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Membrane-bound Adenosine Triphosphatase Activities of Oat Roots[1]

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ABSTRACT

Homogenates of oat (*Avena sativa* cv. Goodfield) roots contained at least five membrane-associated adenosine triphosphatase (ATPase) activities. The membrane-bound ATPases were separated on sucrose gradients and distinguished by membrane density, pH optima, sensitivity to monovalent salts, and substrate specificity.

A membrane fraction sedimenting at low centrifugal force (13,000*g*) contained two ATPase activities at pH 9.0. One membrane ATPase was coincident with cytochrome c oxidase activity and had a density of 1.18 grams per cubic centimeter. This membrane system was identified as mitochondria. The other pH 9.0 ATPase in this fraction occurred at a density of 1.16 grams per cubic centimeter. The identity of this membrane is unknown.

Three additional ATPases were in a membrane fraction sedimenting at high centrifugal forces (13,000–80,000*g*). One membrane ATPase coincided with NADH-cytochrome c reductase activity, had a density of about 1.09 grams per cubic centimeter, and was equally active at pH 6.0 and 9.0. A second membrane ATPase of the 13,000 to 80,000*g* fraction had a density of 1.13 grams per cubic centimeter and was more active at pH 9.0 than at pH 6.0. A third membrane ATPase had greater activity at pH 6.0 than at pH 9.0, and the membrane had an apparent density of 1.17 grams per cubic centimeter on the sucrose gradient. This ATPase was especially sensitive to KCl. The identity of the membranes which contain ATPases is discussed in relation to the distribution of other enzymes on the gradient.

We have previously described an ATPase from roots that was associated with membranes, required Mg^{2+}, and was stimulated by monovalent salts in general but with KCl and RbCl giving the greatest activation (8). A high correlation existed between rates of K^+ or Rb^+ absorption by roots and the amounts of KCl- or RbCl-stimulated ATPases in these roots (9). These results support the concept that ATP is the energy source for ion absorption in roots and that a membrane-bound ATPase participates in the energy transduction process.

For the above concept to be tenable, the ATPase must be associated with either the plasma membrane or tonoplast since these membranes are the major sites of active ion transport in higher plants (20, 23). Indeed, histochemical studies have shown that both the plasma membrane (11, 24) and tonoplast (12, 24) have ATPase activities. However, membrane systems of such structures as mitochondria, nuclei, endoplasmic reticulum, and Golgi also possess ATPase activity (12, 24). Hence, *in vitro* assays of ATPase activity employing a crude preparation of membranes (*i.e.*, a differentially centrifuged fraction) may measure several different ATPases. It would be desirable to know how many membrane-associated ATPases can be distinguished in cell extracts and to identify the various membrane systems containing these enzymes. This was the purpose of the investigations reported here. We show that at least five ATPase activities can be distinguished by density gradient centrifugation of membrane fractions from oat roots. One of these ATPases is associated with mitochondria, and another is associated with plasma membranes (16). The latter enzyme is especially sensitive to KCl. The membranes containing the other ATPases are unidentified.

MATERIALS AND METHODS

Plant Material. Oat seeds (*Avena sativa* cv. Goodfield) were suspended between layers of cheesecloth on stainless steel screens 10 cm above 1 mM $CaSO_4$ (15). They were kept in darkness at 25 ± 3 C with vigorous aeration for 6 days.

Preparation of Membrane Fractions by Differential Centrifugation. Six-day-old roots were excised, washed three times in ice-cold deionized water, and ground in a mortar and pestle without added abrasives as previously described (8, 9). All manipulations were at 0 to 2 C. The grinding medium consisted of 0.25 M sucrose, 3 mM EDTA, and 25 mM tris-MES, pH 7.2. Four milliliters of grinding medium were used per gram fresh weight of root tissue. The brei was strained through four layers of cheesecloth and successively centrifuged at 13,000*g* for 15 min and 80,000*g* for 30 min. Both pellets (13,000*g* and 13,000–80,000*g*) were suspended in fresh grinding medium, pelleted (at same forces as originally used), and suspended in a sucrose solution (2%, w/w, less than the top of the gradient) containing 1 mM $MgSO_4$ and 1 mM tris-MES, pH 7.2.

