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Effects of Germination on Na^+- K^+-stimulated Adenosine 5'-Triphosphatase and ATP-dependent Ion Transport of Isolated Membranes from Cotyledons

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ABSTRACT

A purified membrane fraction featuring ATPase activity was isolated from cotyledon tissue of *Phaseolus vulgaris* at different stages of germination. The fraction is enriched in both basal and Na^+-K^+-stimulated ATPase and is relatively free of contamination by fragments of mitochondrial membrane and microsomes. The isolated membranes have been tentatively identified as partially purified plasma membrane.

The specific activities of the basal- and cation-sensitive ATPases were high in membrane preparations from young cotyledon tissue but decreased with advancing senescence. Electron microscopy of the preparation showed that the isolated membranes were of primarily vesicular conformation. These vesicles proved to be capable of extruding Na^+ and K^+ in the presence of ATP. Moreover, the degree of ATP-dependent extrusion varied during germination in a pattern that resembled variations during the same period in the Na^+-K^+-stimulated ATPase of the isolated fraction. This indicates that as the level of cation-sensitive ATPase on the membrane rises or falls, there is a corresponding change in the ability of the membrane to actively translocate Na^+ and K^+.

Enzymatic hydrolysis of ATP has been documented for quite a number of higher plant tissues (1, 3, 4, 6, 8, 9, 12, 13). In many cases these enzymes have been found to possess distinctive cation sensitivities, particularly for Na^+, K^+, and Mg^{2+}, and in this sense are similar to the cation-stimulated ATPase (EC 3.6.1.3) found on the surface membrane of many mammalian cells (2). However, unlike the mammalian enzyme, which appears to be consistently Mg^{2+}-dependent and activated synergistically by Na^+ and K^+ (22), the plant ATPases feature cation sensitivities of a variable nature. For example, particulate ATPases from oat root (6) and sugar beet root (9) are stimulated by Na^+ or K^+ in the presence of Mg^{2+}, yet a corresponding membrane-bound enzyme from bean cotyledon tissue has been found to be activated by Na^+ or K^+ in the absence of Mg^{2+} (13). Similar differences are apparent for soluble ATPase activities. The enzyme present in the soluble fraction isolated from bean root homogenate is Mg^{2+}-dependent and stimulated by Na^+ and K^+ (8), but soluble ATPases from the storage organs of both beet and carrot have been found to feature sensitivity to Na^+ and K^+ independent of Mg^{2+} (1). Characteristically, plant ATPases do not appear to be synergistically responsive to Na^+ and K^+. Recently, however, Kylin and Gee (12) have reported that the ATPase activity of a microsomal fraction from leaves of *Avicennia nitida* is critically influenced by the total salt concentration of the assay medium. On this basis, they emphasize that any search for Na^+-K^+ synergism in plant systems should be carried out by testing responses to a fine gradation of Na:K ratios at various levels of total salt.

A further distinction between the mammalian and plant ATPases is that the effects of ouabain on the plant enzymes have proven to be erratic, ranging from significant inhibition to marked stimulation of activity (3, 4). This diversity of response has not been fully resolved, but Brown *et al.* (3, 4), on the basis of experiments with the ATPase of *Arachis hypogaea* cotyledon tissue, have suggested that the stimulatory effect of ouabain may be attributable to conformational modification of the enzyme induced by isolation techniques. They found that particulate cation-stimulated ATPase activity was consistently inhibited by ouabain when the membranes featuring the enzyme were structurally intact.

No direct relationship between cation-stimulated ATPase activity and active transport of cations has been unequivocally established for plant tissue. This stems in part from the fact that ouabain, which for the mammalian system inhibits both active cation transport and Na^+-K^+ stimulation of ATPase (22), does not definitively influence either of these phenomena in plant tissue. In addition, activation by cations has been reported for both soluble and particulate plant ATPases, and it is difficult to reconcile the participation of a soluble enzyme in active ion transport. However, as suggested by Greuner and Neuman (8), this could reflect solubilization of a natively bound enzyme incurred during homogenization.

