Plant Physiol. (1984) 76, 498–507
0032-0889/84/76/0498/10/$01.00/0

# Evidence for a KCl-Stimulated, $Mg^{2+}$-ATPase on the Golgi of Corn Coleoptiles[1]

Received for publication April 27, 1984 and in revised form June 28, 1984

ALAIN CHANSON[2], EUGENIA MCNAUGHTON, AND LINCOLN TAIZ*
*Biology Department, Thimann Laboratories, University of California, Santa Cruz, Santa Cruz, California 95064*

## ABSTRACT

**Membranes of corn (*Zea mays*, cv Trojan 929) coleoptiles were fractionated by sucrose density gradient centrifugation and the locations of organelles were determined using marker enzymes and electron microscopy. Latent IDPase (or UDPase) was selected as the Golgi marker and UDPG-sterol glucosyl transferase was selected as the plasma membrane (PM) marker, because they were clearly separable from markers for the other organelles. Golgi-rich and PM-rich fractions were studied in relation to their ATPase activities. The pH optimum of the KCl, $Mg^{2+}$-ATPase of the PM-rich fraction from a step gradient was 6.0 to 6.5, while the Golgi-rich fraction had peaks at pH 6.0 to 6.5 and pH 7.5. It is hypothesized that the peak at pH 6.0 to 6.5 for the Golgi-rich fraction is due to PM-contamination, while the peak at pH 7.5 represents the activity of a Golgi ATPase. To reduce PM contamination, Golgi-rich fractions obtained from step or rate-zonal gradients were recentrifuged isopycnically on linear sucrose gradients. The distribution of KCl, $Mg^{2+}$-ATPase activity was measured at pH 6.5 and 7.5. The pH 6.5 ATPase was coincident with UDPG-sterol glucosyl transferase, a PM marker, while the pH 7.5 ATPase overlapped with latent UDPase, a Golgi marker. These results provide strong evidence for a KCl, $Mg^{2+}$-ATPase, active at pH 7.5, associated with the Golgi membranes of corn coleoptiles.**

Membrane ATPases have been implicated in the transport of ions in plant cells (34). On the basis of electrophysiological and biochemical evidence, the primary transport mechanism on the plasma membrane appears to be an electrogenic $H^+$-ATPase which pumps protons to the cell exterior (41). There is also strong evidence that the tonoplast is the site of a second $H^+$-ATPase which pumps protons into the vacuole (9, 15, 26, 45).

Ion transport in the Golgi has received very little attention, since its primary function is to secrete macromolecules across the plasma membrane. Inasmuch as many of these secreted macromolecules are charged, *e.g.* pectins and glycoproteins, charge balancing and pH adjustment may be required for normal processing of secreted macromolecules by the Golgi. Monensin, which collapses $Na^+/H^+$ gradients, specifically alters Golgi morphology and interferes with secretion (27 and references therein). However, there is little direct evidence bearing on the existence of transport ATPases on Golgi membranes.

West (47) demonstrated ATP-dependent $Ca^{2+}$ accumulation by a Golgi vesicle preparation isolated from lactating rat mammary glands. An electrogenic $H^+$-ATPase has also been detected on the Golgi apparatus of rat liver (14, 48). In plants, Ray *et al.* (38) early reported low levels of ATP-hydrolyzing activity associated with pea stem Golgi membranes purified by a combination of rate-zonal and isopycnic sucrose density gradient centrifugation. However, nonspecific phosphatase contamination was not ruled out. Binari and Racusen (3) reported the presence of a KCl, $Mg^{2+}$-ATPase associated with Golgi secretory vesicles isolated from *Avena* suspension cultured cells. However, Taiz *et al.* (42), working with pea stems, found no evidence for a $Mg^{2+}$-ATPase in Golgi secretory vesicles, although this did not rule out the possibility of an ATPase on the cisternal membranes.

The present work was undertaken in an effort to clarify the status of ATPases on Golgi membranes by careful comparison of ATPase activity and the distribution of various membrane markers on isopycnic and rate-zonal sucrose and dextran gradients. The corn coleoptile was selected because it is a well characterized system which allows reasonably good separation of membrane markers on gradients.

## MATERIALS AND METHODS

**Plant Material.** Corn seeds (*Zea mays* L. cv Trojan 929) were soaked overnight and sown in trays with moist vermiculite. Seedlings were grown in the dark (20–22°C) with 2 h of red light daily to inhibit mesocotyl growth. After 5 to 7 d, coleoptiles (~3 cm) were harvested, debladed, and stored on ice under room lights. Highest yields of Golgi membranes were obtained with young coleoptiles. Homogenization and subsequent treatments were performed at 0 to 4°C.

**Homogenization.** Coleoptiles (13 g) were chopped by hand (10 min) with razor blades in 6 ml of homogenization medium (250 mM sucrose, 2 mM EDTA, 1 mM DTT, 50 mM Tris-Mes, pH 7.8). The tissue was then ground very lightly in a mortar and strained through nylon. The remaining tissue was lightly reground in an additional 6 ml of homogenization buffer. The final homogenate was filtered through nylon and combined with the first homogenate. Unbroken cells, cell wall fragments, starch and nuclei were removed by a 5-min centrifugation at 1000*g* (Sorvall, SS-34 rotor). The pellet (1 KP[3]) and the supernatant (1

[1] Supported by Grant PCM-8301995 from the National Science Foundation.

[2] Recipient of a postdoctoral fellowship from the Swiss National Foundation. Present address: Institut de Biologie et de Physiologie Végétales, Université de Lausanne, Bâtiment de Biologie, 1015 Lausanne, Switzerland.

[3] Abbreviations: KP, 1000*g* pellet; KS, 1000*g* supernatant; NPA, *N*-1-naphthalamic acid; PM, plasma membrane; PnPP, *p*-nitrophenylphosphate; UDPG-ST, UDPG-sterol glucosyltransferase.

KS) were collected. In some experiments, the majority of the mitochondria were eliminated by a 20-min centrifugation at 6000*g* (Sorvall, SS-34 rotor) (30) and the pellet (6 KP) and supernatant (6 KS) were collected.

**Linear Sucrose Gradients.** The 1 KS fraction was layered onto a linear gradient consisting of a 2-ml cushion of 45% sucrose (w/w), 20 ml 15 to 45% sucrose and a 1-ml layer of 15% sucrose (in 0.5 mM EDTA, 2.5 mM Tris-Mes, pH 7.2). The gradients were centrifuged at 80,000*g* for 3 h (Beckman L2-65B, SW28 rotor) (36) and fractionated into 16 fractions of 1.5 ml or 23 fractions of 1 ml.

**Nonlinear Sucrose Gradients.** The 1 KS fraction was layered onto a five-step gradient consisting of a 3-ml cushion of 45% sucrose and 5 ml each of 36, 31, 26, and 18% sucrose (in 0.5 mM EDTA, 2.5 mM Tris-Mes, pH 7.2). The gradients were centrifuged 2 h at 80,000*g* (21). The material at the different sucrose interfaces was collected with a Pasteur pipet (Fig. 1).

**Rate-Zonal Centrifugation.** The 1 KS fraction was layered onto a linear gradient (20 ml, 15–35% sucrose) made in 0.5 mM EDTA, 2.5 mM Tris-Mes (pH 7.2) with a 2-ml cushion of 45% sucrose and a 1-ml 15% sucrose overlay. The gradients were centrifuged 25 min at 30,000*g* (Beckman L2-65B, SW28 rotor) and fractionated into 16 fractions of 1.5 ml.

**Electron Microscopy.** Samples (1 ml) were taken from the peak fractions of the ER (24%), Golgi (29%), PM (35%), and mitochondria (39%) on an isopycnic sucrose gradient similar to Figure 3. One ml of primary fix (2.5% glutaraldehyde, 0.2 M K-phosphate at pH 7.1, and sucrose equal to the concentration of each sample) was added and the samples incubated overnight at 4°C. They were then centrifuged at 80,000*g* for 1 h in nitrocellulose tubes. The bottom portion of the tubes containing the pellets were cut off and washed twice (10 min each) in 0.2 M K-phosphate buffer (pH 7.1). The pellets were secondarily fixed in 1% $OsO_4$ for 30 min, followed by two washes in distilled $H_2O$ (10 min each). The fixed samples were dehydrated in an acetone series: 25, 50, 75, 90% (5 min per step), 100% (2 X 10 min). The dehydrated pellets were then embedded in Polybed/Araldite resin after going through 1:3, 2:2, 3:1 resin:acetone exchanges (1 hr each). The samples were left in 100% resin at room temperature overnight and then polymerized in a 70°C oven for 5 d. Ultrathin sections were made with glass knives and stained with uranyl acetate and lead citrate. Micrographs were taken on a Siemens Electron Microscope.

