the solubilized state. We have previously relied on ionophore stimulation of the ATPase (DuPont et al., 1982*b*) to identify the functionally active $H^+$-ATPase in the presence of other ATP hydrolyzing activities, clearly a property only of the membrane-bound enzyme. Here, continuous sucrose gradients were used to identify an ATPase activity correlated with membranes possessing ATP-dependent $H^+$ transport. Figure 1 shows that when assayed at pH 6.75 three distinct ATPase activities could be identified. Panel *a* (Fig. 1) shows the distribution of ATPase activity which is sensitive to azide. The azide-sensitive ATPase was correlated with cytochrome C oxidase activity ($r=0.85$) and had a peak density of 1.18 g/cc. A lower density peak of azide-sensitive ATPase was consistently observed which was not correlated with cytochrome C oxidase activity. This activity may result from mitochondrial $F_1$-ATPase dislodged during isolation and adhering to lower density membrane fragments. Panel *b* (Fig. 1) shows the distribution of vanadate-sensitive ATPase activity which was well correlated with glucan-synthetase II activity ($r=0.89$) at a peak density of about 1.15 g/cc. These two activities are proposed markers for plasma membrane (Taiz, Jacobs, Gepstein & Mettler, 1981; Ray, 1979). Panel *c* (Fig. 1) shows the distribution of ATP-dependent $H^+$ transport measured by quenching of quinacrine fluorescence, which had a peak density of 1.11 to 1.12 g/cc and relatively broad distribution. Since there has been no specific inhibitor proposed for the $H^+$-ATPase responsible for the observed $H^+$ transport, ATPase activity was assayed in the presence of 3 mM azide, 20 μM vanadate, and 50 mM LiCl. It was reasoned that azide and vanadate would inhibit the other major ATPase activities and LiCl substituted for KCl since $Li^+$ has been shown to only weakly stimulate the plasma membrane ATPase (Perlin & Spanswick, 1981), while we have shown that $Cl^-$-stimulation of the functionally active $H^+$-ATPase is insensitive to the accompanying cation (Bennett & Spanswick, 1983). Under these assay conditions, the ATPase activity showed a distribution similar to ATP-dependent $H^+$ transport ($r=0.95$) and became the standard assay conditions for identifying the ATPase in solubilization experiments.

From the gradients shown in Fig. 1 and similar ones presented by DuPont et al. (1982*b*), it was possible to design a method for collecting a fraction of low density membranes enriched in anion-sensitive $H^+$-ATPase. A three-step discontinuous gradient of 20, 30, and 38% (wt/wt) sucrose was selected and the 20–30% (1.08–1.13 g/cc) interface collected for most experiments. The 30–38% (1.12–1.17 g/cc) was enriched in plasma membrane and was occasionally collected for comparative purposes.

As indicated in Fig. 1, the mitochondrial contamination of the microsomal fraction was quite high. It was found that a second $10{,}000 \times g$ centrifugation resulted in a 70% loss of cytochrome C oxidase activity, a 45% loss of azide-sensitive ATPase, and only 17 and 16% loss of vanadate-sensitive ATPase and ATP-dependent $H^+$ transport activity, respectively, in the microsomal pellet. As shown in Fig. 1, and demonstrated previously (DuPont et al., 1982*b*), ATP-dependent $H^+$ transport activity could be completely separated from mitochondrial markers. Tables 1 and 2 show the distribution of various activities in a routine membrane preparation using two $10{,}000 \times g$ centrifugations and a discontinuous sucrose gradient. Table 1 indicates that ATP-dependent $H^+$ transport associated with putative tonoplast vesicles is separable from both endoplasmic reticu-

**Table 1.** Distribution of protein, ATP-dependent $H^+$ transport, NADPH-cytochrome C reductase, and cytochrome C oxidase throughout a routine membrane preparation

| Fraction | Protein total | ATP-dependent $H^+$-Transport | | NADPH cytochrome C reductase | | Cytochrome C oxidase | |
|---|---|---|---|---|---|---|---|
| | (%) | Specific activity (%Q/mg·min) | Total (%) | Specific activity (μmol/mg·hr) | Total (%) | Specific activity (μmol/mg·hr) | Total (%) |
| ×g | | | | | | | |
| 1. 0–10,000 | 57.6 | 22.8 | 13.2 | 1.36 | 37.2 | 10.40 | 79.5 |
| 2. 10,000–10,000 | 11.7 | 39.7 | 4.6 | 2.18 | 12.0 | 9.62 | 14.8 |
| 3. 10,000–80,000 | 30.4 | 239.9 | 82.2 | 3.53 | 50.8 | 1.41 | 5.6 |
| | 100 | | 100 | | 100 | | 100 |
| Sucrose gradient | | | | | | | |
| 4. 20–30% interface | 21.6 | 271.0 | 60.0 | 3.27 | 22.1 | 0.18 | 2.1 |
| 5. 30–38% interface | 59.3 | 55.1 | 37.7 | 3.72 | 69.0 | 1.32 | 41.7 |
| 6. Pellet | 19.1 | 10.5 | 2.3 | 1.49 | 8.9 | 5.51 | 56.2 |
| | 100 | | 100 | | 100 | | 100 |

Specific activities and total protein are presented for each fraction. Percentages refer to the portion of each activity in the fraction relative to the total collected in the three differential centrifugation pellets or relative to the total collected from the three fractions of the 20–30–38% (wt/wt) discontinuous sucrose gradient. About 60% of the microsomal protein applied to the discontinuous gradient was recovered. Quinacrine was used to monitor ATP-dependent $H^+$ transport.