We have recently described the isolation of membrane fragments from cotyledon tissue of *Phaseolus vulgaris* which are enriched in Na^+-K^+ stimulated ATPase activity (13). The properties of this isolated fraction were such that it was tentatively identified as a preparation of partially purified plasma membrane. In this investigation, the relationship between this cation-sensitive enzyme and active transport of Na^+ and K^+ has been investigated. In addition, the effects of germination on the transport properties of the isolated membranes have been determined with a view to establishing whether the ATPase and active transport change in a parallel fashion during cellular modification.

MATERIALS AND METHODS

Growth Conditions and Isolation of Membranes Possessing ATPase Activity. Seeds of *Phaseolus vulgaris* were germinated and grown in moist vermiculite in the dark at 29 C. The seeds were not soaked prior to planting. Cotyledons were harvested at 2-, 4-, 7-, and 9-day intervals after planting. Purified prepa-

rations of membrane enriched in basal and Na^+-K^+ stimulated ATPase were isolated from the cotyledon tissue at each of these ages according to a procedure described previously (13). This procedure is summarized in Figure 1.

Measurements of Na^+-K^+ Transport by the Isolated Membrane Vesicles. Membrane fragments to be used for ion transport studies were isolated as indicated in Figure 1 except that the 4-hr incubation at 4 C was omitted in order to minimize deterioration of the vesicles. This alteration in the isolation procedure had no effect on the ATPase activity of the purified membrane fraction. Membrane derived from 80 g of tissue was collected from the density gradient centrifugation tubes, diluted to a sucrose concentration of about 0.3 M with 1 mM EDTA, pH 7.5, and centrifuged at 138,000*g* for 1 hr. The pellet was resuspended in 8.5 ml of 0.3 M sucrose-1 mM EDTA solution.

An assessment of Na^+-K^+ translocation attributable to active transport was made by comparing the residual ion content of vesicles which had been incubated with Na^+ and K^+ in the presence of ATP and in the absence of ATP. For this purpose, four 125-ml Erlenmeyer flasks containing 2 ml of membrane suspension, 20 mM tris (pH 7.5 for 2- and 9-day-old tissue and 8.0 for 4- and 7-day-old tissue), 40 mM KCl and 40 mM NaCl in a total volume of 50 ml were set up. The reaction mixtures were adjusted to 0.3 M with sucrose. The flasks were incubated in a reciprocal shaking water bath, rotating at 125 rpm and adjusted to 38 C, for an initial period of 15 min to allow the reaction mixtures to equilibrate. Tris-ATP was then added to two of the flasks to a final concentration of 3 mM, and incubation was continued for an additional 15 min. The reactions were stopped by filtration through 0.45 μ Millipore filters, and the membrane residues remaining on the filters were washed with 100 ml of 0.3 M sucrose-EDTA solution. Each Millipore filter was then cut up, placed in a Kjeldahl flask, and predigested with 1 ml of concentrated nitric acid for 1 hr. After cooling, 1 ml of 70% perchloric acid was added, and digestion was continued for 4 hr. Finally, the volume was made up to 5 ml, and the levels of both Na^+ and K^+ were determined by atomic absorption spectrophotometry.

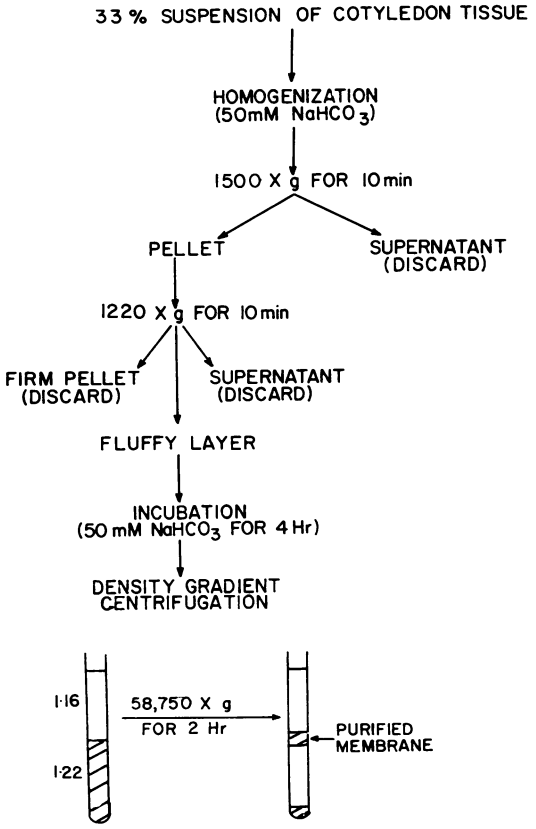

FIG. 1. Flow chart of the procedure for isolating a purified membrane fraction enriched in ATPase activity from cotyledon tissue of *Phaseolus vulgaris*. For the density gradient centrifugation, the bottom portion of the gradient consisted of payload (input of partially purified membranes to the sucrose gradient) suspended in sucrose solution of density 1.22. The gradient was completed by layering sucrose of density 1.16 over the bottom portion.

