Plant Physiol. (1981) 68, 377-381
0032-0889/81/68/0377/05/$00.50/0

Effect of Polar Lipids on ATPase Activity of Membrane Preparations from Germinating Radish Seeds[1]

Received for publication November 4, 1980 and in revised form February 20, 1981

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ABSTRACT

Membrane preparation (sedimenting between 13,000*g* and 80,000*g*) of germinating radish seeds (*Raphanus sativus* L.) was active in hydrolyzing ATP and, to a lesser extent, a variety of other phosphorylated compounds. Dicyclohexylcarbodiimide (DCCD) and diethylstilbestrol significantly inhibited the ATPase activity (40%) while their effect on hydrolysis of other phosphorylated compounds was much less.

The sucrose density gradient analysis of the membrane preparation showed that the position of the DCCD-sensitive K^+-dependent ATPase was similar to that found for plasma membrane of other plant material.

Cholate treatment of membrane preparation removes almost all phospholipids, and ATPase activity is barely detectable. However, the addition of polar lipids completely restores the ATPase activity but does not restore general phosphatase activity.

The ATPase of the polar lipids restored cholate preparation, showed a high sensitivity to DCCD and diethylstilbestrol (up to 90% inhibition), a complete dependence on Mg^{2+}, and a strong dependence on K^+ at low concentration; the pH optimum of ATPase was close to 6.5, and the K_m for ATP-Mg was 0.51 millimolar. ATPase activity was much greater when polar lipids from 24-hour-germinated seeds were added.

Plasma membrane preparation from higher plants contains an ATPase which is thought to have an important role in ion transport (15, 21, 23). This hypothesis is supported by the following: (*a*), the observation that the kinetics of K^+ uptake closely resemble the kinetics of K^+ activation of ATPase (15); (*b*), fusicoccin, a plant toxin which stimulates ion transport, also stimulates ATPase (21); and (*c*), various ATPase inhibitors also inhibit ion transport, though in some cases the correlations may be complex due to their effect on metabolism (2, 4).

Previous work in this laboratory has shown that a crude membrane preparation sedimenting between 13,000*g* and 80,000*g* from germinating radish seeds contains an ATPase showing K^+ dependency, DCCD[2] sensitivity, and kinetics of substrate activation similar to those found with other plant preparations (4, 14, 15, 19). The ATPase activity rapidly increases during the early phases of germination, parallel with the development of the capacity for proton extrusion and K^+ uptake (3, 7). Furthermore, orthovanadate, which is a specific inhibitor of the plasmalemma ATPase of *Neurospora crassa* (5), has recently been shown to inhibit the ATPase of radish seeds as well as to inhibit ion transport without affecting the energy supply (8).

The involvement of polar lipids in the functionality of ATPase has been demonstrated with preparations from animal tissues and microorganisms (9, 17, 24) and, more recently, the effect of various phospholipids on the ATPase has been investigated (6, 11).

In the present paper, we report some properties of the ATPase from a membrane preparation from radish seeds and the effect of phospholipids on its activity in relation to the proposed role in ion movement. Furthermore, we investigated whether the change in polar lipid composition observed during early phases of germination (10, 28) might influence the ATPase activity.

MATERIALS AND METHODS

Plant Material and Incubation Conditions. Radish seeds (*Raphanus sativus* L., c.v. Tondo-Rosso Quarantino, Ingegnoli, Milan, Italy) were used. Germination conditions were those described in a previous paper (7).

Preparation of Membrane Fraction. Samples of germinated or ungerminated, decoated radish seeds (10 g, initial fresh weight) were homogenized in a mortar with 60 ml 0.1 M Tris-Cl (pH 8) buffer containing 0.3 M sucrose, 0.1 mM $MgCl_2$, 1 mM EDTA, 25 mM DTE. The membrane fractions were prepared as described by Hodges *et al.* (16).