**Table 2.** Distribution of ATPase activities throughout a routine membrane preparation

| Fraction | ATPase activity | | | | | |
|---|---|---|---|---|---|---|
| | $\Delta N_3^-$ | | $\Delta$ Vanadate | | +Vanadate $+N_3^-$ +LiCl | |
| | Specific activity (μmol $P_i$/mg·hr) | Total (%) | Specific activity (μmol $P_i$/mg·hr) | Total (%) | Specific activity (μmol $P_i$/mg·hr) | Total (%) |
| ×g | | | | | | |
| 1. 0–10,000 | 1.747 | 70.6 | 0.457 | 51.1 | 1.156 | 56.5 |
| 2. 10,000–10,000 | 1.879 | 15.3 | 0.570 | 12.8 | 1.208 | 11.9 |
| 3. 10,000–80,000 | 0.667 | 14.1 | 0.615 | 36.1 | 1.231 | 31.6 |
| | | 100 | | 100 | | 100 |
| Sucrose gradient | | | | | | |
| 4. 20–30% interface | 0.979 | 16.2 | 1.259 | 23.0 | 3.916 | 39.4 |
| 5. 30–38% interface | 1.523 | 68.8 | 1.320 | 66.2 | 1.929 | 53.1 |
| 6. Pellet | 1.028 | 15.0 | 0.672 | 10.8 | 0.850 | 7.5 |
| | | 100 | | 100 | | 100 |

Vanadate-sensitive ($\Delta$ Vanadate) and azide-sensitive ($\Delta N_3^-$) ATPase activity is calculated as the difference in activity measured in the presence of 5 mM $MgSO_4$ and 50 mM KCl with and without either 20 μM vanadate or 3 mM azide. Specific and total activities are presented for each fraction. Percentages refer to the portion of each activity in the fraction relative to the total collected in the three differential centrifugation pellets or relative to the total collected from the three fractions of the 20–30–38% (wt/wt) discontinuous sucrose gradient. About 50–70% of the activities applied to the discontinuous gradient were recovered.

lum (NADPH cytochrome C reductase) and mitochondrial (cytochrome C oxidase) markers using this fractionation procedure. The two balance sheets (Tables 1 and 2) also indicate that while the 20–30% (wt/wt) sucrose interface is enriched in ATP-dependent $H^+$ transport and the associated anion-sensitive $H^+$-ATPase, there is contamination by vanadate- and azide-sensitive ATPase.

*Solubilization*

Treatment of membranes collected from the 20–30% (wt/wt) sucrose interface with 50 or

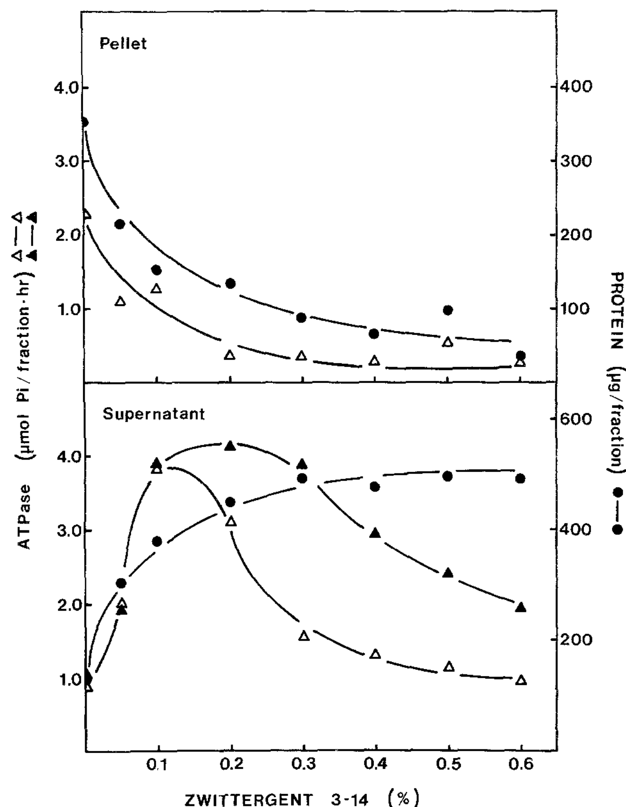


Fig. 2. Solubilization of ATPase and protein with increasing concentration of the detergent, Zwittergent® 3-14. Upper panel shows the release of ATPase (△) and protein (●) from the pellet. Lower panel shows the appearance in the supernatant of protein (●) and ATPase assayed in the absence (△) or presence (▲) of 10 mg/ml phospholipid. ATPase was assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 20 μM vanadate, 3 mM azide, and 50 mM LiCl