Table I. *Relative Enrichments of ATPase Activity in a Purified Membrane Fraction from Cotyledon Tissue of Phaseolus vulgaris*

Purified membrane was isolated as described in Figure 1. The enzyme reaction system included 3 mM ATP (tris salt), 20 mM tris (pH 7.5 for 2- and 9-day old tissue and 8.0 for 4- and 7-day old tissue) and when added 40 mM NaCl and 40 mM KCl. Reaction time was 15 min.

Age of Tissue		Basal ATPase[1]			Na^+-K^+ Stimulation of Basal ATPase[2]		
	Experiment	Homogenate	Purified membrane	Ratio[3]	Homogenate	Purified membrane	Ratio[3]
days		*mg Pi/mg protein·hr*			*mg Pi/mg protein·hr*		
2	A	1.44	2.50	1.7	0.92	2.94	3.2
	B	0.84	2.50	3.0	0.77	2.50	3.3
4	A	0.80	4.62	5.8	1.25	10.34	8.3
	B	0.92	3.92	4.2	1.25	9.48	7.6
7	A	0.71	1.98	2.8	0.41	4.26	10.4
	B	0.40	0.79	2.0	0.50	3.62	7.3
9	A	1.07	1.53	1.4	0.27	0.91	3.4
	B	1.14	2.12	1.8	0.24	1.12	4.7

[1] Activity measured in the absence of added cations.

[2] Activity measured in the presence of added Na^+ and K^+ minus the basal activity.

[3] Enrichment expressed as a ratio of the specific activity in the purified membrane fraction to that of the corresponding homogenate.

Enzymatic and Protein Determinations. ATPase (EC 3.6.1.3) was measured as described previously (6, 13). Glucose-6-phosphatase (EC 3.1.3.9), 5'-nucleotidase (EC 3.1.3.5), and succinate dehydrogenase (EC 1.3.99.1) were assayed according to the methods of Hubscher and West (10), Michell and Hawthorne (18), and Pennington (21), respectively. Protein levels were routinely determined by the method of Lowry *et al.* (17).

Electron Microscopy. Samples of the purified membrane fraction were fixed in glutaraldehyde (3% w/v in 50 mM phosphate buffer, pH 7.4), postfixed in OsO_4 (1% w/v in 50 mM phosphate buffer, pH 7.4), dehydrated in a graded acetone series, and embedded in Vestopal (11). Thin sections were poststained with uranyl acetate (1% w/v aqueous solution) and lead citrate (0.1% w/v in 0.1 M NaOH) and examined in a Philips 300 transmission electron microscope.

RESULTS

Enrichment of Na^+-K^+-stimulated ATPase in the Purified Membrane Fraction. Purified membrane fractions from all ages of tissue proved to be enriched in both basal and Na^+-K^+-stimulated ATPase, although the enrichments were greater for 4- and 7-day-old tissue (Table I). Purifications relative to homogenate on a specific activity basis ranged from about 1.5- to 6-fold for the basal enzyme and from 3- to 10-fold for the Na^+-K^+-stimulated activity (Table I). For individual experi-

Table II. *Recoveries of ATPase Activities in the Fractionation Scheme used for Isolating Purified Membrane*

The fractionation scheme used is described in Figure 1. The enzyme reaction system included 3 mM ATP (tris salt), 20 mM tris (pH 7.5 for 2- and 9-day-old tissue and 8.0 for 4- and 7-day-old tissue) and when added 40 mM NaCl and 40 mM KCl. Reaction time was 15 min.