The membrane fraction sedimenting between 13,000*g* and 80,000*g* showed little contamination by mitochondria. Thus, less than 2% of the total Cyt *c* oxidase activity was associated with the 13,000*g* to 80,000*g* fraction. This membrane fraction was resuspended in 1 mM Tris-Cl (pH 7), 0.25 M sucrose, 0.1 mM $MgCl_2$ resuspension solution and recentrifuged. The washing procedure produced a preparation containing less protein and phospholipid without significant loss of ATPase activity. Further washing produced a slight decrease in ATPase without significantly changing the amount of protein and phospholipid.

A cholate-treated fraction was obtained (26) by resuspending the washed membrane fraction in a solution of 1% Na-cholate, 0.25 M Na_2SO_4, 0.5 M Tris-sulfate (pH 8) to give a final concentration of 0.3 to 0.4 mg of protein/ml. This suspension was stirred at 0 C for 60 min and then centrifuged at 105,000*g* for 30 min; the pellet was resuspended in the resuspension solution.

All fractions were stored at −70 C, and, under these conditions, there was no loss of ATPase activity for several days.

The washed membrane fraction was subjected to sucrose density gradient centrifugation by layering on to a linear density gradient of 20 to 45% (w/w) sucrose in a solution containing 0.1 mM $MgCl_2$, 1 mM Tris-Cl (pH 7). After centrifugation for 120 min in a SW 27 Spinco rotor (Beckman Instruments, Palo Alto, CA) at 27,000 rpm, fractions (1.2 ml) were collected and assayed for ATPase and Cyt *c* oxidase activities, and their refractive index was measured.

Proteins were assayed according to Lowry *et al.* (20) using crystalline serum albumin as standard. Phospholipids were ex-

[1] This work is part of a research program of Consiglio Nazionale delle Ricerche on the biology of reproduction.

[2] Abbreviations: DCCD, dicyclohexylcarbodiimide; DES, diethylstilbestrol; DTE, dithioerythritol; PNPP, p-nitrophenylphosphate; PEP, phosphoenolpyruvate; Glc6P, glucose 6P.

tracted with chloroform-methanol-H_2O (1:1:0.75, v/v/v) according to Wilson and Rennie (27) and assayed for phosphate according to Ames (1).

Total Polar Lipid Extraction. Polar lipids were extracted from germinated or ungerminated radish seeds by the chloroform-methanol-H_2O method (27) and purified on silicic acid-hyflo Supercel (J. T. Baker, Phillipsburg, NJ) column according to Fujimori and Hosoya (13). The amounts of polar lipids were determined gravimetrically, and the total phosphorus content was assayed (1). Polar lipids contained 0.9 (SE = 0.03) μmol of phosphate/mg of polar lipids. Polar lipids were stored in chloroform under nitrogen in sealed ampoules at −70 C.

Polar Lipid Dispersion. The procedure was similar to that used by Papahadjopoulos (22). Vacuum-dried polar lipids were dispersed with a Whirlimixer mechanical shaker (Jencons, England) for 10 min in a solution containing 2 mM histidine, 0.1 mM EDTA, 2 mM Hepes (pH 6.7) to give a final phosphorus concentration of 10 μM. This suspension was sonicated (20 kilo-Hertz; three pulses of 30 s each). All operations during extraction and dispersion of polar lipids were performed under nitrogen.

ATPase Assay. ATPase was assayed by measuring the amount of Pi released at 26 C. This temperature was selected because it is far from the transition temperature of phospholipids, and, at this temperature, inactivation of ATPase and oxidation of polar lipids does not take place.

The membrane fraction in resuspension solution (0.1 ml) was added to 0.8 ml 37.5 mM KCl solution, and the reaction was started by adding 0.1 ml solution containing 30 mM ATP, 30 mM Mg^{2+}, and 45 mM K^+ to give a final pH of 6.4. In this condition, the pH of the incubation medium is buffered by the buffering capacity of ATP; a maximum change of pH of 0.1 unit was observed when ATPase was very active. Hepes 10 mM was introduced in the incubation medium, as specified in the legends of tables and figures, when substrates other than ATP were utilized or when the study of the effect of pH on activity was investigated. The reaction was stopped after 1 h by adding 1 ml ice-cold TCA (10%).

