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Salt-stimulated Adenosine Triphosphatase from Smooth Microsomes of Turnip[1]

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ABSTRACT

The turnip (*Brassica rapa* L.) microsome fraction contains both a Mg^{2+}-inhibited acid phosphatase and a salt-stimulated Mg^{2+}-activated ATPase. However, as the pH optimum of the ATPase was 8.0 to 8.5, the acid phosphatase activity could be eliminated by assaying at or above pH 7.8. The ATPase was concentrated in a fraction equivalent to the smooth microsomal membranes and was not due to fragments of mitochondria. The salt-stimulated activity showed specificity for anions rather than cations. The activity was further stimulated by carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP), 2,4-dinitrophenol, valinomycin, nigericin, and NH_4Cl. There was a synergistic effect between CCCP and valinomycin. Activity was insensitive to oligomycin phlorizin, ouabain, and atractylate. Based on similarity to the chloroplast ATPase, it was proposed that this ATPase was situated on the outside of the vesicle.

It is suggested that the ATPase is involved in the movement of ions, particularly anions, and may be related to the anion accumulation mechanism, which is known to occur across the tonoplast of such tissues.

Salt-stimulated ATPases have been studied in both plant and animal tissue homogenates in an effort to establish the mechanism of cellular ion transport. An animal microsomal ATPase which requires Mg^{2+} and is synergistically stimulated by Na^+ and K^+ has been well characterized (25). However, this activity has now been shown to be concentrated in the plasma membranes which sediment with the endoplasmic reticulum membranes and can be largely separated from them using density gradients (2, 9). Plant workers have concentrated on identifying a corresponding microsomal Na^+-K^+-ATPase activity. Early work was complicated because of a highly active acid phosphatase and the so-called ATPases were generally characterized by (1, 3, 4, 6, 10): (*a*) low pH optima; (*b*) lack of substrate specificity; (*c*) also being present in the soluble supernatant fraction; (*d*) inhibition by Mg^{2+}; (*e*) stimulation (but not synergistically) by Na^+ and K^+; (*f*) insensitivity to ouabain. Atkinson and Polya (1) concluded that the activity in carrot, beet, and *Chara* was entirely due to acid phosphatase.

More recently, specific ATPases which require Mg^{2+} have been reported in particulate fractions from plants. Activity was sometimes stimulated by monovalent ions but was insensitive to ouabain. Hansson and Kylin (11) and Kylin and Gee (12) reported two particulate ATPase activities with acid pH optima, which were stimulated by Na^+ and K^+ depending on the ratio of ions. This appears to be the only report in plants of such a synergistic effect. Sexton and Sutcliffe (24) reported an ATPase in young pea roots with a neutral pH optimum but which was not monovalent-ion stimulated. Reid *et al.* (22), Fisher and Hodges (7), Fisher *et al.* (8), and Lai and Thompson (13) have all reported particulate ATPases with pH optima at about 8.5. All of these were stimulated by monovalent ions (not synergistically) but only the enzymes of Fisher and Hodges (7) and Fisher, *et al.* (8) required both Mg^{2+} and monovalent ion for maximum activity.

This paper describes the separation and properties of an ATPase from the microsomal fraction of turnip, which requires Mg^{2+} and is stimulated by a variety of ions with specificity for the anion. The activity, which is concentrated in the smooth membrane fractions of the microsomes, is interpreted as an ion-translocating ATPase. An attempt is made to correlate the properties of this ATPase with those of the ion accumulation process of intact plant cells.

MATERIALS AND METHODS

Commercially obtained white turnip (*Brassica rapa* L.) (600 g) was homogenized in a Braun juice extractor into 40 ml of 0.3 M sucrose containing 1 g of bovine serum albumin (fraction V powder). The pH was kept at 7.4 during homogenization by the dropwise addition of 1 M tris. The mixture was strained through muslin and centrifuged at 27,000*g* (maximum) for 15 min to remove cell debris, mitochondria, and the larger mitochondrial fragments. The supernatant was then centrifuged at 78,000*g* (average) for 90 min to sediment the microsomes. Fractionation of the microsomes followed the method of Glauman and Dallner (9). The microsomal pellet was resuspended in 90 ml of 0.25 M sucrose containing 15 mM CsCl, and aliquots of 7 ml were layered over 4.5 ml of 1.3 M sucrose containing 15 mM CsCl. Centrifuging at 50,000 rpm (type 50Ti rotor) for 90 min resulted in a pellet (fraction 1) and a layer at the sucrose boundary (fraction 2). The pellet was discarded and the layer resuspended in 0.25 M sucrose, centrifuged at 150,000*g* (average) for 45 min, and finally resuspended in 8 ml of 0.25 M sucrose. All apparatus was prechilled, and all operations were performed at 4 C.