Continuous Gradient Centrifugation. One milliliter of membrane preparation (5–10 mg of protein) was layered onto a 37-ml linear gradient ranging from 20 to 45% (w/w) sucrose in 1 mM $MgSO_4$ and 1 mM tris-MES, pH 7.2, and centrifuged for 3 hr at 95,000*g* in a Spinco SW 27 rotor. The gradient was

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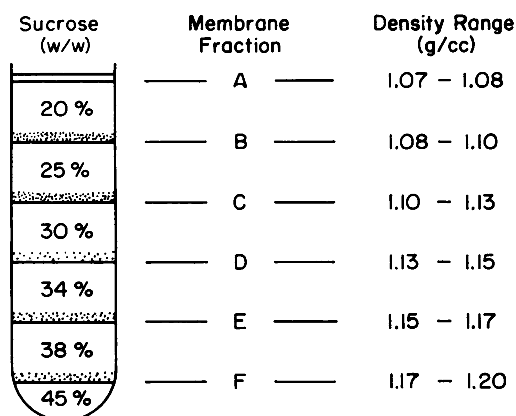

FIG. 1. Discontinuous sucrose gradient used for separation of membrane fractions of oat roots. Density of dots at interfaces depicts relative amounts of protein banded when 13,000*g* for 15 min-80,000*g* for 30 min fraction (see "Materials and Methods") was the overlay.

fractionated with an ISCO model 640 density gradient fractionator into 30 fractions of 1.2 ml.

Discontinuous Gradient Centrifugation. Two milliliters of membrane preparation (10–20 mg of protein) were layered onto a 36-ml discontinuous gradient consisting of 4 ml of 45% (w/w) and 6.4 ml each of 38, 34, 30, 25, and 20% sucrose in 1 mM $MgSO_4$ and 1 mM tris-MES, pH 7.2, and centrifuged for 2 hr at 95,000*g*. Protein bands were removed with a Pasteur pipette and identified as shown in Figure 1.

Enzyme Determinations. Enzyme assays using phosphate substrates were at 38 C in a 1-ml volume containing 1.5 mM $MgSO_4$, 50 mM KCl, and 33 mM tris-MES buffer at the appropriate pH; 10 to 50 μg of membrane protein; and 3 mM substrate. The pH of assay for the various nucleoside phosphate substrates was as follows: nucleoside triphosphates, pH 6 or 9; nucleoside diphosphates, pH 7.5; and nucleoside monophosphates, pH 8.5. Acid and alkaline phosphatase activities were determined using *p*-nitrophenyl phosphate (same reaction components as described above) at pH 5.5 and 9.5, respectively. Glucose 6-phosphatase and pyrophosphatase were determined at pH 6.5. The sodium salt of all substrates was used except for the nucleoside triphosphates, which were converted to the tris salt by Dowex 50 W ion exchange chromatography. After a reaction time of up to 1 hr (depending on amount of substrate hydrolyzed) each assay was terminated by addition of 2 ml of cold 1% (w/v) ammonium molybdate in 2 N sulfuric acid. The P_i released was determined by the Fiske and Subba-Row (10) procedure. Substrate blanks were determined and subtracted to calculate all enzyme activities.

Cytochrome *c* oxidase was assayed spectrophotometrically at room temperature (25 C) by following the oxidation of cytochrome *c* at 550 nm. The 3-ml reaction mixture contained 10 to 50 μg of protein, 15 μM cytochrome *c* reduced with sodium dithionite, 0.01% digitonin, and 50 mM phosphate buffer, pH 7.5. The rate of cytochrome *c* oxidation was calculated according to Smith (29).

NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase were assayed at room temperature by following the reduction of cytochrome *c* at 550 nm. The 3-ml reaction mixture contained 10 to 50 μg of protein, 1.66 mM sodium cyanide, 30 μM cytochrome *c*, 50 mM phosphate buffer, pH 7.5, and 0.1 mM NADH or NADPH. The rate of cytochrome *c* reduction was estimated using an extinction coefficient for cytochrome *c* of 18.5 $mM^{-1}cm^{-1}$ (13).

Proteins were determined by the procedure of Lowry *et al.* (19).