The phosphate released in triplicate samples was determined by the method of Fiske and Subbarow (12).

In the experiments with polar lipid dispersions, the samples were preincubated at 26 C for 10 min. No hydrolysis of phospholipids was found to occur during the assay. The presence of polar lipids did not interfere with the colorimetric assay of phosphate.

Cyt *c* oxidase activity was measured according to Smith (25).

RESULTS

ATPase and other Phosphatase Activities of Washed Membrane Preparations of Germinating Radish Seeds. Table I shows that washed membrane preparations of 24-h germinated radish seeds hydrolyze ATP and a variety of phosphorylated compounds. Two inhibitors of plasma membrane ATPase, DES (2), and DCCD (4, 15, 18) inhibit the release of Pi from ATPase by about 40%, while they have little or no effect on Pi released from other phosphorylated compounds.

Distribution on Density Gradient of K^+-Dependent, DCCD-Sensitive ATPase Activity. The data shown in Table I suggest the presence of at least two components with phosphatase activity, one which utilizes ATP and is sensitive to ATPase inhibitors and another which appears to represent nonspecific phosphatase activity. An attempt to separate these activities was made by density gradient centrifugation.

The analysis of the washed membrane fraction after sucrose density gradient centrifugation showed that ATPase activity in the presence of high K^+ concentration (30 mM) is located in two main regions, one in a low density zone (20 to 22% w/w sucrose) and the other in a high density zone (38 to 41% w/w sucrose). The activity of the low density region (about one-third of total activity) is not stimulated by K^+ and is not inhibited by DCCD (Fig. 1). The evaluation of the K^+ dependence of ATPase was performed by measuring the ATPase activity at K^+ concentration from 4.5 to 30 mM. The ATPase activity of the membrane preparations from the material investigated is activated, to a similar extent, by various monovalent cations. At cation concentrations of 4.5 and 30 mM, the relative ATPase activity (taking as 1 the activity in presence of K^+) was 0.97 in presence of Na^+, 0.97 in presence of NH_4^+, and 0.78 in presence of $Tris^+$. As it is not possible to reduce cation concentration below 4.5 and maintain the pH of the medium at 6.4, we preferred not to introduce cations other than K^+ in the incubation medium.

The K^+-activated DCCD-sensitive ATPase region of the gra-

Table I. *ATPase and Other Phosphatase Activities of the Washed Membrane Preparations from 24-h Germinated Radish Seeds*

Enzymic activities were assayed by measuring the amount of Pi released at 26 C. The assay medium contained 25 mM sucrose, 0.1 mM Tris, 10 mM Hepes, 30 mM KCl, 3 mM $MgCl_2$, and 3 mM substrates (final pH 6.4). DCCD and DES were added 10 min before the addition of substrates and KCl.

	Pi Released		
		+0.3 mM DES	+0.3 mM DCCD
	μmol·h⁻¹·mg⁻¹ protein		
ATP	13.14	7.55 (−43)[a]	7.76 (−41)
ADP	7.36	7.20 (−2)	7.10 (−3)
GTP	5.03	4.68 (−7)	4.32 (−14)
CTP	5.40	5.40 (0)	5.00 (−8)
UTP	6.71	6.08 (−10)	5.73 (−15)
PEP	10.00	10.30 (+3)	10.20 (+2)
AMP	3.28		
Glc6P	3.41		
PNPP	10.60	11.00 (+4)	9.30 (−13)

[a] Numbers in parentheses, percentage of the effect.