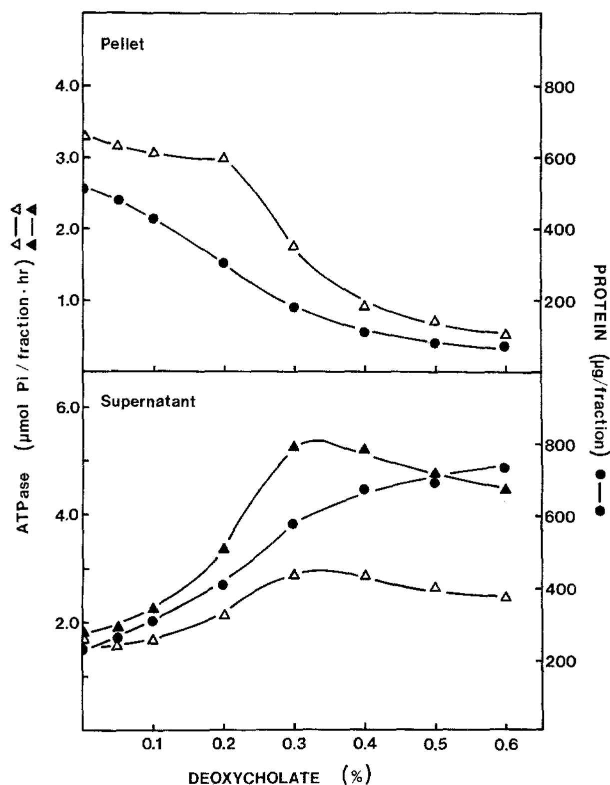


Fig. 3. Solubilization of ATPase and protein with increasing concentrations of the bile salt, deoxycholate. Upper panel shows the release of ATPase (△) and protein (●) from the pellet. Lower panel shows the appearance in the supernatant of protein (●) and ATPase assayed in the absence (△) or presence (▲) of 10 mg/ml phospholipid. ATPase was assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 20 μM vandate, 3 mM azide, and 50 mM LiCl

200 mM KI released 15 and 22% of the membrane associated protein, respectively. When the pellet collected after KI treatment was resuspended and assayed for ATP-dependent $H^+$ transport or ATPase, there was no apparent loss of either activity. The specific ATPase activity increased moderately following KI treatment as a result of the loss of loosely bound protein. The ineffectiveness of KI in releasing ATPase indicated that the $H^+$-ATPase is itself either an integral membrane protein or is tightly attached to an integral membrane protein and would require detergent treatment for effective removal from the membrane.

Two detergents, deoxycholate and Zwittergent® 3-14, were tested for their effectiveness in solubilizing the $H^+$-ATPase associated with membranes collected from the 20–30% (wt/wt) sucrose interface as described above. Since it has been shown that the detergent/protein ratio is important in achieving optimal solubilization (Kyte, 1971), membranes were resuspended at a concentration of 2 mg protein/ml for all solubilization experiments, and only the detergent concentration was varied. It has also been demonstrated that the presence of glycerol during solubilization helps maintain ATPase activity (Kyte, 1971; Bowman, Blasco & Slayman, 1981) and so glycerol was included at a concentration of 40% in the buffers and detergents used for solubilization.

Figures 2 and 3 show the effects of Zwittergent® 3-14 and deoxycholate in releasing ATPase activity and protein to the supernatant of treated membrane samples. Although both detergents effectively solubilized the ATPase in active form, there were interesting differences in the activity of the enzyme after solubilization. The lower panels of Figs. 2 and 3 show the ATPase activity released to the supernatant when assayed in the absence or presence of added phospholipid (10 mg/ml final concentration). At low concentrations of Zwittergent® 3-14 (0.1%), all of the ATPase activity was solubilized in active form and was not dependent on added phospholipid for full activity. At higher concentrations of Zwittergent® 3-14, the solubi-