**Enzyme Assays.** NADH- and NADPH-dependent Cyt *c* reductase activities were determined spectrophotometrically in the presence of 2.5 mM KCN (21). Cyt *c* oxidase was determined spectrophotometrically in the presence of 0.02% Triton X-100 (21). ATP (Tris salt) and PnPP hydrolyzing activity were determined by detection of released Pi, as previously described (26), after a 30-min incubation at 37°C in the presence of 3 mM substrate and 25 mM Tris-Mes, at the pH indicated. For each assay the ATPase activities were determined without added salt, with 50 mM KCl alone, with 3 mM $MgSO_4$ alone, or with 50 mM KCl and 3 mM $MgSO_4$. The $Mg^{2+}$-ATPase activity was calculated as the difference between the activities with and without $MgSO_4$. The KCl,$Mg^{2+}$-ATPase activity was calculated as the difference between the activities with and without KCl.

Latent UDPase and IDPase activities, measured as the difference of activity in the presence and absence of 0.1% digitonin, were determined as released Pi in 2.5 mM substrate after a 20-min incubation at 37°C (2.5 mM $MgSO_4$, 50 mM KCl, 25 mM Tris-Mes, pH 6.5).

The activities of glucan synthases I and II were measured by the procedures of Ray (36) in the presence of 17.5 nCi UDP-[$^{14}$C]glucose (233 Ci/mol; Radiochemical Centre, Amersham, U.K.). Cellobiose (0.3 M) was added to the incubation medium for the glucan synthase II assay (37). In Figure 4, glucan synthase I was assayed by a filtration method (11). UDPG-ST activity was assayed by the method of Hartmann-Bouillon and Benveniste (17) in the presence of 17.5 nCi of UDP-[$^{14}$C]glucose per assay.

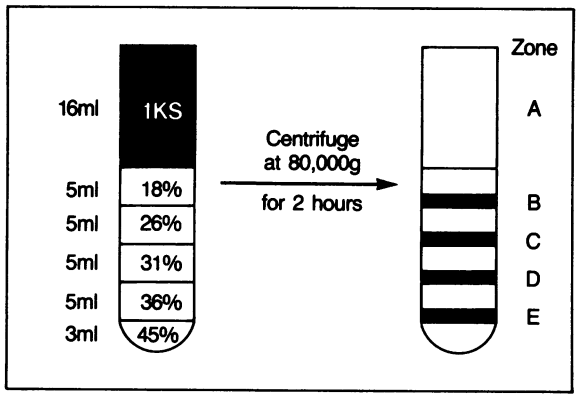


FIG. 1. Sucrose step gradient.

**NPA-Binding Assay.** NPA binding was assayed as specific binding according to the method of Ray (36) using 11.5 nCi [$^3$H] NPA (30 Ci/mmol; CEA, Gif-sur-Yvette, France) ±10 μM unlabeled NPA.

**Other Assays.** Sucrose concentrations were determined refractometrically. Carotenoids were measured as described by Ray (36). Protein was determined according to Lowry et al. (25) after a TCA precipitation and using BSA as a standard.

**Chemicals.** ATP (disodium salt) was purchased from Calbiochem, NPA from Frinton Laboratories (Vineland, NJ), and 1-amino-2-naphthol-4 sulfonic acid from Eastman Kodak Co. SDS was from Bio-Rad Laboratories. All the other chemicals were purchased from Sigma Chemical Company or Mallinckrodt.

## RESULTS

**Membrane Separation and Marker Enzymes.** Preliminary studies testing the effects of an initial centrifugation at 1000*g* *versus* 6000*g* on marker activities were carried out. The 1-KS fraction was centrifuged for 20 min at 6000*g* and the supernatant (6 KS) collected. To avoid pelleting, the 1-KS and 6-KS fractions were then centrifuged onto an 18/45% sucrose step gradient. Table I illustrates the effects of these initial centrifugations on the marker activities of the membranes collected at the 18/45% interface. Most (92%) of the mitochondrial marker, Cyt *c* oxidase, was sedimented by the 6000*g* centrifugation step. However, 37% of the ER marker, NADPH-Cyt *c* reductase, 53% of the Golgi marker, latent UDPase, and 67% of the PM marker, UDPG-ST, were also sedimented. Therefore, to avoid such major losses of Golgi membranes, the 1-KS fraction was routinely applied directly to density gradients.

Figure 2 illustrates the distribution of six marker enzymes from the 1-KS fraction after centrifugation on a linear sucrose gradient. The ER marker, NADPH-Cyt *c* reductase, was located at the light end of the gradient (1.10 g/cm$^3$), while the mitochondrial marker, Cyt *c* oxidase, formed a single peak at 1.17 g/cm$^3$. Latent IDPase, a Golgi marker, formed a single peak at 1.13 g/cm$^3$, while Glucan synthase I, another Golgi marker, was more broadly distributed and was shifted toward the denser region of the gradient. The lack of coincidence between the two Golgi markers has been observed before in monocots (40). NPA-binding, a PM marker, had a major peak at 1.17 g/cm$^3$, coinciding with Cyt *c* oxidase, and a minor peak associated with NADPH-Cyt *c* reductase (1.10 g/cm$^3$). The minor peak of NPA-binding on the ER was present in the very young coleoptiles used in the present study, but was negligible in older coleoptiles

Table I. *Effect of an Initial Centrifugation Used to Separate Mitochondria and Microsomes from a Corn Coleoptile Homogenate*

A 1,000*g* supernatant (1 KS) was prepared and one-half was centrifuged 20 min at 6,000*g* (Sorvall SS-34). The new supernatant (6 KS) was collected; 7 ml of the two supernatants (1 KS and 6 KS) were diluted with homogenization buffer (diluted one time) and layered on the top of two step gradients (5 ml 45% sucrose and 5 ml 18% sucrose). The gradients were centrifuged at 80,000*g* for 3 h and the material at the 18–45% sucrose interfaces was collected and analyzed for marker enzyme activities.

| Fraction | Protein | Cyt *c* Oxidase | | NADPH-Cyt *c* Reductase | | Latent UDPase | | UDPG-Sterol-Glucosyltransferase | |
|---|---|---|---|---|---|---|---|---|---|
| | | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity |
| | *mg* | *μmol/mg prot·min* | *μmol/ frac·min (%)* | *μmol/mg prot·min* | *μmol/ frac·min (%)* | *μmol/mg prot·min* | *μmol/ frac·min (%)* | *cpm/mg prot·min* | *cpm/ frac·min (%)* |
| 1 KS | 5.03 | 0.932 | 4.69 (100) | 0.185 | 0.930 (100) | 0.263 | 1.325 (100) | 222 | 1115 (100) |
| 6 KS | 2.58 | 0.143 | 0.37 (8) | 0.226 | 0.584 (63) | 0.240 | 0.620 (47) | 142 | 367 (33) |