Age of Tissue		Percentages of Recovered Activity			
		A		B	
		Basal[1]	Na^+-K^+ stimulated[2]	Basal	Na^+-K^+ stimulated
days					
2	Supernatants[3]	88.7	81.2	84.0	93.8
	Pellet[4]	3.2	8.8	4.8	2.1
	Payload[5]	8.1	10.0	11.2	4.1
	Recovery[6] (%)	90.0	82.0	114.8	107.0
4	Supernatants	93.5	89.6	95.3	82.9
	Pellet	3.1	5.1	1.6	8.7
	Payload	3.4	5.3	3.1	8.4
	Recovery (%)	96.0	97.0	90.5	70.5
7	Supernatants	88.2	92.0	92.8	87.7
	Pellet	4.9	3.5	2.7	4.1
	Payload	6.9	4.5	4.5	8.2
	Recovery (%)	69.0	95.0	82.5	83.5
9	Supernatants	95.0	91.5	92.0	86.7
	Pellet	1.6	4.8	1.9	7.6
	Payload	3.4	3.7	6.1	5.7
	Recovery (%)	76.0	92.0	90.0	70.0

[1] Measured in the absence of added cations.

[2] Measured in the presence of added Na^+ and K^+.

[3] Combined supernatants arising from the fractionation (see Fig. 1).

[4] Pellet discarded during the fractionation (see Fig. 1).

[5] Input to the density gradient centrifugation (see Fig. 1).

[6] Sum of the activities in the supernatants, pellet, and payload expressed as a percentage of the homogenate activity.

ments, the enrichment of the cation-sensitive ATPase in the membrane preparation was consistently greater than that of the basal activity (Table I). Recoveries of ATPase activities in the various fractions derived directly from the homogenate ranged from 69 to 115% (Table II), and recovery of enzyme activity from the density gradient centrifugation was routinely within the range 100 ± 20%.

Only slight variation in the ATPase pH profiles was apparent among tissues of different ages (Fig. 2). The pH optimum for total activity measured in the presence of Na^+ and K^+ was 7.5 for 2- and 9-day-old tissue and 8.0 for 4- and 7-day-old tissue. All ATPase assays were routinely carried out at the pH optimum which corresponded to the age of tissue being used. It was necessary to measure the Na^+-K^+-sensitive and basal enzymes at the same pH because values for Na^+-K^+ stimulation were determined by subtracting basal activities from the total activity measured in the presence of the ions.

Levels of Contamination in the Purified Membrane Fraction. As previously described (13), contamination in the purified membrane fraction was assessed by measuring succinate dehydrogenase, a marker for mitochondrial membrane fragments, and glucose-6-phosphatase and 5′-nucleotidase, markers for microsomal membranes for this tissue (13, 14). All three enzymes were in most cases not detectable in the isolated fraction for each age of tissue examined. In a few instances glucose-6-phosphatase was present, but its specific activity in the purified membrane fraction was always lower than that of the corresponding homogenate. Thus, in contrast to ATPase, these measurable levels of glucose-6-phosphatase in the isolated fraction did not amount to an enrichment relative to homogenate. This implies that even when glucose-6-phosphatase was detectable, contamination by membrane fragments bearing this enzyme was not significant. It is also clear from the electron micrograph shown in Figure 3 that the preparation is essentially free from recognizable contamination by cytoplasmic organelles.

Behavior of the Membrane-associated ATPase during Germination. The specific activities of the basal and Na^+-K^+-stimulated ATPase in the purified membrane fraction showed rather striking variations during germination. Profiles of these changes are illustrated in Figure 4. Initially, in 2-day-old tissue, the activities of both types of ATPase were relatively low, although there was still a significant stimulation of the basal activity in the presence of added Na^+ and K^+. Thereafter, both enzyme activities increased to reach a peak at 4 days of age. Subsequently, with the onset of senescence they declined, reaching a low level again by day 9. It is also clear from the profiles illustrated in Figure 4 that the degree of cation stimulation of enzyme activity is approximately the same for all ages of tissus examined.