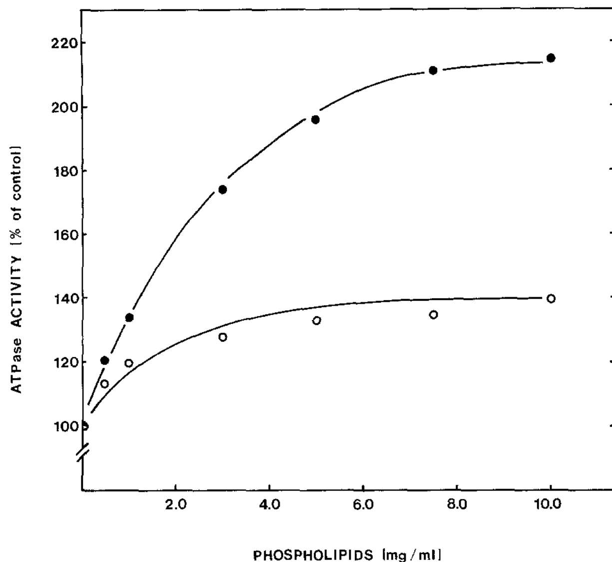


**Fig. 4.** Stimulation of 0.4% deoxycholate-solubilized ATPase activity with increasing concentration of added phospholipid. Sonicated phospholipid was added either directly to the ATPase assay (●) or to an aliquot of solubilized ATPase (o) prior to assaying ATPase activity, at the concentrations indicated. ATPase was assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 20 μM vanadate, 3 mM azide, and 50 mM LiCl and expressed relative to the activity measured in the absence of added phospholipid (100%)

lized ATPase became increasingly dependent on phospholipid for activation, presumably as a result of delipidation of the ATPase at increasing Zwittergent® 3-14 concentrations. With deoxycholate, the ATPase was dependent on added phospholipid for maximal activity at all stages of solubilization. The optimal concentration of deoxycholate for complete solubilization of the ATPase was 0.3 to 0.4%, after which inactivation of the ATPase became partially irreversible by the addition of phospholipid.

*Lipid Activation*

Activation of the 0.4% deoxycholate-solubilized ATPase by added phospholipid was further investigated. Figure 4 shows the effect of increasing phospholipid concentration on ATPase activation. Phospholipid was added at the indicated concentration directly into the assay reaction medium or to solubilized ATPase just prior to the addition of the enzyme to the assay reaction medium. Maximal activation was obtained when the phospholipid was added directly to the assay. In all subsequent assays of deoxycholate-solubilized ATPase, phospholipid was present in the assay at a final concentration of 10 mg/ml.

**Table 3.** Phospholipid activation of deoxycholate-solubilized ATPase activity with purified phospholipids

| Phospholipid added | ATPase activity % of control |
|---|---|
| None | 100 |
| Phospholipids (mixed) | 202.9 |
| Phosphatidylcholine | 152.4 |
| Phosphatidylinositol | 150.4 |
| Phosphatidic acid | 126.9 |
| Phosphatidylethanolamine | 122.1 |
| Phosphatidylglycerol | 115.4 |

Phospholipid was added at a concentration of 7.5 mg/ml as described in Materials and Methods. ATPase was assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 20 μM vanadate, 3 mM azide, and 50 mM LiCl and expressed as a percentage of the activity in the absence of added phospholipid.

Since mixed soybean phospholipid was used to activate ATPase activity after deoxycholate solubilization, it was of interest to see if this activation was the result of an interaction between the $H^+$-ATPase and a specific class of phospholipid. The effects of several purified phospholipids on activating the deoxycholate-solubilized $H^+$-ATPase are shown in Table 3. Phosphatidylcholine (PC) and phosphatidylinositol (PI) gave greater activation than the other purified phospholipids tested but all were partially effective in stimulating activity. None of the purified phospholipids were as effective as mixed phospholipid (PL). All of the lipids tested were unsaturated, but differences in fatty acid composition may have accounted for some differences in degree of ATPase activation. It is likely, however, that the activation by added phospholipid is relatively nonspecific and results from hydrophobic interactions between the $H^+$-ATPase and the lipid environment.

In order to verify that glycerol was required for the maintenance of activity during solubilization, the glycerol concentration was varied during solubilization with both Zwittergent® 3-14 and deoxycholate. The results of this experiment are shown in Table 4 where it is apparent that non-$Mg^{2+}$-requiring activity is relatively insensitive to the presence of glycerol during solubilization. The $Mg^{2+}$-dependent and LiCl-stimulated component of the ATPase activity is, however, strongly dependent on the presence of glycerol. Release of protein and ATPase activity from the membrane pellets was unaffected by the presence of glycerol (not shown). This suggests that the effect of glycerol is in the maintenance of a slightly hydrophobic environment which favors the preservation of ATPase activity following solubilization.