The complete medium for the ATPase assay contained 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.8), 2 mM ATP, 2 mM $MgCl_2$, 50 mM KCl, and 0.1 ml of fraction 2 microsomes (approx. 0.2 mg of protein) in a total volume of 1.0 ml. The reaction was started by the addition of microsomes, shaken for 30 min at 25 C then terminated by the addition of 1.0 ml of 5.5% perchloric acid and diluted to 5.0 ml with water. Inorganic phosphate was estimated on the total sample by the method of Marsh (17) (this method eliminates molybdate

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Table I. *ATPase Distribution among Microsomal Fractions*

Separation of fractions 1 and 2 is described in "Materials and Methods." Fraction 2 was further subfractioned by centrifuging it over 4 ml of 0.9 M sucrose in the presence of 10 mM $MgCl_2$ at 150,000*g* for 90 min. This resulted in a pellet (2 heavy) and a layer (2 light). All three fractions were washed and resuspended in 10 ml of 0.25 M sucrose. ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.8), and 2 mM ATP in a total volume of 1.0 ml.

Fraction	1	2 Light	2 Heavy
	nmoles Pi/min·mg protein		
ATPase			
Control	0.8	2.3	3.0
+ 2 mM $MgCl_2$	2.6	7.0	8.8
+ 50 mM KCl	1.6	3.8	5.4
+ 2 mM $MgCl_2$ + 50 mM KCl	5.6	12.3	13.8
	μg/mg protein		
RNA	669	96	57
	nmoles cyt c reduced/min·mg protein		
NADH-cytochrome *c* reductase	3.2	7.7	20.5

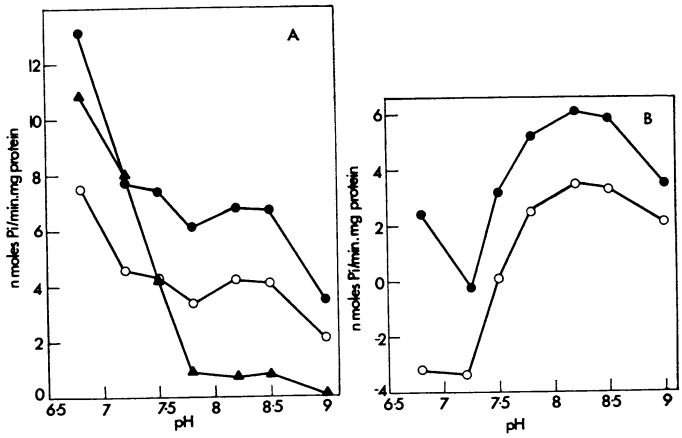

FIG. 1. A: Effect of pH on the ATPase activity in the absence of ions (▲) and in the presence of 2 mM $MgCl_2$ (○) and 2 mM $MgCl_2$ plus 50 mM KCl (●). ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer, and 2 mM ATP in a total volume of 1.0 ml. B: Effect of pH on the ATPase activity in the presence of ions with the control in the absence of ions subtracted.

catalysed acid hydrolysis of ATP). Protein was estimated by the method of Lowry *et al.* (16) using bovine serum albumin as the protein standard and RNA by the method of Loening (15).

Biochemicals were obtained from Sigma Chemical Co. Ouabain was obtained from British Drug Houses Ltd., CCCP[2] and valinomycin from Calbiochem Inc., and phlorizin from Fluka. Nigericin was a gift from Dr. K. R. West.

RESULTS

Subfractionation of Microsomal Membranes. Table I shows the distribution of RNA, antimycin A-insensitive NADH-cytochrome *c* reductase, and ATPase in the various microsomal subfractions. It can be seen that the ATPase activity was concentrated in the fractions low in RNA (*i.e.*, smooth membranes without attached ribosomes). The ATPase was activated by $MgCl_2$, and it was only in the presence of this catalyst that 50 mM KCl caused a large stimulation of activity. In all further experiments, the total smooth fraction 2 (light + heavy) was used. Similar ATPase activity was detected in artichoke but not beetroot microsomes.