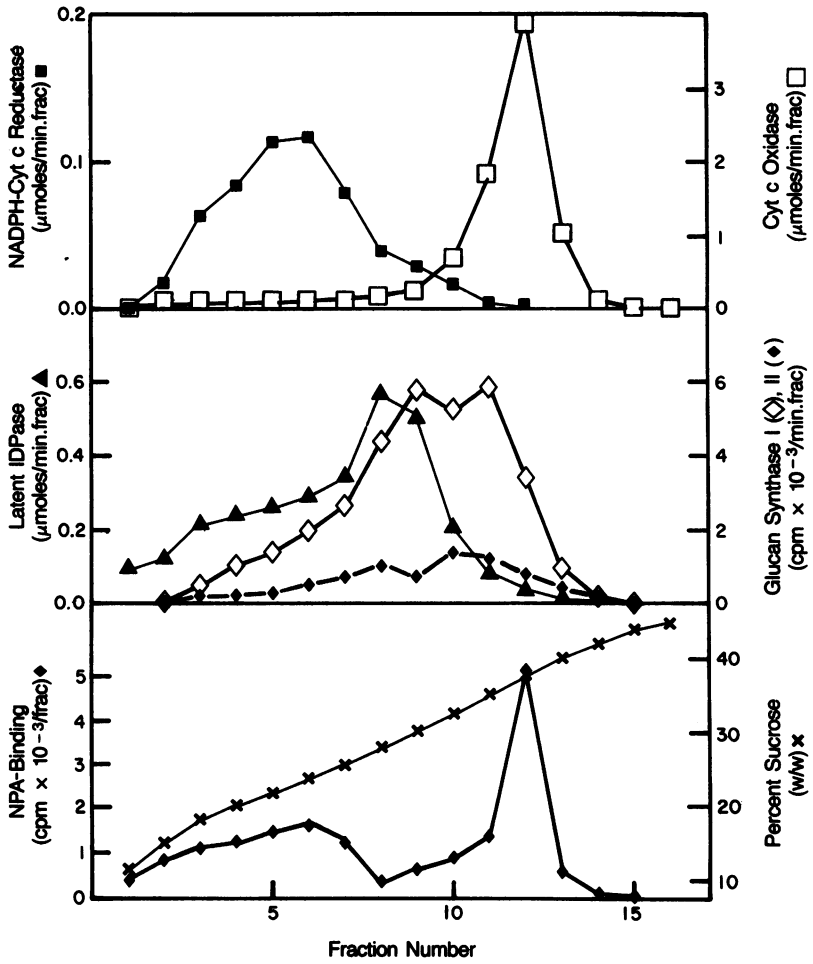


FIG. 2. Linear 15–45% (w/w) sucrose gradient of the 1-KS fraction of a corn coleoptile homogenate centrifuged for 3 h at 80,000*g*. NADPH-Cyt *c* reductase (■), Cyt *c* oxidase (□), latent IDPase (▲), and percent sucrose (×) were analyzed with 50 μl aliquots. Glucan synthase I (◇) and II (◆) were determined on 0.1 ml aliquots and NPA-binding (◆) on 0.4 ml aliquots.

(A. Chanson and L. Taiz, unpublished data). Glucan synthase II, another PM marker, was present in low amounts (relative to Glucan synthase I) and showed a broad distribution which overlapped with the Golgi.

Since neither NPA-binding nor Glucan synthase II had single peaks, an additional PM marker, UDPG-ST, was investigated. As shown in Figure 3, UDPG-ST exhibited a single peak at 1.16 g/cm$^3$ and was well separated from latent IDPase and Cyt *c* oxidase. In subsequent gradients, UDPG-ST was utilized as the PM marker. Total carotenoids, used as a marker for plastid membranes, were assayed on a complete gradient, but were not present in detectable amounts using the method of Ray (36).

Table II shows the distribution of the same marker enzymes from the 1 KS fraction after centrifugation on a sucrose step gradient (Fig. 1). Fraction B was enriched in ER (NADH-Cyt *c* reductase), fraction C in Golgi (latent UDPase and IDPase), fraction D in PM (UDPG-ST), and fraction E in mitochondria (Cyt *c* oxidase). Here again, glucan synthase I had a broader distribution than latent IDPase, and NPA-binding exhibited a second peak coincident with the ER in fraction B. Glucan synthase II was also broadly distributed between fractions C and D, while UDPG-ST was mainly in fraction D.

The distribution of the markers after rate-zonal centrifugation for 25 min is shown in Figure 4. Mitochondria were recovered on the 45% sucrose cushion, while the PM and ER zones were located near the top of the gradient. Latent UDPase exhibited two peaks, the first one at the top of the gradient and the second within the gradient (fractions 7–10). Glucan synthase I, a Golgi

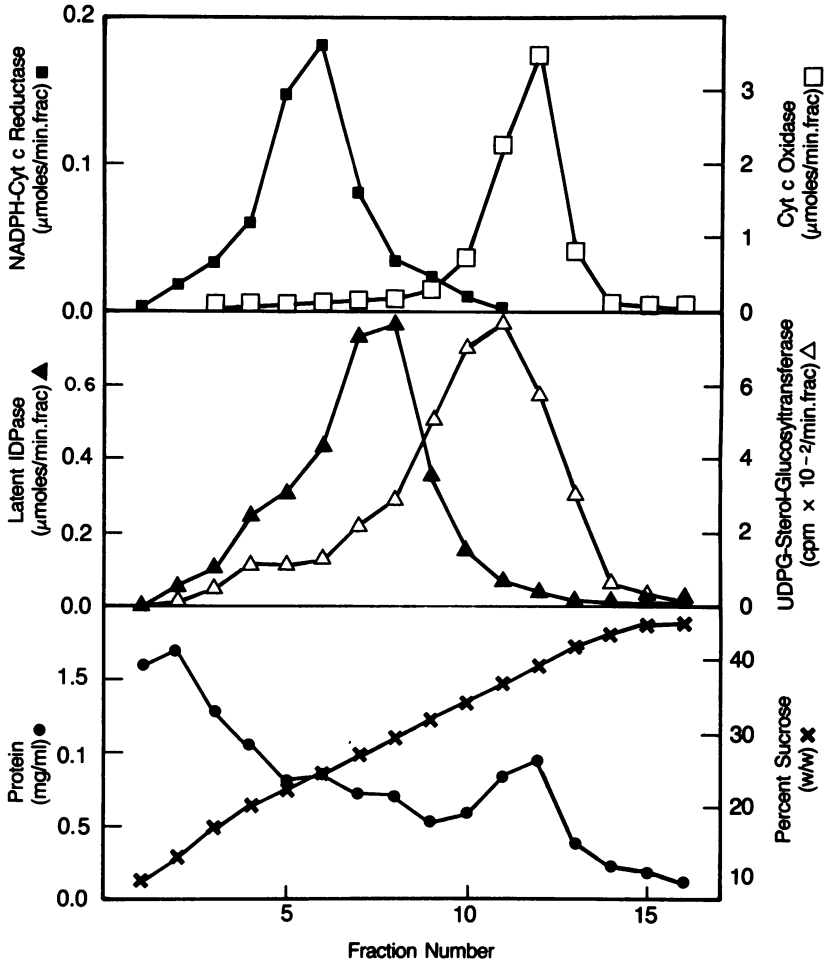


FIG. 3. Linear sucrose gradient as in Figure 2. NADPH-Cyt *c* reductase (■), Cyt *c* oxidase (□), latent IDPase (▲), protein (●) and percent sucrose (×), were analyzed with 50 μl aliquots. UDPG-sterol glucosyltransferase (△) was determined on a 0.5 ml aliquot.

cisternae marker (35, 39, 42) was broadly distributed, but exhibited a major peak which coincided with the faster sedimenting zone of latent UDPase, as well as a shoulder which appeared to overlap with the PM. As noted previously by others (37, 40, 46) glucan synthases I and II may not be specific markers for the Golgi and PM, respectively, in monocots. However, the coincidence of the major peak of glucan synthase I with the heavier band of latent UDpase suggests that this zone consists mainly of Golgi cisternae and attached vesicles, as was previously found to be the case in renografin rate zonal gradients of pea stem homogenates (42).

**Electron Microscopy.** The localization of Golgi membranes on isopycnic sucrose gradients was verified by electron microscopy. As shown in Figure 5A, the peak of latent IDPase is clearly enriched in Golgi membranes, consisting largely of isolated cisternal membranes and their attached vesicles. Golgi membranes were generally absent from the ER- (Fig. 5B), PM- (Fig. 5C), and mitochondria-rich (Fig. 5D) fractions.

**ATPase Activity.** As a precaution against interference by nonspecific phosphatases, all ATPase assays contained 1 mM sodium molybdate (12). The presence of 1 mM molybdate strongly inhibited PnPPase activity and ATPase (minus Mg) activities, and promoted $Mg^{2+}$-ATPase in the plasma membrane- (D) and Golgi-enriched (C) fractions taken from a sucrose step gradient (data not shown). The KCl-dependent $Mg^{2+}$-ATPase activities of the two fractions were unaffected by the presence of molybdate.