**Table 4.** Effect of glycerol on the maintenance of ATPase activity during solubilization

| % Glycerol | ATPase activity (μmol $P_i$/mg·hr) | | | % Cl-stimulation |
|---|---|---|---|---|
| | −Mg | +$MgSO_4$ | +$MgSO_4$ +LiCl | |
| 0.1% Zwittergent 3-14 solubilized | | | | |
| 0 | 1.12 | 0.67 | 0.78 | 16 |
| 20 | 1.10 | 3.02 | 3.90 | 29 |
| 40 | 0.44 | 4.62 | 5.77 | 25 |
| 0.4% Deoxycholate solubilized | | | | |
| 0 | 0.84 | 0.53 | 0.53 | |
| +PL | 0.80 | 0.69 | 0.72 | 5 |
| 20 | 0.75 | 0.75 | 1.04 | |
| +PL | 0.87 | 1.07 | 1.37 | 28 |
| 40 | 0.75 | 2.02 | 2.89 | |
| +PL | 0.79 | 3.37 | 4.71 | 40 |

Aliquots of membrane suspension from the 20–30% (wt/wt) sucrose interface were pelleted and resuspended in buffer containing either 0, 20 or 40% glycerol and solubilized with either 0.1% Zwittergent® 3-14 or 0.4% deoxycholate. ATPase activities were measured in the solubilized supernatant fraction. Deoxycholate solubilized ATPase was assayed in the absence and presence of 10 mg/ml phospholipid (PL). $MgSO_4$ and LiCl were added at final concentrations of 5 and 50 mM, respectively.

**Table 5.** Effect of inhibitors on membrane-bound (native) and solubilized ATPase activity

| Additions | | Native | ATPase activity (μmol $P_i$/mg·hr) (%) | |
|---|---|---|---|---|
| | | | Deoxycholate solubilized | Zwittergent 3-14 solubilized |
| None | | 4.71 (100) | 3.87 (100) | 6.33 (100) |
| $Na_3VO_4$ | (20 μM) | 4.20 (89.1) | 3.20 (82.6) | 4.91 (77.6) |
| $NaN_3$ | (3 mM) | 4.22 (89.7) | 3.52 (90.9) | 5.68 (89.7) |
| DCCD | (50 μM) | 1.68 (35.6) | 3.59 (92.7) | 3.17 (50.1) |
| DES | (50 μM) | 1.92 (40.8) | 3.85 (99.6) | 2.61 (41.2) |

Membranes from the 20–30% (wt/wt) sucrose interface were solubilized either with 0.4% deoxycholate, or 0.1% Zwittergent 3-14 and assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 50 mM LiCl, and the indicated inhibitor. Deoxycholate-solubilized ATPase was assayed in the presence of 10 mg/ml phospholipid, and all assays contained 2 μM gramicidin to ensure maximal activity in the native membranes.

### *Characterization of Solubilized ATPase*

It was also important to determine that the characteristics of the solubilized ATPase were similar to those of the membrane-bound enzyme. Table 5 lists the effects of various inhibitors on the native and solubilized ATPase. We have previously shown that $H^+$ transport and the gramicidin-stimulated ATPase activity are insensitive to vanadate and azide but very sensitive to N,N′-dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol (DES) (DuPont et al., 1982*a*). ATPase activity of native membrane showed approximately 10% inhibition by vanadate and azide, indicating the level of contamination of the membrane preparation by plasma membrane and mitochondrial ATPase, respectively. Inhibition by vandate was slightly greater in the solubilized fractions. This could result from the release of some intravesicular factor which rendered the anion-sensitive $H^+$-ATPase insensitive to vanadate as has been described for the $Ca^{2+}$-ATPase from sarcoplasmic reticulum (O'Neal, Rhoads & Racker, 1979), or from the exposure of cryptic vanadate-sensitive ATPase in sealed outside-out plasma membrane vesicles. We feel the latter interpretation is more likely, due to the relatively small increase in vanadate sensitivity. The lack of inhibition by DCCD and DES for deoxycholate-solubilized ATPase is attributed to the presence of 10 mg/ml phospholipid in the assay as required for full activation of the ATPase. Since DCCD and DES are lipid soluble, it might be expected that a majority of the inhibitor would partition into the phospholipid and be unavailable to inhibit the ATPase. This interpretation is supported by the strong inhibition by DCCD and DES of Zwittergent® 3-14 solubilized ATPase, which does not require added lipid in the assay for activation (see Fig. 2).

The effect of salts on ATPase activity of native and deoxycholate-solubilized membranes was also investigated and the results presented in Table 6. Stimulation of ATPase activity by salts was similar for both the native and solubilized ATPase. $K_2SO_4$ gave a slight stimulation of activity although $Cl^-$ salts were clearly more effective in stimulating the ATPase. The small effect of cations, especially $K^+$, may reflect contaminating plasma membrane ATPase known to be present in this membrane fraction. The strong inhibition by $KNO_3$ in the absence or presence of KCl is notable because inhibition by $NO_3^-$ has been observed for ATPase activity associated with isolated vacuoles (Walker & Leigh, 1981) but not for ATPase associated with plasma membranes (Perlin & Spanswick, 1981).