RESULTS

Separation of ATPase Activities on Continuous Sucrose Gradients. Membrane fractions (13,000*g* and 13,000–80,000*g*) obtained from oat root homogenates by differential centrifugation were layered onto continuous sucrose gradients (20–45% w/w) and centrifuged for 3 hr. The choice of differential centrifugation fractions and of the 20 to 45% sucrose gradient was based on preliminary experiments in which these membrane preparations and range of sucrose densities gave the maximal separation of the enzymes being considered. Preliminary studies also showed a pH optimum of 9.0 for mitochondrial ATPase and a broad activity curve with an optimum between 6.0 and 7.0 for ATPase of the microsomal fraction. Hence, gradient fractions were assayed at pH 6.0 and 9.0 to distinguish ATPases of mitochondrial and microsomal origin.

Several different membrane-associated ATPases were detected (Fig. 2, A and B). In the 13,000*g* fraction, two ATPases (pH 9.0) were associated with membranes having densities of about 1.16 and 1.18 g/cc (Fig. 2A). Little ATPase activity at pH 6.0 was detected. In a 13,000 to 80,000*g* fraction from the same homogenate, three ATPases were distinguished (Fig. 2B). One ATPase, at a density of about 1.09 g/cc, was equally

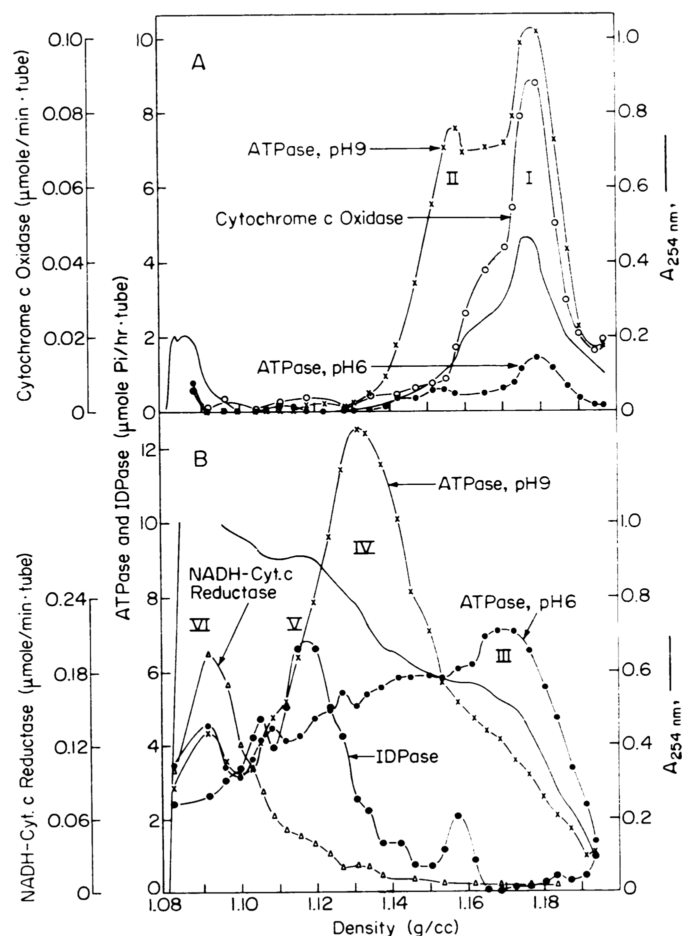

FIG. 2. Distribution of ATPase and marker enzyme activities on linear 20 to 45% (w/w) sucrose gradients. A: 13,000*g* for 15 min fraction from oat root homogenate. B: 13,000*g* for 15 min-80,000*g* for 30 min fraction from same homogenate.

active at both pH 6.0 and 9.0 (peak VI). A second ATPase was distinguished at pH 9.0 (peak IV) and was associated with membranes having a density of about 1.13 g/cc. And a third ATPase, with maximal activity at pH 6.0 and broadly distributed throughout the gradient, showed a distinct peak at an apparent density of 1.17 g/cc (peak III). The density of this membrane was similar to that of membranes with ATPase activity in the 13,000*g* fraction (peaks I and II, Fig. 2A), but these membranes are apparently different because the associated ATPases have different pH optima. Thus, by using two differential centrifugation fractions and a continuous sucrose gradient and assaying at two pH levels, five different membrane-bound ATPases could be distinguished.