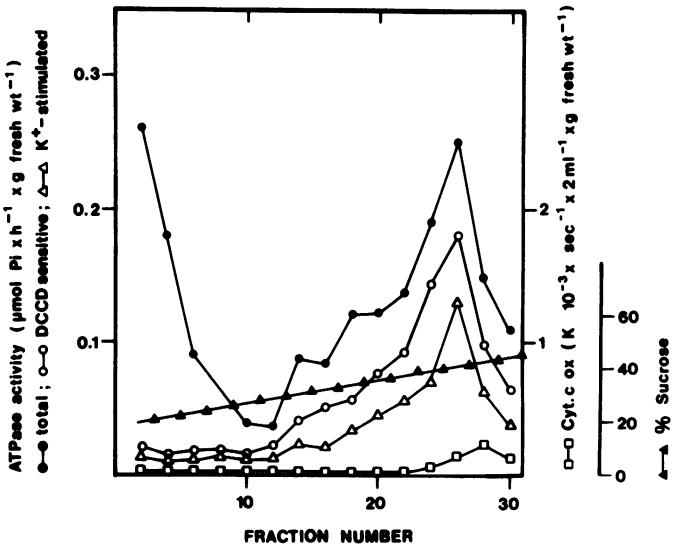


FIG. 1. Distribution of ATPase activity present in washed membrane fraction from 24-h germinated radish seeds on linear sucrose density gradient. Total amount of Cyt *c* oxidase activity present in the crude membrane fraction sedimenting between 13,000*g* and 80,000*g* was less than 2% of the total activity. DCCD-sensitive ATPase activity is expressed as the difference between the activity assayed in the absence and that assayed in the presence of 0.3 mM DCCD. K^+-stimulated ATPase activity is expressed as the difference between the activity assayed in the presence of 4.5 and 30 mM K^+.

dient is at a similar density (38 to 41% w/w sucrose) as that tentatively identified with the plasma membrane of other plant material (4, 14–16).

Figure 1 also shows that Cyt *c* oxidase activity, which is a marker of the inner mitochondrial membrane which partially contaminates (see "Materials and Methods") the washed membrane fraction, is detectable in a gradient region of higher density than that in which ATPase activity peak is found. This suggests only modest contamination by mitochondrial ATPase in this region of the density gradient.

Effect of Removal and Subsequent Addition of Phospholipids on ATPase Activity. Treatment of the washed membrane fractions with a high concentration of cholate removes almost all the phospholipids and about 50% of the protein, and it produces an almost total loss of ATPase activity (Table II). The subsequent addition of polar lipids extracted from radish seeds to the cholate-treated fraction restored the ATPase activity with a doubling of specific activity.

The restored ATPase activity was much more sensitive to DCCD and DES than the activity present in the washed membrane preparation. One possible explanation of the increased sensitivity is that the cholate treatment removed nonspecific phosphatases and activated the ATPase. This interpretation is consistent with other data for nonplant material (17, 24). Confirmation of this explanation was obtained by comparing the general phosphatase and ATPase activity of the washed membrane fraction (Table I) with that of the lipid-activated cholate-treated preparation (Table III). This table also shows that the residual nonspecific phosphatase activity was insensitive to DCCD and DES.

The effect of polar lipid from germinated and ungerminated seeds on the ATPase activity of washed membrane and cholate-treated fractions from 24-h germinated and ungerminated seeds was examined. The results (Fig. 2) show that the lipids from germinated seeds are more effective in increasing the ATPase activity than are those from ungerminated seeds. On an absolute basis, the lipid-induced increase in activity is far higher (about double) in the cholate-treated than in the washed membranes. This strongly suggests that the increase depends on a restoration of the cholate-inactivated activity rather (or much more) than on an activation of some lipid-sensible fraction of the ATPase activity already present in the washed membranes. The finding that polar lipids also increase the ATPase activity of the washed membranes may be due to the restoration of some mechanical injury or of some enzymic damage (phospholipase) during the membrane preparation.

Some Characteristics of the ATPase Activity of Lipid-Activated Cholate Preparation from 24-h Germinated Radish Seeds.

Effect of Mg^{2+} and K^+. Table IV shows the effect of K^+ and Mg^{2+} on the ATPase activity of washed membrane and lipid-activated cholate preparation. The addition of lipids did not significantly modify the sensitivity of washed membrane preparations to either K^+ or Mg^{2+} (data not shown). The lipid-activated cholate-treated preparations, which are stimulated by a variety of monovalent cations (data not shown), show a much greater Mg^{2+} dependence than does the washed membrane preparation, possibly due to the removal of Mg^{2+} by the cholate treatment or, alternatively, to the removal of Mg^{2+}-insensitive ATPase activity.