The results presented thus far indicate that the ATPase activity associated with membranes collected from a 20–30% (wt/wt) discontinuous sucrose gradient have properties similar to those we have previously described for an anion-sensitive membrane-bound $H^+$-ATPase (Bennett & Spanswick, 1983). Additionally, this ATPase can be

**Table 6.** Effect of salts on membrane-bound (native) and solubilized ATPase activity

| Additions | ATPase activity (μmol $P_i$/mg·hr) (%) | |
|---|---|---|
| | Native | Deoxycholate solubilized |
| None | 0.43 | 0.48 |
| + $MgSO_4$ | 3.18 (100) | 2.03 (100) |
| + $K_2SO_4$ | 3.44 (108.3) | 2.31 (113.8) |
| + KCl | 4.39 (138.2) | 2.86 (140.9) |
| + RbCl | 4.33 (136.2) | 2.86 (140.9) |
| + NaCl | 4.19 (131.8) | 2.83 (139.7) |
| + LiCl | 3.98 (125.1) | 2.64 (130.3) |
| + $KNO_3$ | 1.59 (49.9) | 1.36 (67.4) |
| + KCl + $KNO_3$ | 1.67 (52.5) | 1.39 (68.5) |

Native or 0.4% deoxycholate-solubilized ATPase was assayed in the presence of 5 mM ATP, 20 μM vanadate, 3 mM azide, 5 mM $MgSO_4$ (when present), and the indicated monovalent salts. Monovalent salts were added to give a final concentration of 50 mM cation, except when KCl and $KNO_3$ were added together to give 100 mM cation. Deoxycholate-solubilized ATPase was assayed in the presence of 10 mg/ml phospholipid, and all assays contained 2 μM gramicidin to ensure maximal activity in the native membranes.

**Table 7.** Ionophore-stimulation of ATPase activity associated with native, 0.4% deoxycholate-solubilized, and reconstituted membranes

| | ATPase activity (μmol $P_i$/mg·hr) | | | |
|---|---|---|---|---|
| | −G | +G | $\Delta$G | % $\Delta$G |
| Native | 5.01 | 6.92 | 1.91 | 38.2 |
| Deoxycholate solubilized | 4.60 | 4.55 | −0.05 | −1.1 |
| Reconstituted | 7.12 | 9.04 | 1.92 | 27.0 |

ATPase activity was assayed in the presence of 5 mM ATP, 5 mM $MgSO_4$, 50 mM LiCl, and in the absence or presence of 2 μM gramicidin (G). Deoxycholate-solubilized ATPase was assayed in the presence of 10 mg/ml phospholipid.

readily solubilized, in the presence of glycerol, in active form by the detergent Zwittergent 3-14 or in a form requiring added phospholipid for full activity by deoxycholate.

*Reconstitution*

Reconstitution of ATP-dependent $H^+$ transport by the solubilized $H^+$-ATPase required removal of the detergent and vesicle reformation. Since Zwittergent® 3-14 has a relatively low critical micelle concentration and forms large micelles, it was expected that this detergent would be difficult to remove by dialysis or gel filtration. Preliminary experiments confirmed this expection and further attempts to reconstitute $H^+$ transport activity were confined to the deoxycholate-solubilized $H^+$-ATPase.

Prolonged dialysis of deoxycholate-solubilized $H^+$-ATPase with added phospholipids did not reconstitute ATP-dependent $H^+$ transport. It was presumed that during the 48-hr dialysis the ATPase became inactivated and more rapid methods of detergent removal were adopted. Gel filtration through Bio-Gel P-10, as used by Allen, Romans, Kercret and Segrest (1980) and more recently Perlin and Slayman (1982) for removal of deoxycholate, gave encouraging results, with reconstitution of approximately 30% of transport activity as judged by the recovery of gramicidin-stimulated ATPase activity (not shown). It was determined that the presence of glycerol in the column equilibration and elution buffers prevented reconstitution of ATP-dependent $H^+$ transport. It was also found that addition of phospholipids (10 mg/ml final concentration) to the solubilized ATPase greatly enhanced reconstitution and that unlike other reports (Chiesi, Peterson & Acuto, 1978), the addition of 0.2 M KCl did not enhance reconstitution. During the course of these experiments, an ultraviolet absorbance monitor was used to identify the cloudy void volume, and it was noticed that reconstitution of $H^+$ transport was less effective when this monitor was in use. Subsequent experiments indicated that ultraviolet (290 nm) irradiation abolished approximately 50% of $H^+$ transport activity in reconstituted vesicles. In later experiments, the cloudy void volume was detected visually.