To identify the membranes with ATPase activities, the distribution of other enzymes on these gradients was determined (Fig. 2, A and B). Cytochrome *c* oxidase, a marker for mitochondria (1,17), was coincident with the pH 9.0 ATPase that occurred at a density of 1.18 g/cc (peak I). Of the total membrane-bound cytochrome *c* oxidase activity in oat roots, 89.5% was in the 13,000*g* pellet (16). Hence, we concluded that the high pH ATPase activity at a density of 1.18 g/cc (Fig. 1A) is associated with intact mitochondria.

NADH-cytochrome *c* reductase is believed to be associated with either endoplasmic reticulum or outer mitochondrial membrane in animals (17) and has been ascribed to the tonoplast in plants (22, 28). The activity of this enzyme was low in the 13,000*g* fraction and is not shown, but it was present in significant amounts in the 13,000 to 80,000*g* fraction (Fig. 2B). The peak of NADH-cytochrome *c* reductase activity was coincident with the ATPase having equal activity at pH 6.0 and 9.0, and an apparent density of 1.09 g/cc (peak VI).

It has been shown by both histochemical (6, 24) and biochemical (26) procedures that IDPase activity is associated with Golgi apparatus membranes. In addition, Ray *et al.* (26) reported that IDPase of Golgi membranes from pea seedlings is latent (*i.e.*, activity increased with storage of the membranes for 4 days at 0–2 C). Latent IDPase was found principally in the 13,000 to 80,000*g* fractions from oats (Fig. 3), and its distribution on the continuous gradient is shown in Figure 2B.

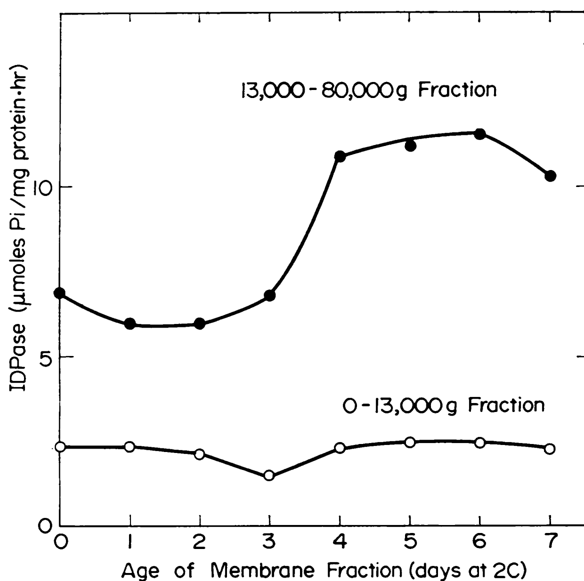

FIG. 3. IDPase activity of oat root membrane fractions (see "Materials and Methods") stored at 2 C for various periods of time.

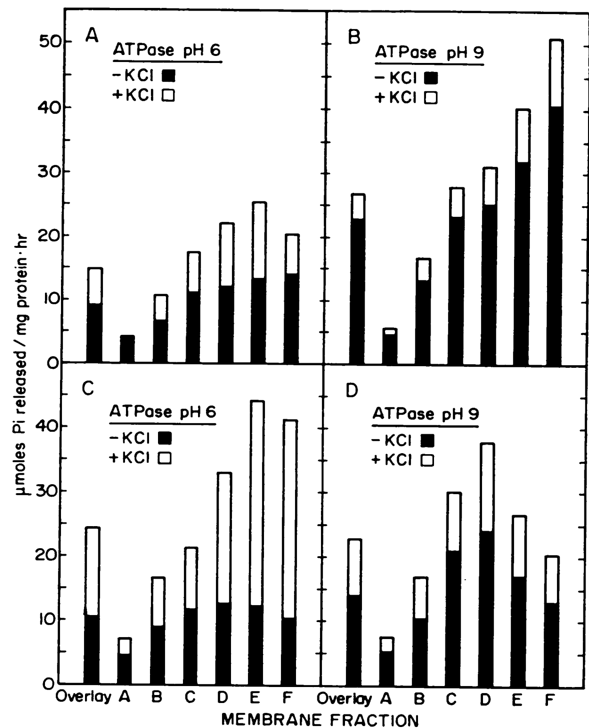

FIG. 4. Distribution of ATPase activity on discontinuous sucrose gradient of Figure 1. A and B: 1,000*g* for 10 min-80,000*g* for 30 min fraction from oat root homogenate was the overlay. C and D: 13,000*g* for 15 min-80,000*g* for 30 min fraction from same homogenate was the overlay.