Conditions for Optimal Activity. The pH profiles for phosphatase activity with ATP, in the presence of $MgCl_2$ and $MgCl_2$ + KCl, are shown in Figure 1, A and B. At low pH, activity was probably due to acid phosphatase which was partially inhibited by Mg^{2+} (1). Only at or above pH 7.8 where the acid phosphatase was inactive, did the KCl-stimulated Mg^{2+}-ATPase become evident. At pH 7.5, $MgCl_2$ had no effect, since inhibition of acid phosphatase and stimulation of ATPase were equal. All subsequent reactions were assayed at pH 7.8.

Figure 2 shows that 2 mM $MgCl_2$ was the most effective concentration for activation in the presence of 50 mM KCl. In the absence of KCl, the increase in activity with higher concentrations of $MgCl_2$ was probably due to the increasing concentrations of Cl^- (Fig. 3). Ca^{2+} and Mn^{2+} did not substitute for Mg^{2+} (Table II). Also Ca^{2+} partially inhibited and Mn^{2+} severely inhibited the KCl-stimulated activity in the presence of Mg^{2+} (Table II). Figure 3 shows that 50 mM was the most effective concentration of KCl in the presence of 2 mM $MgCl_2$. At higher concentrations there was considerable inhibition. Optimum ATP concentration in the presence of 2 mM $MgCl_2$ and 50 mM KCl was about 6 mM.

Specificity of Salt Stimulation. The specificity of salt (50 mM) in stimulating the Mg^{2+}-ATPase is shown in Table III. With the exception of NH_4Cl and CsCl, there was very little difference in the effects of the various cations (with Cl^- as the anion) (Table III, A). Even choline and tris chlorides were as effective as KCl. The smaller stimulation by Na^+ than by K^+ was not reproducible (see Table V), and there was no significant synergistic effect between Na^+ and K^+. Table III, B, however, shows that there was considerable variation in activity with different anions (with Na^+ and K^+ as the cations). This

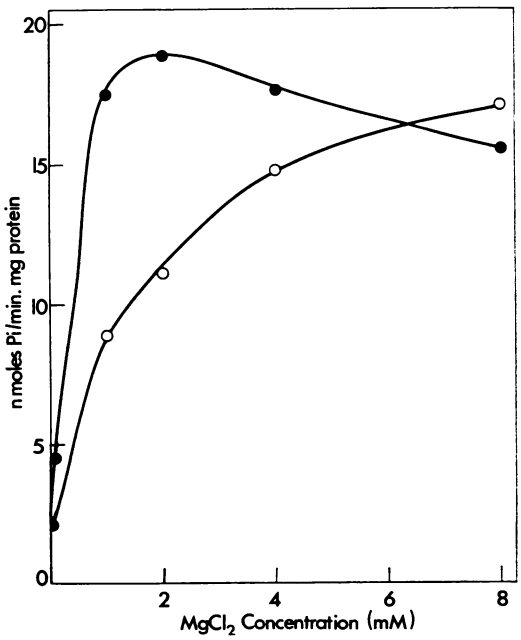

FIG. 2. Effect of $MgCl_2$ on the ATPase activity in the absence (○) and presence (●) of 50 mM KCl. ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.8), and 2 mM ATP in a total volume of 1.0 ml.

[2] Abbreviations: CCCP: carbonylcyanide *m*-chlorophenylhydrazone; DNP: 2,4-dinitrophenol.

Table II. *Effect of Divalent Cations on the ATPase Activity*

ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.8), and 2 mM ATP in a total volume of 1.0 ml.

Divalent Cation	ATPase Activity	
	−KCl	+50 mM KCl
	nmoles Pi/min·mg protein	
Control	2.06	4.46
2 mM $MgCl_2$	8.41	17.00
2 mM $CaCl_2$	3.26	3.09
2 mM $MnCl_2$	2.06	1.03
2 mM $MgCl_2$ + 2 mM $MgCl_2$	11.51	15.98
2 mM $MgCl_2$ + 2 mM $CaCl_2$	8.93	11.82
2 mM $MgCl_2$ + 2 mM $MnCl_2$	2.92	2.75