ATP-dependent Extrusion of Na^+ and K^+ by the Purified Membrane Fraction. The fragments of membrane in the isolated fraction were primarily of vesicular conformation and therefore, theoretically capable of either accumulating or extruding ions by active means. In order to test this, the vesicles were incubated in a reaction mixture containing Na^+, K^+, and tris in the proportions and at the pH routinely used for the ATPase assay. After an equilibration period, ATP was added, and, at the end of the reaction, the ion content of the vesicles was compared with a control which had been treated in ex-

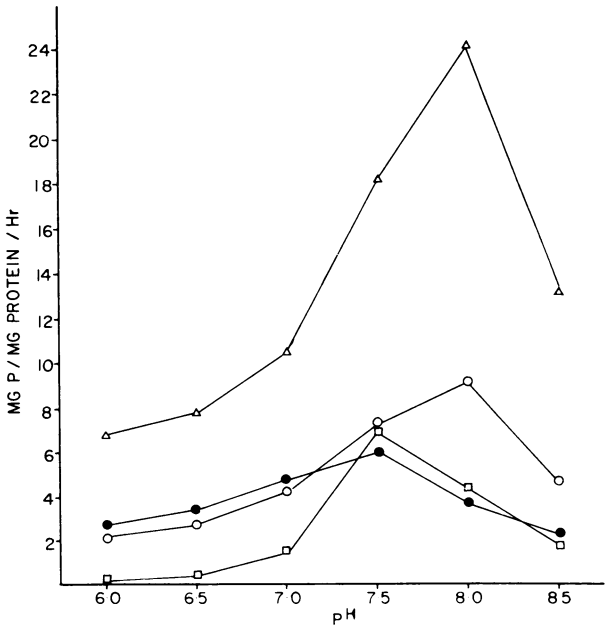

FIG. 2. The pH profiles for Na^+-K^+-stimulated ATPase activity (total activity measured in the presence of added Na^+-K^+) of a purified membrane fraction from cotyledon tissue of *Phaseolus vulgaris;* ●: 2-day-old tissue; △: 4-day-old tissue; ○: 7-day-old tissue □: 9-day-old tissue. The enzyme reaction system included 3 mM ATP (tris salt), 20 mM tris (adjusted to the required pH value), 40 mM NaCl and 40 mM KCl. Reaction time was 15 min.