Most IDPase activity (assayed after storage for 4 days at 2 C) was at a density of about 1.12 g/cc and was not coincident with any of the ATPase peaks, although significant ATPase activity at both pH 6.0 and 9.0 was found in this area of the gradient.

Purification of ATPase Activities on Discontinuous Sucrose Gradients. To further characterize and identify the membranes in the 13,000 to 80,000*g* fraction, a discontinuous gradient was devised to obtain fractions enriched in the various enzymes. Based on the membrane densities on the continuous gradient (Fig. 2B), fraction B (of discontinuous gradient, see Fig. 1) should be rich in NADH-cytochrome *c* reductase, C in IDPase, D in the pH 9.0 ATPase, and E and F in the pH 6.0 ATPase. These enzyme enrichments were obtained (Figs. 4, C and D, and 5). Since the discontinuous gradient gives nearly the same separation of enzymes as the continuous gradient and is easier to use, we employed this gradient for further characterization studies.

We investigated the sensitivity of the various ATPases to KCl since the KCl- or RbCl-stimulated component of ATPase activity correlated highly with K^+ or Rb^+ transport into roots (9, 18). With a 1,000-80,000*g* overlay (Fig. 4, A and B) the greatest enrichment in ATPase activity at pH 6.0 was in fraction E of the gradient, and KCl stimulated this ATPase activity by 101% (Fig. 4A). At pH 9.0 (Fig. 4B) the greatest ATPase activity was in fraction F, and the addition of KCl gave only a 23% stimulation (Fig. 4B). This ATPase activity is largely due to mitochondria since the pH optimum for mitochondrial ATPase is 9.0 (unpublished results, see also Fig. 2A), and the density of mitochondria is such that they would collect in this area of the gradient (see cytochrome *c* oxidase distribution on continuous gradient of Fig. 2A).

When mitochondria were removed from the membrane

fraction placed onto the gradient (*i.e.*, 13,000–80,000*g* overlay), the distribution of pH 9.0 ATPase was different (compare Fig. 4, B and D). Without mitochondria, ATPase activity at pH 9.0 in fraction F was considerably reduced, and the highest specific activity was found in fraction D. This supports the above contention that mitochondria were responsible for most of the pH 9.0 ATPase activity in fraction F (Fig. 4B).

The distributions of ATPase activity at pH 6.0 on the discontinuous gradient, using either the 1,000 to 80,000*g* overlay or the 13,000 to 80,000*g* overlay, were qualitatively similar.