Figure 6 shows the effect of pH on the $Mg^{2+}$ and KCl, $Mg^{2+}$-ATPase of Golgi-rich and a PM-rich fractions obtained from a linear sucrose gradient. In the absence of KCl, the pH optima of the $Mg^{2+}$-ATPase was 7 to 7.5 for both fractions. In the presence of KCl the two fractions had a pH optimum of 6.0 to 6.5. However, the Golgi-rich fraction exhibited a second peak at pH 7.5. We hypothesized that the main peak at pH 6.0 of the Golgi-rich fraction might represent PM contamination and that the pH 7.5 peak was due to a Golgi ATPase.

The Golgi-rich fractions (zone C) from two sucrose step gradients were pooled and recentrifuged on a linear sucrose gradient (15–45%) for 3 h. Figure 7 shows the distribution of the marker enzymes. The activities of NADPH-Cyt *c* reductase and Cyt *c* oxidase were very low compared to their activities in a complete gradient (Fig. 3). In addition, the peak of Cyt *c* oxidase was shifted to 31% sucrose. This is most likely due to the fact that zone C of the step gradient contains submitochondrial particles rather than intact mitochondria. The $Mg^{2+}$-ATPase activity had the same distribution at pH 6.5 and 7.5 and coincided with UDPG-ST. The KCl, $Mg^{2+}$-ATPase had the same distribution as UDPG-ST when assayed at pH 6.5, but was coincident with latent UDPase when assayed at pH 7.5.

An isopycnic gradient was also carried out using a Golgi-rich fraction prepared from two rate-zonal gradients. To minimize contamination from the ER and PM, the faster sedimenting peak (fractions 8–10 of Fig. 4), consisting of Golgi cisternae and their attached vesicles, was pooled and recentrifuged. Here again (Fig. 8), the activities of NADPH-Cyt *c* reductase and Cyt *c* oxidase were very low, but in this case, the peaks were recovered at their normal densities (1.10 and 1.17 g/cm$^3$). The $Mg^{2+}$-ATPase activities at pH 6.5 and 7.5, as well as the KCl, $Mg^{2+}$-ATPase activity at pH 6.5, had the same distribution as the PM marker, UDPG-ST. The pH 7.5 KCl, $Mg^{2+}$-ATPase again had a distribution similar to the Golgi marker, latent UDPase.

Table II. *Distribution of Marker Enzymes from the 1-KS Fraction after Centrifugation on a Sucrose Step Gradient*

A homogenate of corn coleoptiles was prepared (see "Materials and Methods") and centrifuged at 1000*g* for 5 min. The pellet (1 KP) and the supernatant (1 KS) were collected and two step gradients of 1 KS were prepared (see Fig. 1). The material at the different interfaces was collected and each identical fraction combined. The different fractions were analyzed for marker enzyme activities. The relative amounts of activity in the gradient fractions are expressed as the percent of the total activity recovered in the gradient. The 1 KS was inhibitory to two of the markers resulting in greater than 100% recovery relative to the 1 KS.

| Fraction | Protein | NADH-Cyt *c* Reductase | | Latent IDPase | | Latent UDPase | | Glucan Synthase I | |
|---|---|---|---|---|---|---|---|---|---|
| | | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity |
| | *mg* | *μmol/mg prot·min* | *μmol/ frac·min (%)* | *μmol/mg prot·min* | *μmol/ frac·min (%)* | *μmol/mg prot·min* | *μmol/ frac·min (%)* | *cpm/mg prot·min* | *cpm/ frac·min (%)* |
| Homogenate | 85.5 | 0.314 | 26.813 | 0.132 | 11.324 | 0.123 | 10.564 | 117 | 10032 |
| 1 KS | 77.5 | 0.324 | 25.086 | 0.133 | 10.286 | 0.124 | 9.620 | 123 | 9571 |
| 1 KP | 8.5 | 0.0 | 0.0 | 0.034 | 0.286 | 0.023 | 0.198 | 106 | 904 |
| A | 44.1 | 0.013 | 0.585 (6.5) | 0.0 | 0.000 (0) | 0.0 | 0.000 (0) | 51 | 2244 (10) |
| B | 3.0 | 1.903 | 5.708 (63.3) | 0.283 | 0.848 (30.3) | 0.372 | 1.116 (33.1) | 1110 | 3331 (14.8) |
| C | 2.1 | 0.939 | 1.972 (21.9) | 0.699 | 1.468 (52.5) | 0.834 | 1.752 (52.0) | 3839 | 8061 (35.9) |
| D | 2.1 | 0.357 | 0.750 (8.3) | 0.164 | 0.344 (12.3) | 0.175 | 0.368 (10.9) | 2933 | 6160 (27.4) |
| E | 2.8 | 0.0 | 0.000 (0) | 0.049 | 0.136 (4.9) | 0.047 | 0.132 (3.9) | 953 | 2667 (11.9) |

| Fraction | Glucan Synthase II | | NPA-Binding | | UDPG-sterol glucosyltransferase | | Cyt *c* Oxidase | |
|---|---|---|---|---|---|---|---|---|
| | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity | Specific activity | Total activity |
| | *cpm/mg prot·min* | *cpm/ frac·min (%)* | *cpm/mg protein* | *cpm/frac (%)* | *cpm/mg prot·min* | *cpm/ frac·min (%)* | *μmol/mg prot·min* | *μmol/ frac·min (%)* |
| Homogenate | 611 | 52227 | 1078 | 92150 | 49 | 4155 | 0.392 | 33.516 |
| 1 KS | 551 | 42673 | 1015 | 78625 | 47 | 3626 | 0.413 | 31.998 |
| 1 KP | 213 | 1809 | 345 | 2930 | 27 | 227 | 0.212 | 1.799 |
| A | 19 | 839 (6.5) | 19 | 850 (4.6) | 2 | 102 (2.2) | 0.008 | 0.354 (3.4) |
| B | 528 | 1584 (12.3) | 1730 | 5190 (27.9) | 190 | 569 (12.1) | 0.076 | 0.228 (2.2) |
| C | 1636 | 3435 (26.6) | 1471 | 3090 (16.6) | 549 | 1153 (24.6) | 0.168 | 0.353 (3.4) |
| D | 1909 | 4008 (31.0) | 2386 | 5010 (26.9) | 900 | 1889 (40.3) | 0.873 | 1.834 (17.7) |
| E | 1089 | 3048 (23.6) | 1600 | 4480 (24.0) | 348 | 975 (20.8) | 2.718 | 7.611 (73.3) |

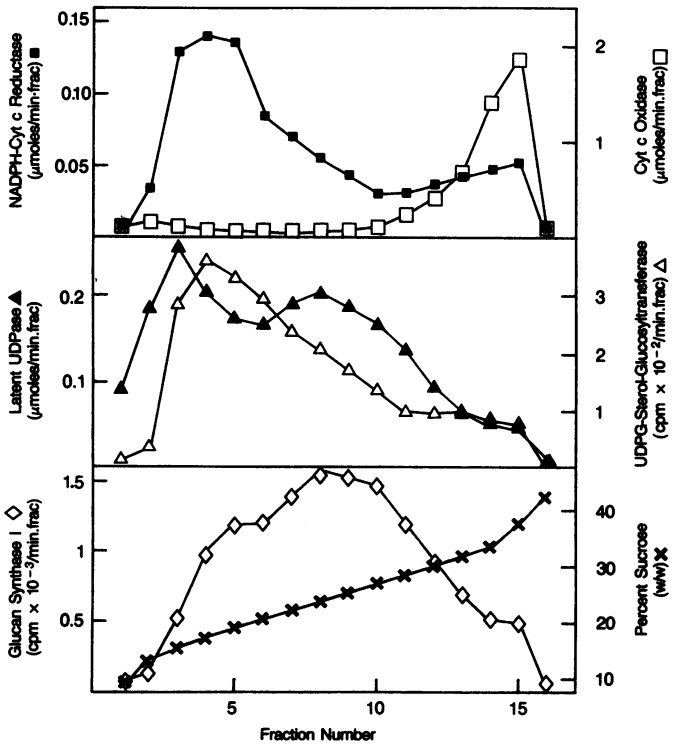


FIG. 4. Rate-zonal sedimentation of the 1-KS fraction, centrifuged for 25 min at 30,000*g*. NADPH-Cyt *c* reductase (■); Cyt *c* oxidase (□); latent UDPase (▲), percent sucrose (×), glucan synthase I (◇), and UDPG-sterol glucosyltransferase (△).