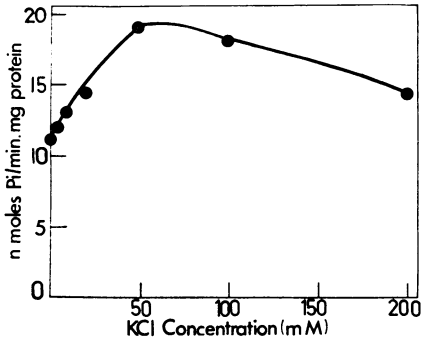

FIG. 3. Effect of KCl on the ATPase activity. ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.8), 2 mM $MgCl_2$, and 2 mM ATP in a total volume of 1.0 ml.

indicated that it was the anion rather than the cation species that was important in salt stimulation. Table III, C, shows that a range of similar 4-carbon organic anions all stimulated the Mg^{2+}-ATPase and, with the exception of succinate, to a greater degree than KCl.

Effect of Membrane Disruption. Electron micrographs (unpublished) showed that fraction 2 microsomes consisted of smooth, single membrane vesicles. The effect of the detergents, digitonin, and Triton X-100, on the microsomes was tested to determine if such a structure limited ATPase activity (Fig. 4). While very low concentrations of Triton stimulated activity 6-fold, slightly higher concentrations inhibited activity. Digitonin, however, stimulated activity only slightly, at the same concentrations, demonstrating the different action of these two detergents on microsomal membranes. As the acid phosphatase activity was unaffected by Triton X-100, it was possible to minimize further acid phosphatase contamination by assaying with a very low concentration of microsomes in the presence of Triton.

Relation to other ATPases. Table IV lists the sensitivity of the KCl-stimulated Mg^{2+}-ATPase to a number of compounds. The anions which inhibited the Mg^{2+}-ATPase (Table III, B) also partially inhibited (at 5 mM) the KCl-stimulated Mg^{2+}-ATPase. The marked inhibition by ADP (not observed with the acid phosphatase) supports the idea of a specific ATPase. The lack of effect of NADH + NADP indicated that the plant microsomal ATP-dependent NADH-NADP transhydrogenase was not involved (20). The activity was insensitive to ouabain, an inhibitor of active transport in animal cells (25).

The ATPase was also insensitive to inhibitors of the mitochondrial (oligomycin) and chloroplast (phlorizin) ATPases but was stimulated by the uncouplers CCCP and DNP, as well as valinomycin and nigericin. Table V shows that there was a synergistic effect between CCCP and valinomycin such that the stimulation due to their combination was greater than the sum of the individual effects. There was a partial specificity for K^+ over Na^+ with valinomycin.

Attempts were made to detect changes in ATPase activity in aging tissue slices that could be correlated with the well established increase in ion accumulation (14). In turnip slices, KCl accumulation increased 8-fold, to a maximum, after approximately 60 hr aging (unpublished results). However, there was not a corresponding change in the ATPase activity which only increased from 15.7 nmoles Pi/min·mg protein (in fresh slices) to 25.7 (in 60-hr aged slices).

DISCUSSION

Relation to Na-K ATPases. Previously microsomal ATPase activity had only been detected in tissues which were apparently low in acid phosphatase (7, 8, 11–13, 24). In tissues with high acid phosphatase, specific ATPase activity could not be

Table III. *Effect of Salts on the ATPase Activity in Presence and Absence of $MgCl_2$ of Varying Cation, Varying Anion, varying 4-carbon organic anion*

ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.8) and 2 mM ATP in a total volume of 1.0 ml.

Salt	ATPase Activity			Stimulation by Salt in Presence of $MgCl_2$
	−$MgCl_2$	+2 mM $MgCl_2$	Net	
50 mM	*nmoles Pi/min·mg protein*			%
A. Varying cation				
Control	0.64	6.05	...	...
KCl	0.64	9.09	3.04	49
NaCl	0.11	8.23	2.18	35
NaCl + KCl	0.38	9.47	3.42	56
Tris-HCl	0.78	9.47	3.42	56
Choline-Cl	1.13	9.19	3.14	51
CsCl	0.64	7.46	1.41	23
RbCl	0.87	9.09	3.04	49
LiCl	0.27	8.44	2.39	39
NH_4Cl	1.13	14.01	7.96	130
B. Varying anion				
Control	2.73	5.71	...	...
KCl	3.40	8.99	+3.28	+57
NaBr	3.14	7.31	+1.60	+28
KI	1.09	3.66	−2.05	−36
$NaHCO_3$	1.99	8.47	+2.76	+48
K_2SO_4	1.79	5.39	−0.32	−6
KNO_3	1.79	3.20	−2.50	−44
$NaC_6H_5SO_3$	1.48	3.91	−1.80	−32
KCH_3COO	3.59	7.76	+2.05	+36
NaF	2.12	6.86	+1.15	+20
C. Varying 4-carbon anion				
Control	1.13	5.17	...	...
KCl	0.97	8.90	3.73	72
Na malate	0.97	12.46	7.29	141
Na succinate	0.65	7.76	2.59	50
Na fumarate	1.29	10.20	5.03	97
Na tartrate	1.29	11.81	6.64	129
Na aspartate	1.29	9.95	4.38	85
Na oxaloacetate	1.29	16.18	11.01	218