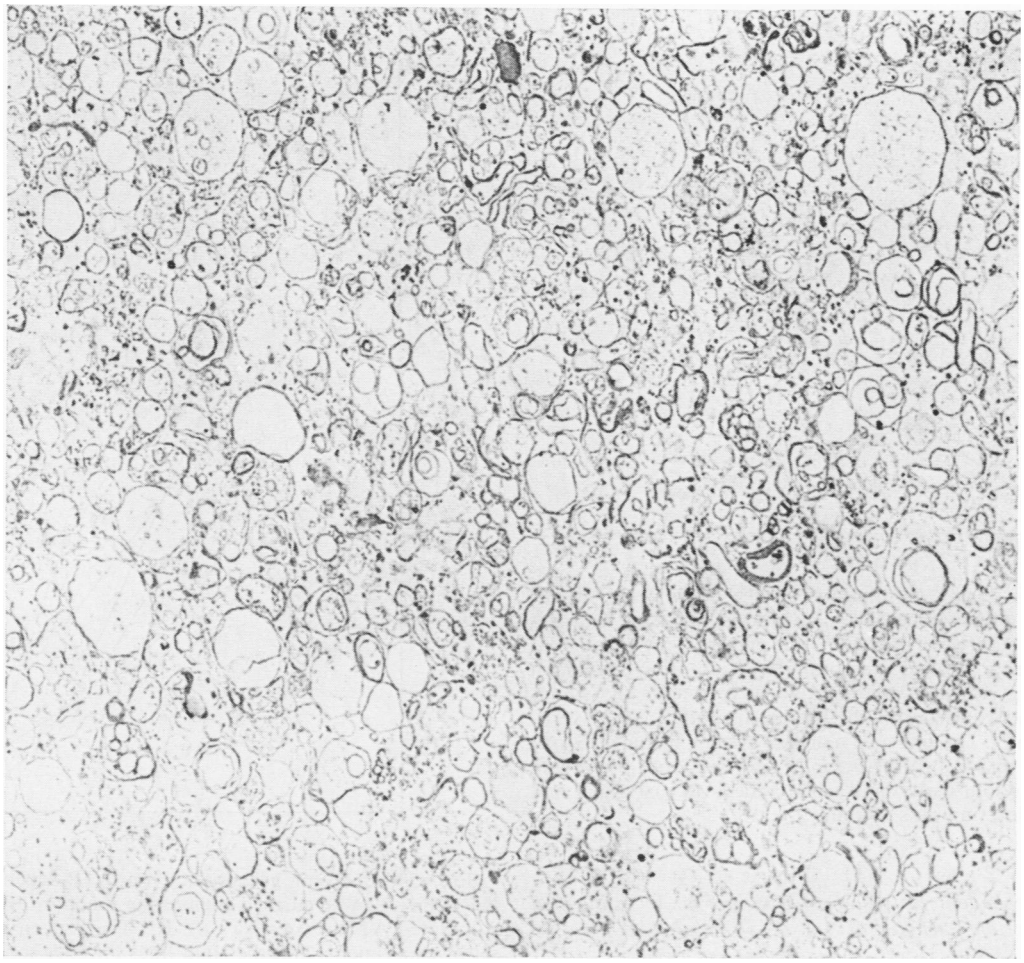

FIG. 3. Electron micrograph of a purified membrane fraction from 4-day-old cotyledon tissue of *Phaseolus vulgaris*. × 9372.

actly the same manner except that ATP was absent from the reaction mixture.

This procedure was carried out with isolated membrane from 2-, 4-, 7-, and 9-day-old tissue. The results were most pronounced for 4-day-old cotyledons (Table III). Both control (no ATP added) and experimental (ATP added) vesicles were found to contain Na^+ and K^+. However, for 4-day-old tissue the levels of both ions were lower in the experimental vesicles implying that the addition of ATP had caused Na^+ and K^+ to be actively extruded. The amount of extrusion ranged from 35 to 53% for membrane from 4-day-old tissue.

At 2 days of age, the extent of extrusion was found to be variable, ranging from 20 to 75% (Table III). This variability has been interpreted as indicating that the capacity for active ion transport is increasing very rapidly during the second day of germination. Although the rates of germination were basically reproducible from experiment to experiment, small differences in rate of growth were unavoidable and would be expected to give rise to variability in measurements of a rapidly changing phenomenon.

With the onset of senescence, the capacity of the vesicles to transport ions actively declined (Table III). For 7- and 9-day-old tissue, extrusion of both Na^+ and K^+ was either very low or not detectable. This pattern of decline is more clearly depicted in Figure 5.

Levels of ions per milligram of protein varied among experiments for any one age of tissue, but this presumably reflects expected variation from experiment to experiment in the proportions of membrane in the fraction which are vesiculated and hence capable of accumulating ions.

DISCUSSION

As discussed previously (13), the enrichment of ATPase in the purified membrane fraction and in particular the increased enrichment of the net (Na^+-K^+) stimulated activity (Table I), a property normally associated with the plasmalemma, indicate that it is a preparation of purified plasma membrane. Contamination by other types of membrane, in particular fragments of mitochondrial membrane and microsomes, appears to be minimal, for succinate dehydrogenase, glucose-6-phosphatase, and 5′-nucleotidase are essentially absent from the preparation. The effects on the basal ATPase activity of Na^+, K^+, and Mg^{2+} in various combinations have been previously shown to be neither additive nor synergistic (13). Consistently, however, Na^+ and K^+ in the absence of Mg^{2+} gave the highest stimulation and hence this combination of ions was used routinely in establishing the enrichment of the capacity for cation sensitive ATP hydrolysis in the purified membrane fraction.

The rise and fall during germination in the level of ATPase activity associated with the purified membranes parallels in principle alterations in other types of metabolic activity that have been shown to characterize cotyledon tissue during this period. For example, subsequent to hydration, there is a progressive and quite rapid increase in carbohydrate metabolism, lipid metabolism and mitochondrial activity in cotyledon tissue