## DISCUSSION

**Membrane Isolation Procedure.** Most procedures for separating microsomal membranes include a low speed initial centrifugation step for removing the mitochondria from the homogenate (16). Nagahashi and Hiraike (30) showed that an optimal separation of mitochondria and microsomes in corn roots could be achieved by an initial centrifugation of 6000*g* for 20 min. They reported that 93% of the mitochondria (Cyt *c* oxidase) were pelleted under these conditions, together with 35% of the PM (pH 6.5 $K^+$-stimulated ATPase) and 28% of the ER (Antimycin A-insensitive NADH-Cyt *c* reductase). Under the same conditions (Table I), we pelleted 92% of the mitochondria, in agreement with Nagahashi and Hiraike (30), but substantially more PM (67% UDPG-ST), ER (37%) NADPH-Cyt *c* reductase), and Golgi (53% latent UDPase) were also removed. These differences could be due to several factors. First, the marker enzymes were not the same in the two studies. Second, the grinding method of Nagahashi and Hiraike (30) would probably result in smaller membrane fragments than the chopping technique used in the present study (19, 29, 38). Third, the densities of the membranes may be slightly different in roots and coleoptiles. Similar losses of membranes during the precentrifugation step have been reported previously (1, 10, 17, 19, 22, 23, 28, 29, 31). To maximize our yields of Golgi membranes we adopted the procedure of precentrifuging the homogenate at 1000*g* for 5 min only, and layering the supernatant directly onto density gradients. This procedure also avoids pelleting and resuspension of the microsomes, which can damage or aggregate organelles, particularly the Golgi (1, 17, 28, 36).

**Selection of Markers.** Because identification of organelles relies so heavily on the distribution of marker enzymes, some

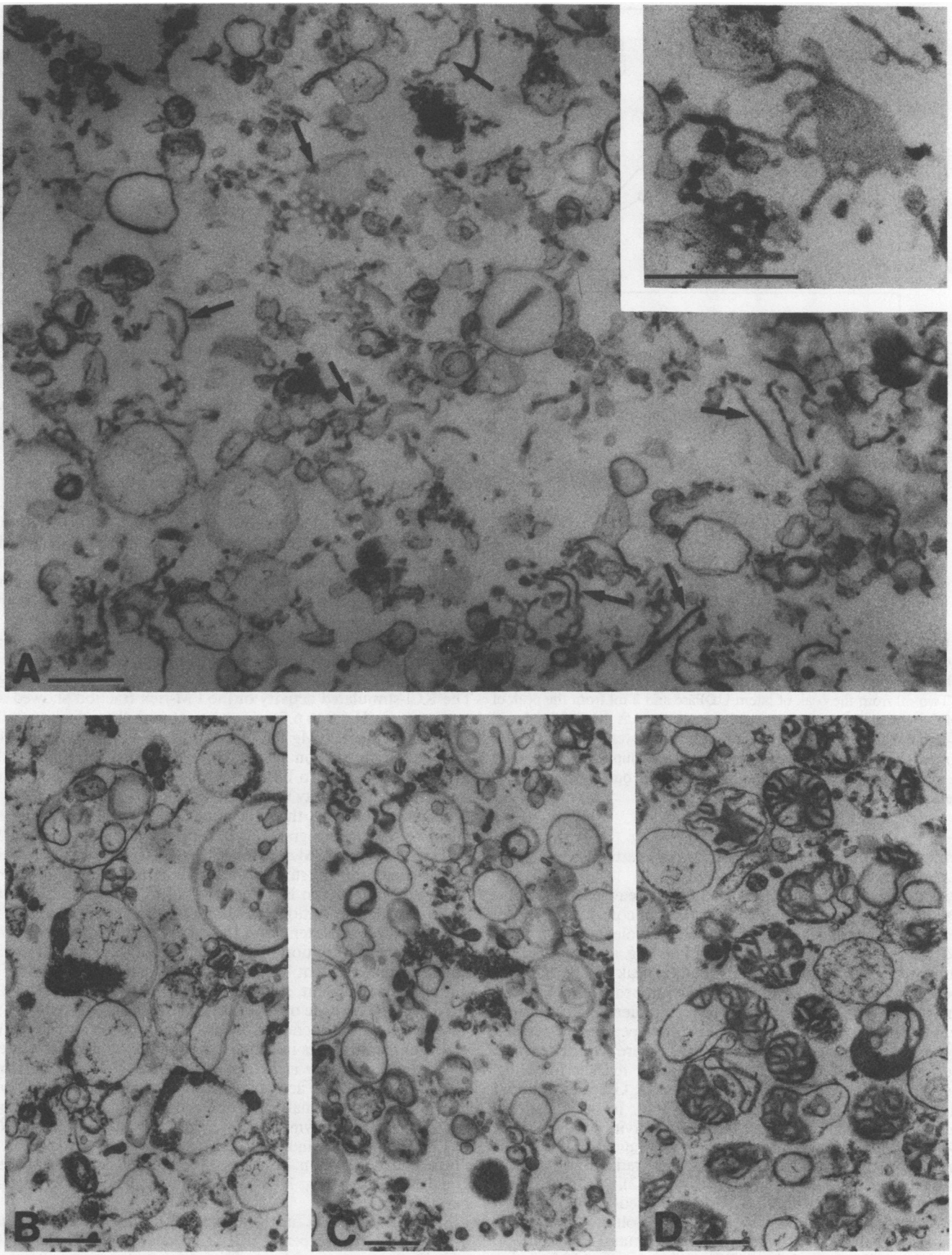


FIG. 5. Electron micrographs of peak fractions taken from a linear sucrose gradient as in Figure 2. A, Golgi, showing isolated cisternae (arrows); surface view of a Golgi cisterna with associated vesicles is shown at higher magnification in the inset of 5A; B, ER; C, PM; D, Mitochondria. Bar, 0.5 $\mu$m.

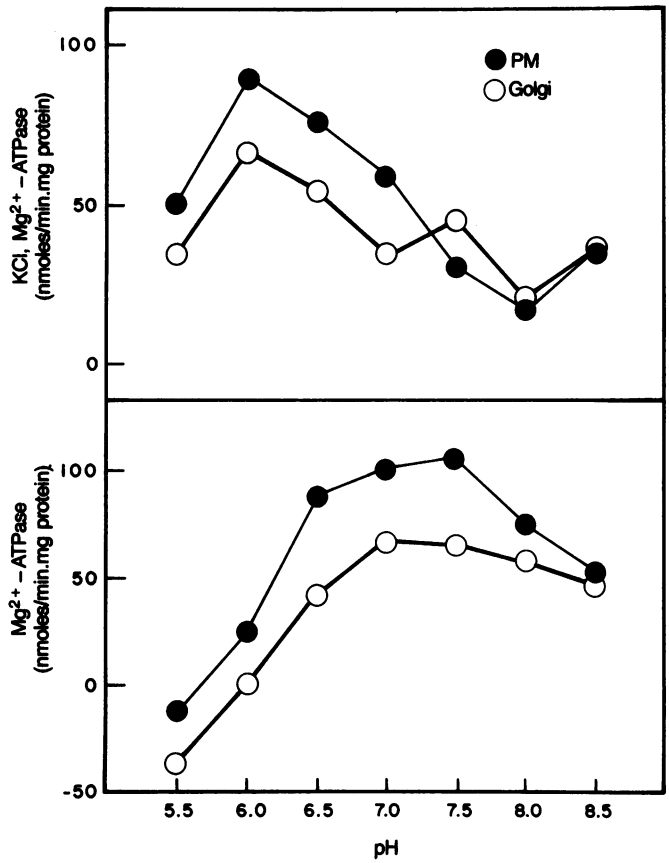


FIG. 6. Effect of pH on the $Mg^{2+}$-ATPase and the KCl, $Mg^{2+}$-ATPase activities of a Golgi-rich fraction and a PM-rich fraction. A linear sucrose gradient was prepared (see Fig. 2) and fractionated into 23 fractions of 1 ml. Two ml from the peak of latent UDPase and 2 ml from the peak of UDPG-sterol glucosyltransferase activity were used as the Golgi-rich and PM-rich fractions, respectively. The two fractions were diluted with 1.5 ml of gradient buffer. Each point is the average of duplicate samples and the curve shown is a representative experiment out of three similar experiments.

justification of their selection is necessary, particularly those of the plasma membrane and Golgi.