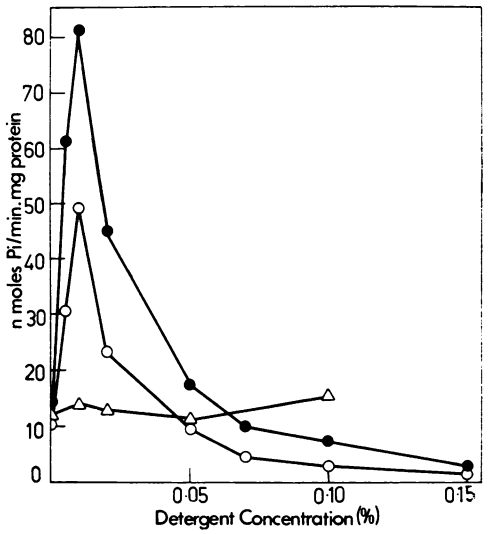

FIG. 4. Effect of digitonin in the presence of 50 mM KCl (▲) and Triton X-100 in the absence (○) and presence (●) of 50 mM KCl on ATPase activity. (The detergents were included in the assay system.) ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.8), 2 mM $MgCl_2$, and and 2 mM ATP in a total volume of 1.0 ml.

Table IV. *Sensitivity of the ATPase to Various Compounds*

ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.8), 2 mM $MgCl_2$, 50 mM KCl, and 2 mM ATP in a total volume of 1.0 ml.

Compound	Final Concn	% Inhibition (−) or Stimulation (+)
K_2SO_4	5×10^{-3} M	−8
$NaHCO_3$	5×10^{-3} M	−5
KNO_3	5×10^{-3} M	−22
$NaC_6H_5SO_3$	5×10^{-3} M	−12
Na succinate	5×10^{-3} M	−2
NADH + NADP	7×10^{-4} M	−5
ADP	2×10^{-3} M	−57
ADP	4×10^{-3} M	−75
Mersalyl	10^{-6} M	−44
Ouabain	10^{-5} M	−6
Atractylate	5×10^{-5} M	−13
Oligomycin	1 μg/ml	+12
Phloridzin	6×10^{-4} M	0
DNP	5×10^{-4} M	+19
CCCP	2×10^{-6} M	+51
Valinomycin	10^{-6} M	+11
Nigericin	1.75×10^{-8} M	+44
Ethyl alcohol	1%	−26

Table V. *Effect of CCCP and Valinomycin on the ATPase in the Presence of Either 50 mM KCl or 50 mM NaCl*

ATPase was assayed in a medium containing 0.25 M sucrose, 20 mM TES-tris buffer (pH 7.2), 2 mM $MgCl_2$, and 2 mM ATP in a total volume of 1.0 ml. CCCP and valinomycin were used at the concentrations which individually gave maximum stimulation.

Salt	Control	2×10^{-6} M CCCP	10^{-6} M Valinomycin	CCCP + Valinomycin
	nmoles Pi/min·mg protein			
KCl	11.25	16.97	12.47	24.95
NaCl	11.45		12.59	21.80

detected at pH 4.5 (1), 7.2 (4), and 7.5 (3). This paper shows that in the turnip microsome fraction specific ATPase activity can be assayed in the presence of high acid phosphatase activity by working at or above pH 7.8. The high pH optimum of the turnip microsomal ATPase is in agreement with the activities from young barley roots (7) and bean cotyledons (13) and suggests alkaline phosphatase activity. However, lack of activity with β-sodium glycerophosphate (10 mM) and inhibition by ADP (Table IV) and by activators of alkaline phosphatase (Ca^{2+} and Mn^{2+} [5]) (Table II) does not support this. The distribution of ATPase activities (Table I) would also indicate no relation with the ribosomal ATPase reported in pea seedlings (18).