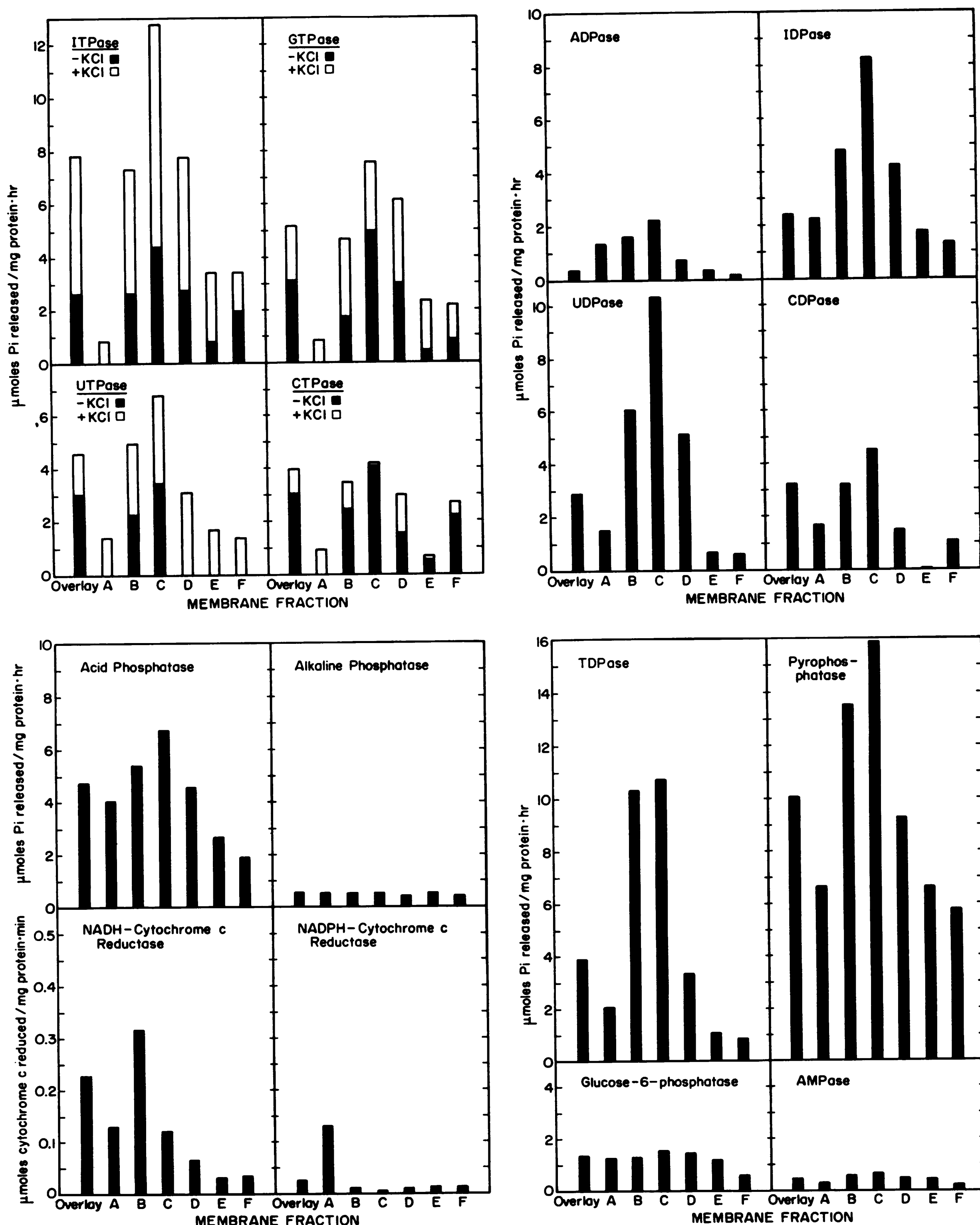

FIG. 5. Distribution of various enzyme activities on discontinuous sucrose gradient of Figure 1. The 13,000 to 80,000*g* fraction (see "Materials and Methods") was the overlay.

However, the 13,000 to 80,000g overlay yielded fractions with much higher specific activities, and this was particularly apparent in fractions E and F (KCl stimulations of the ATPase of fractions E and F were 256 and 295%, respectively). Hence, removal of mitochondria and other membranes with the initial 13,000g centrifugation is important for further separation and purification of the KCl-stimulated ATPase using density gradient centrifugation.

The distribution of enzymes on the discontinuous gradient using other phosphate substrates was substantially different from that using ATP as the substrate (compare Figs. 4, C and D, and 5). Fraction C was enriched, in addition to IDPase, in all the nucleoside tri- and diphosphatases tested, pyrophosphatase, and acid phosphatase (Fig. 5). In contrast, activity in fractions E and F was markedly reduced with these substrates (note the different scales in Figs. 4C and 5), indicating that the KCl-stimulated ATPase in fractions E and F is quite specific (16).

The activity of glucose 6-phosphatase, a marker for endoplasmic reticulum in animals (4, 14) was low in all membrane fractions, as was the activity of 5'nucleotidase (AMPase), the classic marker for animal plasma membranes (7) (Fig. 5). IMPase, GMPase, CMPase, UMPase, and TMPase activities in the membrane fractions were also determined (unreported) and were virtually identical to those for AMPase.