Nagahashi and Kane (31) showed that a detergent-stimulated UDPase provided a reliable Golgi marker in corn root homogenates. Numerous authors working with corn coleoptiles have also used latent IDPase as a Golgi marker (*e.g.* 6, 26, 37). We confirmed by electron microscopy that the peak of latent IDPase on an isopycnic sucrose gradient was enriched in Golgi membranes. However, on rate zonal sucrose gradients, two peaks of latent UDPase occur. The faster sedimenting peak coincides with another Golgi marker, Glucan synthase I. There is evidence from other systems that Glucan synthase I is a marker for Golgi cisternae (35, 38, 42), although, in monocots, Glucan synthase I activity is also associated with the PM (40). In our rate-zonal gradients, a shoulder of Glucan synthase I activity coincided with UDPG-ST, but the majority migrated with latent UDPase. The band of latent UDPase at the top of the gradient presumably represents isolated Golgi secretory vesicles (42).

In 1975, Gardiner and Chrispeels (13) used UDPG-glucosyl transferase, which transfers glucose to lipid soluble products, as a marker for the PM of carrot roots. Hartmann-Bouillon and Benveniste (17) reported that UDPG-ST was a good PM marker in corn coleoptiles. Baydoun and Northcote (1), working on the same material, reported high UDPG-ST activity in a PM-enriched fraction prepared from a step gradient. Buckhout *et al.* (7) used UDPG-ST as a PM marker in cress roots. However, Lercher and Wojciechowski (24) found a high level of UDPG-ST in two Golgi-rich fractions prepared from onion stems. Unfortunately, they gave no indication of the PM contents of these fractions. Bowles *et al.* (5) claimed that UDPG-ST is Golgi-associated, but were unable to separate the Golgi and PM on linear sucrose gradients (4). A reanalysis of their data, however, shows a good correlation between a pH 6.0 ATPase and UDPG-ST, which argues in favor of a PM localization of UDPG-ST.

NPA-binding has also been employed as a PM marker (16, 35). We consistently found a minor second peak of NPA-binding associated with the ER. Similar results were reported by Thom *et al.* (43) using suspension-cultured sugarcane cells and by Hartmann-Bouillon and Benveniste (17) in corn coleoptiles. The main peak of NPA-binding coincides with the peak of Cyt *c* oxidase on our isopycnic sucrose gradients. However, NPA-binding is not associated with the mitochondria, since we were able to separate the two on linear dextran gradients (data not shown). NPA-binding may be a marker for a heavy domain of the PM (35).

Although carotenoids were not detectable in our gradients we cannot rule out the possibility of contamination by proplastid or amyloplast membranes, which are low in carotenoids. Tonoplast membranes were not monitored in this study, but it is well established that they are light membranes with a density of 1.11 $g/cm^3$ (26). We have recently used nitrate-sensitive, $Mg^{2+}$-ATP-dependent proton pumping to localize the tonoplast at this density under our centrifugation conditions (A. Chanson and L. Taiz, unpublished data). The peak of nitrate-sensitive proton pumping is clearly separable from Golgi markers, and we conclude that the tonoplast is not a significant contaminant of the Golgi fraction.

**The Golgi KCl, $Mg^{2+}$-ATPase.** A comparison of the pH curves of the activity in the Golgi-rich and PM-rich fractions provided the first hint of an ATPase activity associated with the Golgi. The KCl-stimulated activity of the PM-rich fraction showed an optimum at pH 6.0 to 6.5, in agreement with the value generally reported for the $K^+$, $Mg^{2+}$-ATPase of the PM (2, 12, 20, 32, 44). The Golgi-rich fraction also exhibited a peak at pH 6.0 to 6.5, which might be due to PM-contamination. However, a second, smaller peak of activity was found at pH 7.5.

It seemed possible that the putative Golgi pH 7.5 ATPase might be obscured in gradients of total homogenates by the PM ATPase. To reduce PM contamination, Golgi-rich fractions were prepared either from step- or rate-zonal gradients and recentrifuged isopycnically on linear gradients. A close correlation was found in the distributions of UDPG-ST and the pH 6.5 KCl, $Mg^{2+}$-ATPase, consistent with their use as PM markers. On the contrary, the distribution of the pH 7.5 KCl, $Mg^{2+}$-ATPase was similar to that of latent UDPase. This was true only of the KCl-stimulated component, since the peak of pH 7.5, $Mg^{2+}$-ATPase activity in the absence of KCl overlapped with the PM.

A review of the literature on plant microsomal ATPases revealed numerous cases in which a Golgi ATPase may have been detected. In fact, Ray *et al.* (38) were the first to report a low level of $Mg^{2+}$-ATPase activity co-purifying with the Golgi of pea stem tissue in rate-zonal and isopycnic linear sucrose gradients. Fortuitously, the enzyme was assayed at pH 7.5, although KCl was omitted. Cross and Briggs (8) reported two peaks of $K^+$-stimulated ATPase on isopycnic gradients of corn coleoptiles. The lighter peak was at 30% sucrose, the same density as Glucan synthase I.

Beffagna *et al.* (2), using a 13,000 to 80,000*g* microsomal pellet, identified two peaks of KCl-stimulated ATPase (pH 7.0) on a linear sucrose gradient. The main peak was recovered at 38% sucrose and was probably associated with the PM. No comments were made concerning the lighter peak at 28% sucrose. Pierce and Hendricks (33) reported multiple sites of $K^+$-