In characterizing the plant particulate ATPase, other workers have tended to concentrate on the effects of monovalent cations with no attempt to test for the effect of anions. However, the results in Table III indicate that the salt stimulation of the turnip microsomal Mg^{2+}-ATPase is specific for anions rather than cations. In fact, most workers have assayed for salt stimulation in the presence of tris-HCl buffer which itself could activate the ATPase and minimize further effect of salts. This may explain the lack of salt stimulation of pea root ATPase (24). The activity reported by Fisher and Hodges (7) in barley roots most closely resembles the activity in turnip microsomes. However, the two activities differ markedly in their sensitivity to NH_4Cl, $MnCl_2$, and oligomycin. This may be explained by the fact that, although ATPase activity was detected in cell wall, mitochondrial, and microsomal fractions, it was the nonvesicular cell wall activity which Fisher and Hodges (7) characterized. In fact, they showed that the vesicular microsomal activity was considerably less oligomycin-sensitive but this activity was not further characterized.

With some exceptions (*e.g.*, NO_3^-, I^-) the anions (inorganic and organic) which stimulate the ATPase are those which are generally accumulated by intact plant cells, while those that inhibit the ATPase are not accumulated (Table III). This suggests that the ATPase could well be involved in anion accumulation by plants.

Relation to other ATPases. The effect of the uncouplers (proton carriers) and the ionophorous antibiotics (cation carriers) would suggest that ion movements across a vesicular membrane may be coupled to and limit the ATPase activity. This is reminiscent of the energy-conserving ATPases of mitochondria and chloroplasts which can use electron transport energy or ATP to move ions across their respective membranes. However, the possibility of these fractions being submitochondrial particles can be eliminated because (*a*) oligomycin had no effect on the ATPase; (*b*) there was no succinate-cytochrome *c* reductase in spite of the presence of an active NADH-cytochrome *c* reductase; (*c*) the NADH-cytochrome *c* reductase was insensitive to antimycin A (and therefore probably not of the inner membrane type).

If this is an ion-translocating ATPase (19), there should be a sidedness to the membranes with respect to site of ATP hydrolysis and pH gradient. The stimulation of activity with NH_4Cl would suggest a parallelism to the ATPases of chloroplast thylakoids (23), *i.e.*, the ATP reacts on the outside and, in effect, protons are moved into the internal space while the outside goes alkaline. The inward movement of anions (Cl^-) would maintain electrical neutrality. Subsequent exchanges of cations (*e.g.*, K^+ for H^+) would remove the pH gradient. So compounds which promote these cation exchanges (CCCP, DNP for H^+, valinomycin for K^+ or nigericin for both) should stimulate the ATPase activity—hence the synergistic effect of CCCP and valinomycin (Table V). However, this activity did

differ from the chloroplast ATPase in that it was insensitive to phoridzin.

Attempts were made to measure microsomal swelling and both K^+ and Cl^- accumulation by the vesicles (by following decrease in outside ion concentration using K^+ and Cl^- electrodes). These were unsuccessful either because the membranes were too leaky for the low rates of ion movement (despite the resistance to K^+ movement indicated in Table V) or because the sidedness of the membranes when forming vesicles was too random.

Origin of the ATPase. The origin of the two smooth membrane fractions in which the ATPase is concentrated (Table I) is not clear. The lighter of the two fractions may consist of plasma-membranes (9) but whether this is plasmalemma, tonoplast, or both and which way they are folded is not clear. However, it is tempting to suggest that the ATPase is located on the outside of the tonoplast membrane, *i.e.*, the cytoplasmic side of the tonoplast, because it (*a*) showed specificity for anions (21) and was maximally stimulated by organic anions likely to be accumulated in the vacuole; (*b*) was saturated at high salt concentration (50 mM) in accordance with the (system 2) ion pump proposed on the tonoplast (14).

As little change in turnip microsomal ATPase was observed with aging of tissue, the development of ion accumulation is probably not due to the synthesis of such an ion pump. Table III, C, shows that the ATPase favors organic anions and may normally move such ions in intact tissue. Tissue aging induces a depletion of the organic substrates by increased respiration and washing out. Under these conditions of low organic anion concentration, the cell may accumulate inorganic anions.

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