NADPH-cytochrome *c* reductase is associated with endoplasmic reticulum in animal cells (4), while its localization in plant cells is unreported. The activity of this enzyme was specifically rich in fraction A (Fig. 5), which contains membranes and solubilized proteins remaining in the overlay after centrifugation on the gradient.

DISCUSSION

Five membrane-associated ATPases were distinguished by density gradient centrifugation of membrane fractions from oat root homogenates. A sixth ATPase activity occurred in the soluble fraction (*i.e.,* nonsedimentable material at 200,000g for 12 hr) of similar oat root homogenates (8). This soluble ATPase activity was not stimulated by KCl (8), had an acid pH optimum, and had little substrate specificity (unpublished). We feel that this soluble activity is due to a nonspecific acid phosphatase similar to that reported by Atkinson and Polya (2) in beet tissue.

Among the membrane-bound ATPases distinguished here, one was associated with mitochondria. It sedimented with low centrifugal force (13,000g $\times$ 15 min); it had an apparent density of 1.18 g/cc, which is typical for both plant (3, 26, 27) and animal mitochondria (17); it was coincident with cytochrome *c* oxidase activity, which is a classic marker enzyme for mitochondria. This mitochondrial ATPase showed maximal activity at pH 9.0.

A second high pH ATPase was in the 13,000g fraction. It had an apparent density of 1.16 g/cc and showed little cytochrome *c* oxidase (Fig. 2A), NADH-cytochrome *c* reductase, or IDPase (unpublished) activity. The membrane possessing this ATPase is undefined.

The 13,000 to 80,000g fraction contained three ATPases which were distinguishable on continuous sucrose gradients. One was coincident with NADH-cytochrome *c* reductase at a density of about 1.09 g/cc and had equal ATPase activity at pH 6.0 and 9.0. In animal cells NADH-cytochrome *c* reductase is variously ascribed to the endoplasmic reticulum or outer mitochondrial membrane (17). In plants this enzyme has been found in both mitochondrial and microsomal fractions (21, 25), and it has been suggested to be on both inner and outer mitochondrial membranes (5) and the tonoplast (22, 28). Hence, based on the presence of NADH-cytochrome *c* reductase, this portion of the gradient may be rich in tonoplast, endoplasmic reticulum, or mitochondrial membranes. Unambiguous marker enzymes for plant membranes are needed to solve the problem. NADPH-cytochrome *c* reductase, which has been rigorously shown to be associated with endoplasmic reticulum of rat liver (4), was not helpful since it was not enriched in this area of the gradient but rather in a fraction consisting of small membrane fragments and solubilized proteins that did not enter the gradient (fraction A, Fig. 5).

A second ATPase was distinguished at pH 9.0 when the 13,000 to 80,000g overlay was separated by density gradient centrifugation (Fig. 2B, peak IV). None of the enzymes assayed associated predominantly with this membrane fraction (Figs. 2B and 5). The possibility that it derives from Golgi is unlikely since a latent IDPase (Fig. 3), which is thought to be a Golgi-associated enzyme (26), was associated with membranes of lower density (Figs. 2B and 5).

The third ATPase of the 13,000 to 80,000g fraction was more active at pH 6.0 than at pH 9.0, and the membrane had a peak density of 1.17 g/cc. Of most interest was the sensitivity of this ATPase to KCl (Fig. 4C) which is that aspect of ATPase shown to be correlated with ion transport (9, 18). Hence, it is likely that this ATPase is associated with either tonoplast or plasma membrane. Indeed, the principal membrane component in this area of the gradient has been shown to be plasma membranes (16). Characterization of the plasma membrane ATPase with respect to monovalent salt specificity and kinetics of ATP, Mg^{2+}, and KCl activation is in progress and will be published later.

The presence of several membrane-bound ATPases in extracts of roots is undoubtedly responsible for the confusing and apparently contrasting reports concerning plant ATPases (2, 8, 9, 18 and references therein). We hope that this illustration of the complexity of the problem will lead to clarification of these reports. Even with our preliminary characterizations, it is now possible to dictate partially which ATPase is being measured in crude preparations by choice of differential centrifugation fraction, assay pH, and the presence or absence of monovalent ions. Additional characterization, using these partially purified membrane preparations, should further clarify the differences between the various ATPases.

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