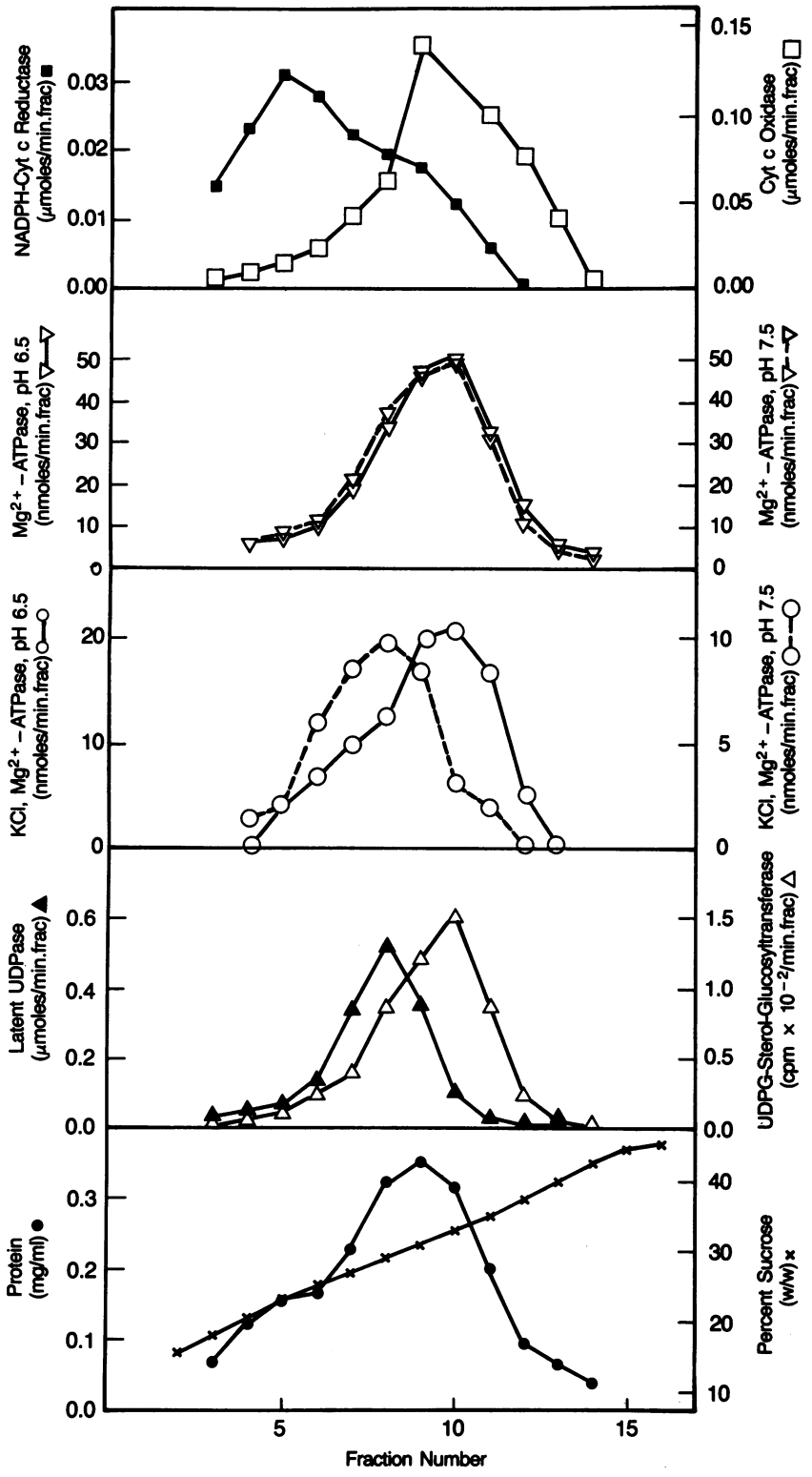


FIG. 7. Linear sucrose gradient of a Golgi-rich fraction centrifuged for 3 h at 80,000*g*. Two sucrose step gradients were prepared as in Figure 1 and Table II. The two Golgi-rich bands (zone C) were pooled (7 ml), diluted with 10 ml of gradient buffer, and layered onto the top of a linear gradient. NADPH-Cyt *c* reductase (■), Cyt *c* oxidase (□), $Mg^{2+}$-ATPase at pH 6.5 (▽——) and at pH 7.5 (▽– – –), KCl, $Mg^{2+}$-ATPase at pH 6.5 (○——) and at pH 7.5 (○– – –) latent UDPase (▲), protein (●), percent sucrose (×), UDPG-sterol glucosyltransferase (△). All ATPase assays were in the presence of 1 mM Na molybdate.

stimulated ATPase (pH 6.0) on linear gradients of pea stem homogenates. The authors found a very close correlation of PM (1.13 g/cm$^3$) and Golgi (1.12 g/cm$^3$) with the $K^+$-stimulated ATPase. Dupont *et al.* (11) also found multiple peaks of KCl,$Mg^{2+}$-ATPase overlapping the Golgi on linear sucrose gradients of corn root microsomes.

Recently, Binari and Racusen (3) found that secretory vesicles isolated from liquid-cultured *Avena* cells possessed cation-stimulated ATPase activity. This ATPase had enzymic properties similar to the PM ATPase, including a pH optimum of 6.0 to 6.5. Unfortunately, the extent of PM contamination was not monitored. In contrast, Taiz *et al.* (42) found no evidence for a pH 6.5 ATPase associated with the Golgi secretory vesicles of pea stems. In the present study, following rate-zonal centrifugation of the 1 KS as in Figure 4, which separates the Golgi membranes into cisternae-rich (Glucan synthase I) and vesicle-rich (latent UDPase) fractions (42), it was not possible to localize the pH 7.5 $Mg^{2+}$-ATPase due to the high levels of contamination

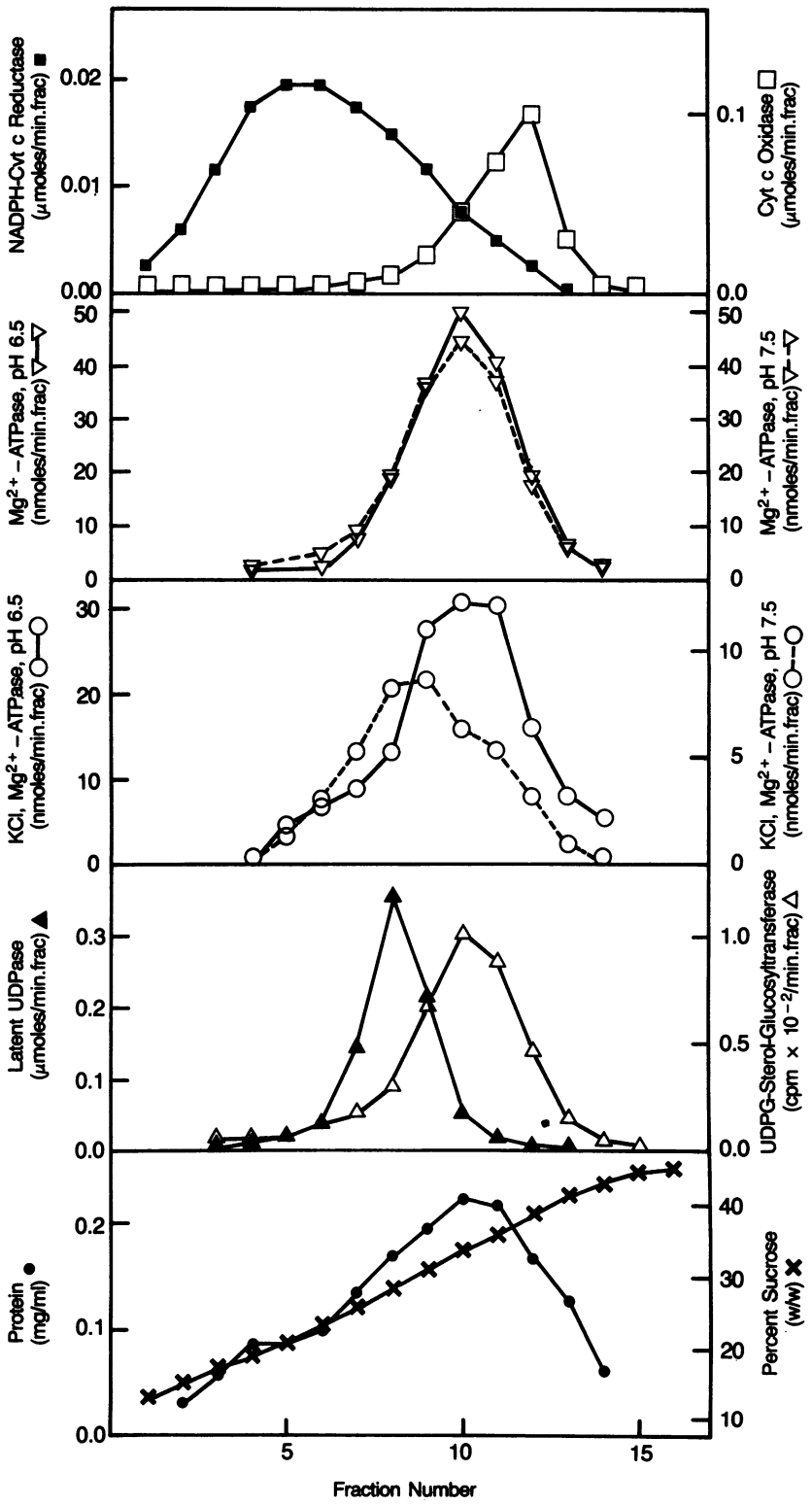


FIG. 8. Linear sucrose gradient of a Golgi-rich fraction. Two rate-zonal gradients were prepared and fractionated as previously described (see Fig. 4). Fractions 8–10 were pooled and diluted with 9 ml of gradient buffer and layered onto the top of a linear gradient. The markers are as shown in Figure 7.

from other ATPases (data not shown). Thus, the results presented here do not allow us to discriminate between Golgi cisternae or secretory vesicles as the probable site of the Golgi ATPase.

The function of the Golgi ATPase in plant cells remains to be determined. In animal tissues, Golgi ATPases have been implicated in calcium (47) and proton (14, 48) transport. We have recently identified an ATP-dependent proton pump on the Golgi of corn coleoptiles with properties identical to the Golgi ATPase. The Golgi $H^+$-pump is insensitive to azide and vanadate, is partially inhibited by nitrate, and appears to be localized on the Golgi cisternae (A. Chanson and L. Taiz, in preparation). The possible role of the Golgi $H^+$-ATPase in regulating secretion and cellular development is an exciting area for further research.

*Acknowledgments*—We wish to express our appreciation to the Pfizer-DeKalb Company for supplying us with Trojan 929 corn seeds. We thank S. Mandala for valuable discussions and for reviewing the manuscript.