The effect of K^+ concentration on the ATPase activity is shown in Figure 3. The monovalent cation concentration cannot, for the technical reason discussed earlier, be reduced below 4.5 mM when using an ATP concentration of 3 mM. The results show a significant stimulation by K^+ in the range of 4.5 to 15 mM.

Activation by lower concentrations of K^+ can be observed by reducing the ATP concentration to 1 mM. Under these concentrations, the increased K^+ concentration (1 mM to 30 mM) produces a 7-fold increase in ATPase activity.

Effect of pH. A characteristic bell-shaped pH curve with an optimum at pH 6.5 was observed at two concentrations of K^+ (Fig. 4).

Effect of Mg-ATP Concentration. Figure 5 presents the double reciprocal plot of ATPase activity against the Mg-ATP concentration for two K^+ concentrations. From the linear plot, the K_m for Mg-ATP is 0.51 mM at both concentrations of K^+, although V_{max} is, of course, K^+-dependent.

DISCUSSION

Membrane preparations from germinating radish seeds are able to hydrolyze ATP and a number of other phosphorylated compounds. DCCD and DES inhibit the release of Pi from ATP by about 40%, while their effect on other phosphorylated compounds was much less. The sucrose density gradient studies indicated that the K^+-dependent DCCD sensitive ATPase, which is present in washed membrane, was not due to contamination of mitochondrial ATPase, and its position in the gradient was similar to that found for plasmalemma ATPase of other plant material (4, 14–16).

The cholate treatment removes phospholipids and ATPase activity is barely detectable. However, the restoration of the polar lipids completely restores the ATPase activity, with a doubling of the specific activity, but it does not restore general phosphatase activity. The lipid-restored preparation is highly sensitive to DCCD and DES (up to 90% inhibition) and is also activated by Mg^{2+} and K^+. There is an almost complete dependence on Mg^{2+},

Table II. *Effects of Cholate Treatment on the Protein and Phospholipid Contents and on the Total and DCCD-Sensitive ATPase Activity of Membrane Preparations from 24-h Germinated Radish Seeds*

DCCD-sensitive ATPase activity is expressed as the difference between the activity assayed in absence and in presence of 0.3 mM DCCD. One unit of ATPase activity is defined as the amount liberating 1 μmol Pi × h^{-1} at 26 C. The data are the average of 6 experiments; SE did not exceed 4.3% for proteins, 3.1% for phospholipids, and 2.8% for ATPase activity.

			ATPase Activity			
	Proteins	Phospholipids	Specific Activity		Total Activity	
			Total	DCCD-Sensitive	Total	DCCD-Sensitive
	mg/g fresh wt	*μmol/g fresh wt*	*units/mg protein*		*units/g fresh wt*	
Washed membrane fraction	0.095	0.086	11.59	5.45	1.101	0.517
Cholate-treated preparation	0.046	0.001	0.29	0.14	0.013	0.006
Cholate preparation + polar lipids from germinated seeds	0.046	0.330[a]	22.25	20.03	1.020	0.921

[a] Polar lipids from 24-h germinated seeds were added as described in "Materials and Methods."

Table III. *ATPase and Other Phosphatase Activities of the Lipid-Activated Cholate Preparations from 24-h Germinated Radish Seeds*

Conditions of measurements as in Table I, except that polar lipids from 24-h germinated seeds (10 mg/mg of proteins) were added, as described in "Materials and Methods."

	P_i released		
		+0.3 mM DES	+0.3 mM DCCD
		μmol·h⁻¹·mg⁻¹ protein	
ATP	21.56	2.70 (−87)[a]	2.00 (−90)
ADP	0.87	0.90 (+3)	0.72 (−17)
GTP	1.00	0.72 (−28)	0.84 (−16)
CTP	2.00	1.80 (−10)	1.80 (−10)
UTP	1.70	1.70 (0)	1.72 (+1)
PEP	0.68	0.58 (−14)	0.58 (−14)
AMP	1.05	0.94 (−7)	0.85 (−19)
Glc6P	1.17		
PNPP	0.10	0.10	0.10