Gel filtration with Sephadex G-200 was later tested and reconstitution of ATP-dependent $H^+$ transport approached 90–100% as judged by gramicidin stimulation of ATPase activity (Table 7) and quenching of acridine orange fluorescence (Fig. 5). Improved reconstitution with Sephadex G-200 may be simply related to the inherently lower flow rates obtained with Sephadex G-200 as compared with Bio-Gel P-10. The detailed protocol giving consistently high levels of reconstitution such as those shown in Fig. 5 and Table 7 is described in the Materials and Methods section and was adhered to for subsequent experiments. In order to be sure that we had in fact reconstituted the anion-sensitive $H^+$-ATPase and not some other contaminating ATPase, comparisons of substrate specificity, salt stimulation, and inhibitor sensitivity between the native and reconstituted vesicles were performed. Figure 6 shows the comparison between native and reconstituted vesicles

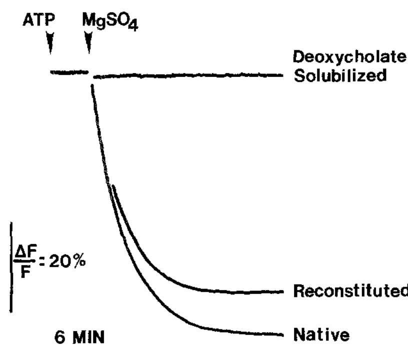


**Fig. 5.** ATP-dependent $H^+$ transport with native, 0.4% deoxycholate-solubilized, and reconstituted membranes. Equal amounts of protein (175 μg) of either native, deoxycholate-solubilized, or reconstituted membranes were assayed for ATP-dependent quenching of acridine orange fluorescence as in Materials and Methods. Active $H^+$ influx was initiated by the sequential addition of ATP and $MgSO_4$ at final concentrations of 5 mM at the indicated arrows

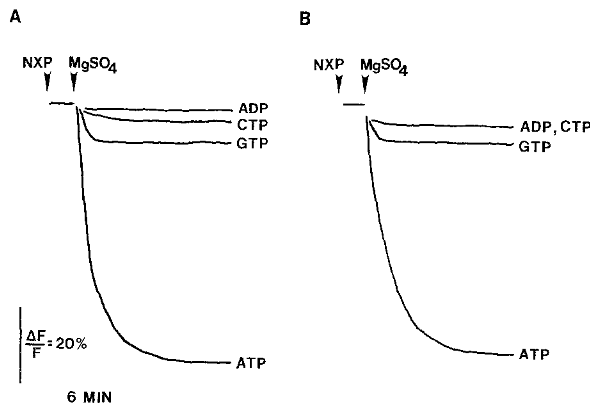


**Fig. 6.** Comparison of various nucleoside phosphates (ATP, GTP, ADP, CTP) to support $H^+$ transport in native and reconstituted vesicles. Fluorescence quenching of acridine orange was measured as in Materials and Methods. Nucleoside phosphate (NXP) and $MgSO_4$ were added to final concentrations of 5 mM at the indicated arrows. Fluorescence intensity was adjusted after the addition of nucleoside phosphate (NXP) to give the same fluorescence reading in the presence of all substrates. Fluorescence quenching was measured with either native (*A*) or reconstituted membrane vesicles (*B*)

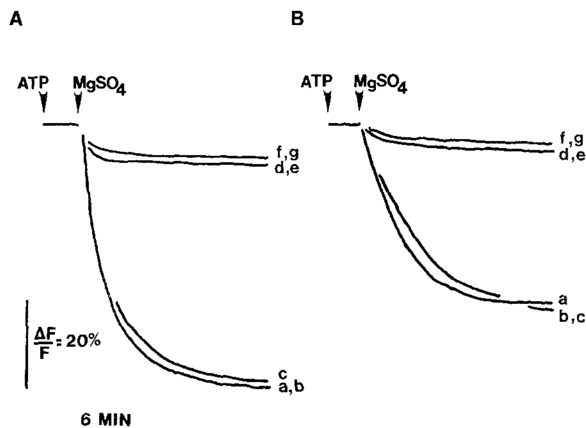


**Fig. 7.** Effect of salts on ATP-dependent $H^+$ transport in native and reconstituted vesicles. Fluorescence quenching of acridine orange was measured as in Materials and Methods with the following salts added: 50 mM KCl (*a*), 50 mM choline Cl (*b*), 50 mM LiCl (*c*), 50 mM $KNO_3$ (*d*), 50 mM $KNO_3$ + 50 mM KCl (*e*), 25 mM $K_2SO_4$ (*f*), none (*g*). ATP and $MgSO_4$ were added at final concentrations of 5 mM at the indicated arrows. Fluorescence quenching was measured with either native (*A*) or reconstituted membrane vesicles (*B*)