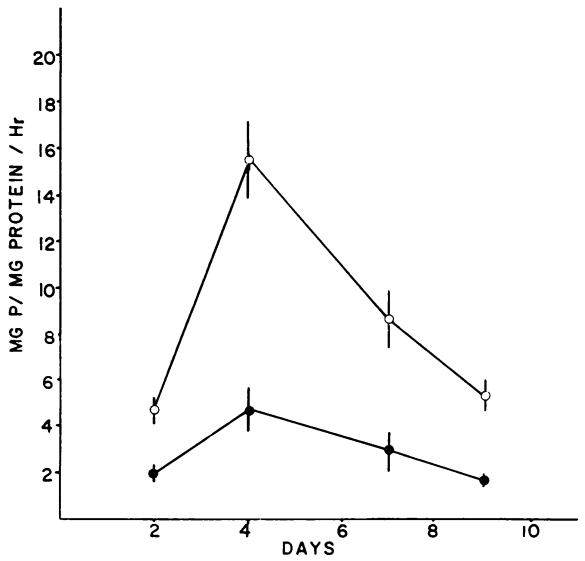

FIG. 4. Changes during germination in the specific activity of basal (measured in the absence of added cations) and total Na^+-K^+-stimulated (total activity measured in the presence of added Na^+ and K^+) ATPase in a purified membrane fraction from cotyledon tissue of *Phaseolus vulgaris;* ●: basal activity; ○: Na^+-K^+-stimulated activity. Standard errors of the means are indicated by vertical lines; n = 3 to 5. The enzyme reaction system included 3 mM ATP (tris salt), 20 mM tris (pH 7.5 for 2- and 9-day-old tissue and 8.0 for 4- and 7-day-old tissue) and when added, 40 mM NaCl and 40 mM KCl. Reaction time was 15 min.

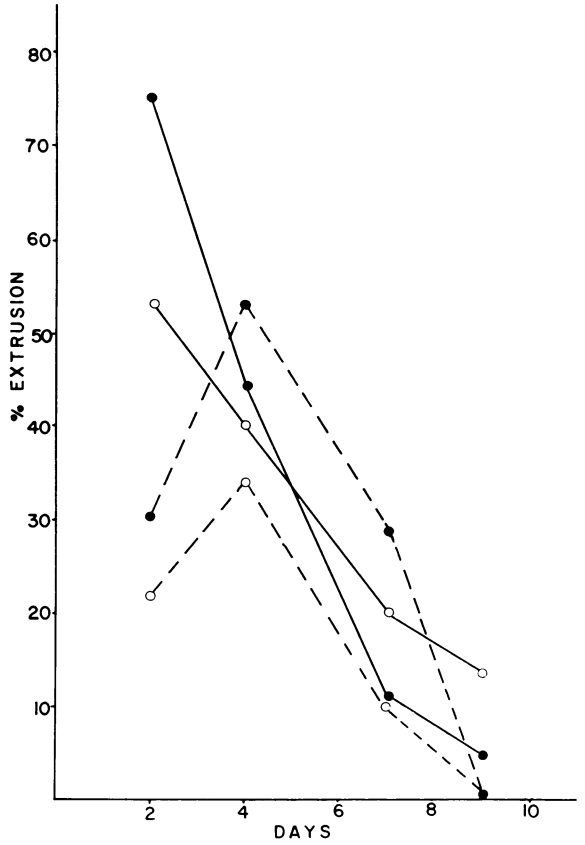

FIG. 5. Effects of germination on ATP-dependent extrusion of Na^+ and K^+ by a purified membrane fraction enriched in Na^+-K^+-stimulated ATPase; ●: Na^+; ○: K^+. The broken lines illustrate one set of experiments and the solid lines another set. The reaction system included 20 mM tris (pH 7.5 for 2- and 9-day-old tissue and 8.0 for 4- and 7-day-old tissue), 40 mM NaCl, 40 mM KCl and when added 3 mM ATP (tris salt).

Table III. *ATP-dependent Extrusion of Na^+ and K^+ by a Purified Membrane Fraction Enriched in Na^+-K^+-stimulated ATPase*

Purified membrane was isolated as described in Figure 1 except that the 4-hr incubation in 50 mM $NaHCO_3$ was omitted. The reaction system included 20 mM tris (pH 7.5 for 2- and 9-day-old tissue and 8.0 for 4- and 7-day-old tissue), 40 mM NaCl and 40 mM KCl. ATP (tris salt) was absent from the control flasks but present in the experimental flasks at a concentration of 3 mM.