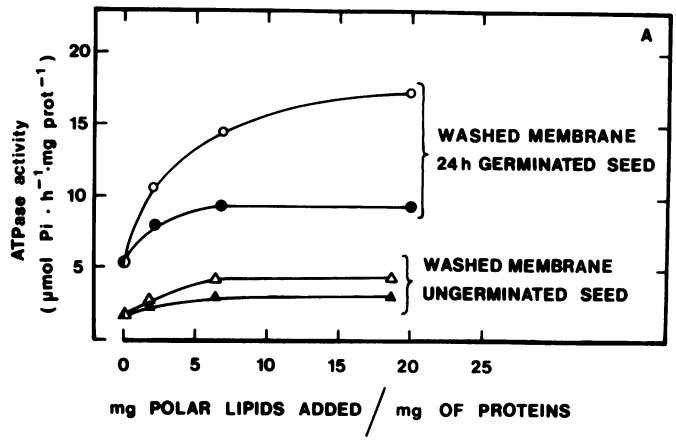
[a] Numbers in parentheses, percentage of the effect.

Table IV. *Effects of Mg^{2+} and K^+ on the ATPase Activity of Washed Membranes and of Lipid-Activated Cholate Preparations from 24-h Germinated Radish Seeds*

ATPase activity was assayed as described in "Materials and Methods," except for Mg^{2+} and K^+ concentrations.

	ATPase Activity			
	Mg^{2+}, 0.01 mM		Mg^{2+}, 3.01 mM	
	K^+, 10.5 mM	K^+, 30 mM	K^+, 4.5 mM	K^+, 30 mM
	μmol Pi·h⁻¹·mg protein⁻¹			
Washed membranes	5.9	7.8	8.4	13.7
Cholate preparation + polar lipids[a]	0.8	1.1	14.7	19.8

[a] Polar lipids (9 to 10 mg) from 24-h germinated seeds/mg of protein were added.



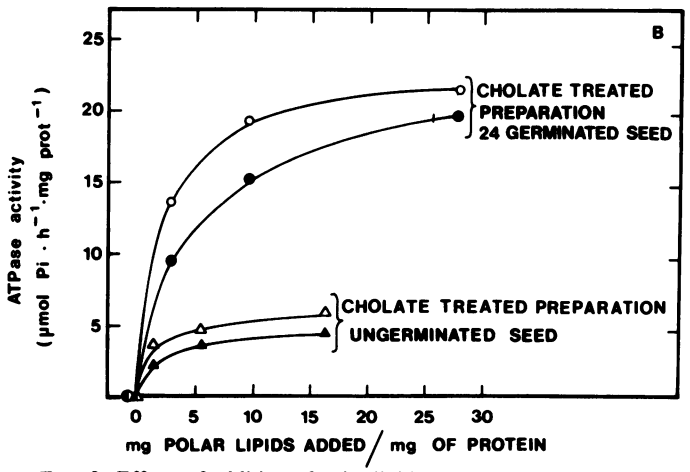


FIG. 2. Effects of addition of polar lipids on DCCD-sensitive ATPase of the washed membrane fraction (A) and of the cholate-treated preparations (B). DCCD-sensitive ATPase activity is expressed as the difference between the activity assayed in the presence and in the absence of 0.3 mM DCCD. ● and ▲, polar lipids from ungerminated seeds; ○ and △, polar lipids from 24-h germinated seeds.

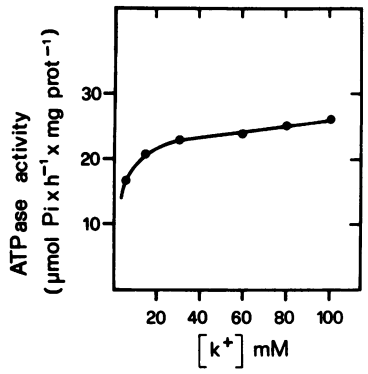


FIG. 3. Effect of K^+ concentration on ATPase activity of a polar lipid-activated cholate preparation. ATPase activity was measured as described in "Materials and Methods," except that K^+ concentrations (KCl) were as indicated. Polar lipids (9 to 10 mg/mg protein) were added.