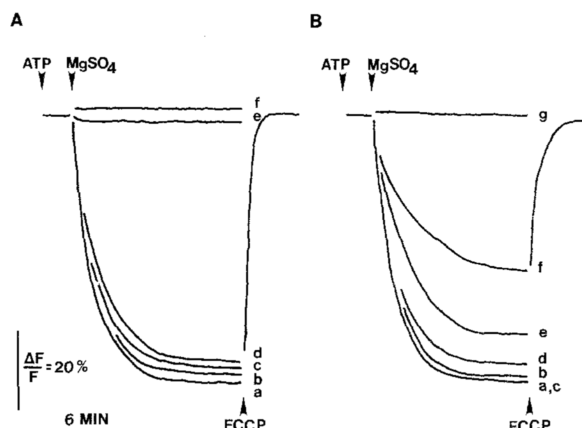


**Fig. 8.** Effects of inhibitors on ATP-dependent $H^+$ transport in native and reconstituted vesicles. Fluorescence quenching of acridine orange was measured as in Materials and Methods with the following inhibitors added: 50 μM orthovanadate (*b*), 5 μg/ml oligomycin (*c*), 200 μM orthovanadate (*d*), 50 μM DCCD (*e*), 50 μM DES (*f*), and 200 μM DES (*g*). Control traces are labeled (*a*). ATP and $MgSO_4$ were added at final concentrations of 5 mM and FCCP at a concentration of 2 μM at the indicated arrows. Fluorescence quenching was measured with either native (*A*) or reconstituted membrane vesicles (*B*)

in the ability of various nucleoside phosphates to support $H^+$ transport. It is apparent that ATP:Mg is the preferred substrate with GTP:Mg, and ADP:Mg supporting only low rates of $H^+$ transport in both native and reconstituted vesicles. While the corn root anion-sensitive $H^+$-ATPase is in many ways similar to the lysosomal, chromaffin granule, and yeast vacuolar $H^+$-ATPases (Schneider, 1981; Pollard, Zinder, Hoffman & Nikodejevic, 1976; Kakinuma, Ohsumi & Anraku, 1981), the inability of GTP:Mg to support $H^+$ transport in corn root vesicles is a major point of difference with respect to the other $H^+$-ATPases.

The effects of various salts in stimulating ATP-dependent $H^+$ transport is shown in Fig. 7. In the presence of $MgSO_4$, 25 mM $K_2SO_4$ has no effect on $H^+$ transport, whereas all of the 50 mM $Cl^-$ salts strongly stimulate $H^+$-transport. The addition of 50 mM $KNO_3$ strongly inhibits $H^+$ transport even in the presence of KCl, which is in agreement with the effects of $KNO_3$ on ATPase activity shown in Table 6. Again the salt effects for the native and solubilized vesicles are similar.

The effects of various inhibitors on $H^+$ transport are shown in Fig. 8. Oligomycin (5 μg/ml) and vanadate (50 and 200 μM) had only a small effect on ATP-dependent $H^+$ transport. The slight inhibition by oligomycin (5 μg/ml) was consistent and confirms the report by Hager and Helmle (1981) of moderate inhibition of ATP-dependent $H^+$ transport in similar membrane vesicles from corn coleoptiles, by high concentrations of oligomycin. DCCD (50 μM) and DES (50 μM) strongly inhibit transport in the native vesicles, but when tested with reconstituted vesicles, they had a much smaller inhibitory effect. We interpret this result as we did the results of inhibitor sensitivity of the solubilized ATPase. Reconstituted vesicles were prepared in the presence of 10 mg/ml added phospholipid, some portion of which is present in the fluorescence assay. The inhibitors DCCD and DES presumably partition into this excess lipid, reducing their effective concentration in free solution. This interpretation is supported by the ability of higher concentrations of DES (200 μM) to abolish ATP-dependent $H^+$ transport.

Overall, Figs. 6–8 indicate identical characteristics for ATP-dependent $H^+$ transport in native and reconstituted membrane vesicles. This suggests that in the reconstituted system only one ATPase has been functionally reconstituted, and it is the anion-sensitive $H^+$-ATPase identified in low density membrane vesicles. The characteristics shown in Figs. 6–8 also confirm our previous characterization of this $H^+$-ATPase (Bennett & Spanswick, 1983; DuPont et al., 1982*a*).

## Conclusions

We have previously reported characteristics of the anion-sensitive $H^+$-ATPase from corn roots (Bennett & Spanswick, 1983) which indicated a striking similarity to the chromaffin granule $H^+$-ATPase. This is of interest here since evidence has been presented suggesting that the chromaffin granule $H^+$-ATPase has structural similarities with the mitochondrial $F_1$-ATPase (Apps & Schatz, 1979), and it seems likely that the anion-sensitive $H^+$-ATPase from corn roots, studied here, would share structural similarities with the chromaffin granule $H^+$-ATPase. Inhibition of ATPase activity by $NO_3^-$ has been reported for the mitochondrial $F_1$-ATPase (Ebel & Lardy, 1975), and the plant cell tonoplast $H^+$-ATPase (Table 5; Walker & Leigh, 1981), suggesting similarities between these ATPases. The relative insensitivity of the non-mitochondrial ATPase to oligomycin, however, indicates a major difference between these enzymes. It is hoped that the results presented in this paper will lead rapidly to the purification of a reconstitutively active anion-sensitive $H^+$-ATPase from corn roots, which will be useful for detailed structural analysis.

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