## LITERATURE CITED

1. BAYDOUN EAH, DH NORTHCOTE 1980 Isolation and characterization of membranes from the cells of maize root tips. J Cell Sci 45: 147–167

2. BEFFAGNA N, E MARRE, SM COCUCCI 1979 Cation-activated ATPase activity of plasmalemma-enriched membrane preparations from maize coleoptiles. Planta 146: 387–391
3. BINARI LLW, RH RACUSEN 1983 Membrane-associated ATPases in isolated secretory vesicles. Plant Physiol 71: 594–597
4. BOWLES DJ, H KAUSS 1976 Characterization, enzymatic and lectin properties of isolated membranes from *Phaseolus aureus*. Biochim Biophys Acta 443: 360–374
5. BOWLES DJ, L LEHLE, H KAUSS 1977 Glucosylation of sterols and polyprenolphosphate in the Golgi apparatus of *Phaseolus aureus*. Planta 134: 177–181
6. BRUMMER B, RW PARISH 1983 Identification of specific proteins and glycoproteins associated with membrane fractions isolated from *Zea mays* L. coleoptiles. Planta 157: 446–453
7. BUCKHOUT TJ, L HEYDER-CASPERS, A SIEVERS 1982 Fractionation and characterization of cellular membranes from root tips of garden cress (*Lepidium sativum* L.). Planta 156: 108–116
8. CROSS JW, WR BRIGGS 1976 An evaluation of markers for plasma membranes in membrane fractions from *Zea mays*. Carnegie Inst Wash Year Book pp 379–383
9. CHURCHILL KA, B HOLAWAY, H SZE 1983 Separation of two types of electrogenic $H^+$-ATPases from oat roots. Plant Physiol 73: 921–928
10. DOHRMANN U, R HERTEL, H KOWALIK 1978 Properties of auxin binding sites in different subcellular fractions from maize coleoptiles. Planta 140: 97–106
11. DUPONT FM, AB BENNETT, RM SPANSWICK 1982 Localization of a proton-translocating ATPase on sucrose gradients. Plant Physiol 70: 1115–1119
12. GALLAGHER SR, RT LEONARD 1982 Effect of vanadate, molybdate, and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots. Plant Physiol 70: 1335–1340
13. GARDINER M, MJ CHRISPEELS 1975 Involvement of the Golgi apparatus in the synthesis and secretion of hydroxyproline-rich cell wall glycoproteins. Plant Physiol 55: 536–541
14. GLICKMAN J, K CROEN, S KELLY, Q AL-AWQATI 1983 Golgi membranes contain an electrogenic $H^+$ pump in parallel to a chloride conductance. J Cell Biol 97: 1303–1308
15. HAGER A, R FRENZEL, D LAIBLE 1980 ATP-dependent proton transport into vesicles of microsomal membranes of *Zea mays* coleoptiles. Z Naturforsch 35: 783–793
16. HALL JL 1983 Plasma membrane *In* JL Hall, AL Moore, eds, Isolation of Membranes and Organelles from Plant Cells. Academic Press, London, pp 55–81
17. HARTMANN-BOUILLON MA, P BENVENISTE 1978 Sterol biosynthetic capability of purified membrane fractions from maize coleoptiles. Phytochemistry 17: 1037–1042
18. HENDRIKS T 1978 The distribution of glucan synthetase in maize coleoptiles: a comparison with K-ATPase. Plant Sci Lett 11: 261–274
19. HENDRIKS T 1978 Isolation of plasma membrane from a maize coleoptile homogenate: possible use of a mitochondrial fraction. Z Pflanzenphysiol 89: 461–465
20. HODGES TK 1976 ATPases associated with membranes of plant cells. *In* U Luttge, MG Pitman, eds, Transport in Plants II, Part A, Cells. Springer-Verlag, Berlin, pp 260–283
21. HODGES TK, RT LEONARD 1972 Purification of a plasma membrane-bound adenosine triphosphatase from corn roots. Methods Enzymol 32: 392–406
22. JESAITIS AJ, PR HENERS, R HERTEL, WR BRIGGS 1977 Characterization of a membrane fraction containing a *b*-type cytochrome. Plant Physiol 59: 941–947
23. LEONARD RT, WJ VANDERWOUDE 1976 Isolation of plasma membranes from corn roots by sucrose density gradient centrifugation. An anomalous effect of Ficoll. Plant Physiol 57: 105–114
24. LERCHER M, ZA WOJCIECHOWSKI 1976 Localization of plant UDP-glucose:sterol glucosyl-transferase in the golgi membranes. Plant Sci Lett 7: 337–340
25. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
26. MANDALA S, IJ METTLER, L TAIZ 1982 Localization of the proton pump of corn coleoptile microsomal membranes by density gradient centrifugation. Plant Physiol 70: 1743–1747
27. MOLLENHAUER HH, DJ MORRE, R DROLESKY 1983 Monensin affects the trans half of *Euglena* dictyosomes. Protoplasma 114: 119–124
28. MORRE DJ, TJ BUCKHOUT 1979 Isolation of Golgi apparatus. *In* E Reid, ed, Plant Organelles. Horwood, Chichester, pp 117–134
29. NAGAHASHI J, L BEEVERS 1978 Subcellular localization of glycosyl transferases involved in glycoprotein biosynthesis in the cotyledons of *Pisum sativum* L. Plant Physiol 61: 451–459
30. NAGAHASHI J, K HIRAIKE 1982 Effects of centrifugal force and centrifugation time on the sedimentation of plant organelles. Plant Physiol 69: 546–548
31. NAGAHASHI J, AP KANE 1982 Triton-stimulated nucleoside diphosophatase activity: subcellular localization in corn root homogenates. Protoplasma 112: 167–173
32. PERLIN DS, RM SPANSWICK 1980 Labeling and isolation of plasma membranes from corn leaf protoplasts. Plant Physiol 65: 1053–1057
33. PIERCE WS, DL HENDRIX 1979 Utilization of enzyme markers to determine the location of plasma membrane from *Pisum* epicotyls on sucrose gradients. Planta 146: 161–169
34. POOLE RJ 1978 Energy coupling for membrane transport. Annu Rev Plant Physiol 29: 437–460
35. QUAIL P 1979 Plant cell fractionation. Annu Rev Plant Physiol 30: 425–484
36. RAY PM 1977 Auxin binding sites of maize coleoptiles are localized on membranes of the endoplasmic reticulum. Plant Physiol 59: 594–599
37. RAY PM 1979 Maize coleoptile cellular membranes bearing different types of glucan synthetase activity. *In* E Reid, ed, Plant Organelles. Horwood, Chichester, pp 135–146
38. RAY PM, TL SHININGER, MM RAY 1969 Isolation of $\beta$-glucan synthetase particles from plant cells and identification with Golgi membranes. Proc Natl Acad Sci USA 64: 605–612
39. RAY PM, WR EISINGER, DG ROBINSON 1976 Organelles involved in cell wall polysaccharide formation and transport in pea cells. Ber Dtsch Bot Ges 89: 121–146
40. ROBINSON DG, R GLAS 1983 The glucan synthases from suspension-cultured sugar cane cells. J Exp Bot 34: 668–675
41. SPANSWICK RM 1981 Electrogenic ion pumps. Annu Rev Plant Physiol 32: 267–289
42. TAIZ L, M MURRAY, DG ROBINSON 1983 Identification of secretory vesicles in homogenates of pea stem segments. Planta 158: 534–539
43. THOM M, WM LAETSCH, A MARETZKI 1975 Isolation of membranes from sugarcane cell suspensions: evidence for a plasma membrane enriched fraction. Plant Sci Lett 5: 245–253
44. VARA F, R SERRANO 1982 Partial purification and properties of the proton-translocating ATPase of plant plasma membranes. J Biol Chem 257: 12826–12830
45. WALKER RR, RA LEIGH 1981 Characterization of a salt-stimulated ATPase activity associated with vacuoles isolated from storage roots of red beet (*Beta vulgaris* L.). Planta 153: 140–149
46. WALTON JD, PM RAY 1982 Inhibition by light of growth and Golgi-localized glucan synthetase in the maize mesocotyl. Planta 156: 302–308
47. WEST DW 1981 Energy-dependent calcium sequestration activity in a Golgi apparatus fraction derived from lactating rat mammary glands. Biochim Biophys Acta 673: 374–386
48. ZHANG F, DL SCHNEIDER 1983 The bioenergetics of Golgi apparatus function: evidence for an ATP-dependent proton pump. Biochem Biophys Res Commun 114: 620–625