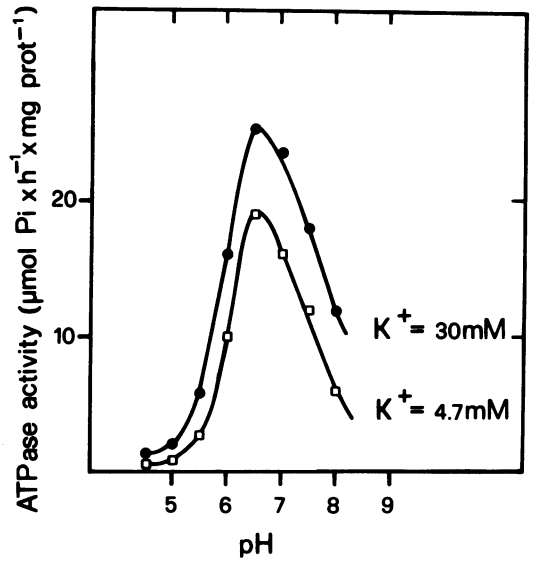
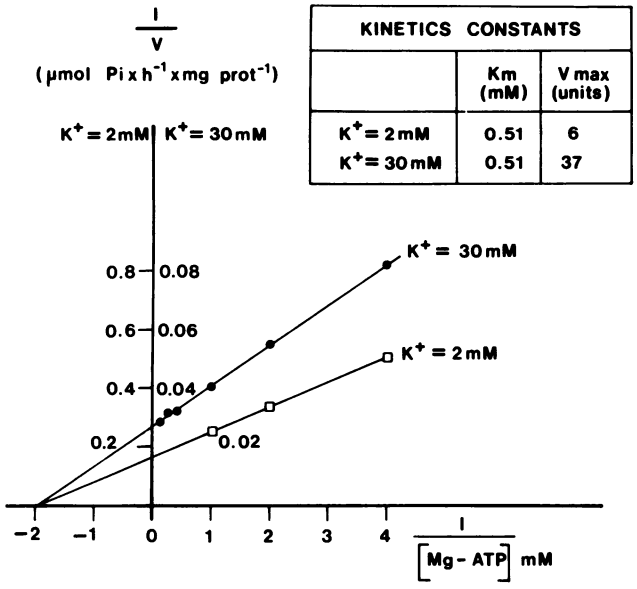


FIG. 4. Effect of pH on ATPase activity of a polar lipid-activated cholate preparation. ATPase activity was measured as described in "Materials and Methods." Polar lipids (9 to 10 mg/mg protein) were added. ●—●, 30 mM K^+; □—□, 4.7 mM K^+.



KINETICS CONSTANTS		
	Km (mM)	V max (units)
K^+ = 2mM	0.51	6
K^+ = 30mM	0.51	37

FIG. 5. Effect of ATP concentration on the ATPase activity of polar lipid-activated cholate preparations at two concentrations of K^+. ATPase activity was measured as described in "Materials and Methods." Polar lipids (9 to 10 mg/mg protein) were added. One unit of ATPase activity is defined as the amount liberating 1 μmol $Pi \cdot h^{-1} \cdot mg^{-1}$ protein. Each point is the average of three different experiments (SEs do not exceed 2.6%).

and a strong dependence on K^+ can be demonstrated at low concentrations of ATP. The pH optimum of ATPase activity was close to 6.5, similar to that reported for other plant material (4, 18, 19), and the K_m for Mg-ATP was 0.51 mM.

These results show that the cholate treatment is an effective method for purifying the K^+-stimulated ATPase from radish seeds. The much greater activation of ATP observed with preparation from germinated and ungerminated seeds when polar lipids from germinated seeds were added suggests that the observations are physiologically significant.

Acknowledgments—The authors are grateful to Professor D. D. Davies of the University of East Anglia and to Professor E. Marrè of the University of Milan for many helpful discussions and suggestions in this work.

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