Age of Tissue	Experiment	Ion	Ion Extrusion		
			Control	Experimental	Extrusion[1]
days			*μg per mg protein*		%
2	A	Na^+	105	49	53.5
		K^+	48	12.2	75
	B	Na^+	232	181	21.8
		K^+	134	94	30
4	A	Na^+	174	104	40
		K^+	88	49	44.5
	B	Na^+	118	77.5	34.3
		K^+	57.4	27.0	53
7	A	Na^+	280	223	20
		K^+	103	94	11
	B	Na^+	261	235	10
		K^+	113	80	29
9	A	Na^+	343	295	14
		K^+	192	184	4.4
	B	Na^+	196	202	0
		K^+	112	123	0

[1] Percentage of extrusion is the difference in ion content between the experimental and control expressed as a percentage of the control value.

(5, 7, 16, 19). After reaching a peak and particularly with the actual onset of senescence, metabolic activity in general declines, ultimately reaching a very low level in extensively senescent tissue (19). The patterns of change for both basal and Na^+-K^+-stimulated ATPase in the isolated membrane fraction do not, however, simulate exactly those for total levels of ATPase in the cotyledon tissue. The specific activities of the membrane associated enzymes reach a peak at 4 days of age (Fig. 4), while the levels of total basal and Na^+-K^+-stimulated ATPase are highest in 2-day-old tissue (15). The reason for this discrepancy is not unequivocally apparent. However, in view of the preponderance of protein bodies in the storage cells of 2-day-old tissue (20), it may very well be that the isolated membrane fraction from tissue of this age is contaminated with non-membranous protein. Contamination of this nature is much less likely in preparations from older tissue because the protein bodies are rapidly digested (20). The presence of nonmembranous protein in the fraction isolated from 2-day-old tissue would give rise to erroneously low values for specific activities of enzymes associated exclusively with the isolated membrane. That this may be so is further apparent from the lower enrichments relative to homogenate of Na^+-K^+-stimulated ATPase in membrane from 2-day-old tissue as compared with those for 4- and 7-day-old tissue. It is quite possible, therefore, that in the membrane preparations from 2-day-old tissue higher specific activities of both basal and cation sensitive ATPase were masked by contaminating protein. The de-

cline in the ATPase activities with advancing germination (Fig. 4) is consistent with the deterioration of membrane function and structure that would be expected to occur as a result of senescence and is presumably due either to inactivation of the enzyme or actual digestion of membranous enzymatic protein.

The ability of the isolated membrane vesicles to extrude Na^+ and K^+ in the presence of ATP suggests that in the intact cell, the Na^+-K^+-stimulated ATPase facilitates active transport of these ions across the plasma membrane. This is further borne out by the similarity in patterns of change during germination for ion extrusion and the Na^+-K^+-stimulated ATPase associated with the purified membranes (Figs. 4 and 5). Agreement between these profiles is even closer upon consideration of the possibility that values for the specific activity of Na^+-K^+-stimulated ATPase may be erroneously low for membrane from 2-day-old tissue. While it is true that ATP can act as a chelator, it seems very unlikely that such chelation could have significantly influenced the levels of ions within the vesicles. For one thing, the concentration of ATP in the reaction mixtures for the ion extrusion studies was markedly lower than that of the ions, meaning that only a relatively small proportion of the ions could have been chelated. Moreover from an estimate of the size of a centrifuged pellet of the membranes it can be calculated that the volume of vesicles in the final reaction mixture was only about $\frac{1}{500}$ of the total reaction mixture volume. Thus even after the initial 15-min equilibration period, the proportion of total salt remaining external to the vesicles was always far in excess of that inside the vesicles. This indicates that chelation by ATP in the external medium would be contributing very little if any to the observed ATP-dependent extrusion of ions.

It would appear, therefore, that as the level of cation-sensitive ATPase on the membrane rises or falls, there is a corresponding change in the ability of the membrane to actively translocate Na^+ and K^+. The ions were consistently extruded by the vesicles in the presence of ATP rather than actively taken up. Granted the assumption that the vesicle surfaces correspond to plasma membrane surfaces, this is of significance for it is in line with the predominantly export activity that characterizes storage cells of cotyledon tissue during germination. It is also equally clear that Na^+ and K^+ can be passively translocated across the isolated membranes, for substantial levels of both ions were consistently present in vesicles which had not been exposed to ATP (Table III).

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