Characterization of a Membrane Fraction Containing a b-type Cytochrome1, 2

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ABSTRACT

The various components obtained from etiolated corn (Zea mays L.) coleoptiles were fractionated by differential or sucrose gradient centrifugation. The endoplasmic reticulum, plastids, Golgi, and mitochondria were localized by enzymic or other markers in the various fractions. A fifth fraction was also characterized. It contains gluan synthetase II activity, binding sites for N-naphthylphthalamic acid, NADH dehydrogenase activity which is both antimycin A- and cyanide-insensitive, and a b-type cytochrome. It is possible that this fraction is plasma membrane and that it may contain the blue-ultraviolet photoreceptor for phototropism in corn.

The action spectra for a wide variety of photoresponses in higher plants and fungi all show a single broad band with a maximum near 370 nm and a band between 400 and 500 nm showing considerable fine structure (4). In those systems in which it was measured (Phycomyces [7, 8], Mycobacterium [14]), a sharp action maximum near 280 nm also appears. These action spectra suggest carotenoproteins or flavoproteins as the responsible pigments (4, 9). Orientation and dichroism of the photoreceptor pigments in a variety of such systems provide strong evidence for localization of these pigments on the plasma membrane (15-17, 32). The absence of any other optically polarizing structure which maintains a constant and stable positional relationship with the cell wall but precludes any other possibility. Hence it was of considerable interest to attempt identification and characterization of the plasma membrane (and other membrane fractions) from phototropically sensitive corn coleoptiles.

In the present work, subcellular particulate fractions from coleoptiles of etiolated Zea mays L. seedlings were characterized following differential or isopycnic sucrose gradient fractionation. The various membrane fractions were identified by assays for established marker enzymes: ER, NADH-dependent cytochrome c reductase (22), G4, b-glucan synthetase I (28), and M,

Cyt c oxidase (1). Proplastids were identified by carotenoid content (10). In addition, the flavin, total Cyt, NADH dehydrogenase, and total protein were determined for each fraction. A fifth particulate fraction was also characterized. It contained the highest specific activity for b-glucan synthetase II and for the binding of NPA. Various workers have used either NPA binding (12, 20) or b-glucan synthetase II activity at high UDPG concentrations (13, 21) as markers for plasma membrane. Inasmuch as the strict specificity of these markers for plasma membrane is open to debate (see under “Discussion”) we shall retain quotes and refer to it as “plasma membrane” (“PM”). This fraction also contains a b-type Cyt.

Muñoz and Butler (24) have proposed that photoreduction of a flavin moiety, leading to reduction of a b-type Cyt may represent reactions of the blue light photoreceptor in Neurospora. Brain et al. (3) report that a Neurospora fraction enriched for “PM” is enriched for the blue-induced Cyt reduction which Muñoz and Butler described from in vivo studies. That this fraction may actually contain the blue light photoreceptor is supported by the recent observation (2) that a Neurospora mutant deficient in b-type Cyt shows impaired photosensitivity. The similarities between the “PM” membrane fraction described here and that from Neurospora, and the possible relationships between these fractions and photoreception for blue and UV-sensitive processes will be considered elsewhere (3).

MATERIALS AND METHODS

Chemicals. 1-N-Naphthylphthalamic acid (NPA) was generously provided by K. St.-Thomson. Uridine-5-diphosphoglucose (UDPG) and reduced b-nicotinamide dinucleotide were obtained from Boehhringer GmbH, Mannheim; Cyt c from SERVA, Feinbiochemica, Heidelberg. Uridine-5-diphospho-4C-glucose (4C-UDPG, ammonium salt, 248 mCi/mmol) was purchased from Amersham/Buchler-Braunschweig, FRB; 2,3,4,5-4H-naphthylphthalamic acid (4H-NPA, 16.4 Ci/mmol) from CEA-Grance, Gif-sur-Yvette, France.

Buffer Composition. Extraction medium contained 0.25 M sucrose, 50 mM tris-acetate, 1 mM EDTA, and 0.1 mM MgCl2 adjusted to pH 8. Final pH of the initial extract was then 7. Gradient medium contained 10 mM tris-acetate, 1 mM EDTA, 1 mM KCl, and 0.1 mM MgCl2, adjusted to pH 7 to avoid pH change. Resuspension medium for the assay of NPA binding contained 0.25 M sucrose, 10 mM Na-citrate, and 5 mM MgSO4, adjusted to pH 5.3 as specified in the assay (20).

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4 Abbreviations: AM: antimycin A; DT: dithionite; G: Golgi; K: n

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Plant Material. Seeds of Z. mays L. (WF9 × Bear 38) were obtained from the Bear Hybrid Co., Decatur, Ill. Seedlings were grown for 5 days in a controlled environment dark room (25 °C, 80% relative humidity) following overnight soaking in running tap water. The seedlings received 2 hr of red light every night except the last to suppress mesocotyl growth. Apical sections of coleoptiles 1 to 2 cm in length and free of primary leaves were harvested under room light, and placed directly on ice, as described previously (11). Homogenization and subsequent treatments were performed at 0 to 4 °C.

Homogenization and Fractionation. Coleoptiles (10–30 g) were chopped with razor blades in 1 volume of extraction medium equal to their weight (ml/g), and then ground in a chilled mortar. Following filtration through fine mesh nylon cloth, unbroken cells, starch grains, wall fragments, and nuclei were removed by centrifugation of the homogenate at 500g for 5 min. Differential centrifugations followed the scheme as Figure 1. Various precentrifugation and sucrose gradient protocols were applied to separate the various particulate fractions. The differing methods are given with each gradient in the Results section.

Assays. NPA-binding activity was measured following the technique of Lembi et al. (20), scaled down to 2-ml samples in resuspension medium. H-NPA (0.6 μM) was assayed with or without unlabeled NPA (6 μM) was added to the homogenates or fractionated particles. Samples were made up to 2 ml, and contained at least 1 ml of the resuspension medium to yield a final pH of 5.5. These samples were centrifuged in 2 ml cellulose nitrate tubes or 8 ml polycarbonate tubes at 100,000g for 20 min in a Beckman Spinc0 Ti-50 rotor. The pellets in the cellulose nitrate tubes were collected by cutting off the bottom of the tubes after draining, and transferring both bottom and pellet to vials with 9 ml Bray solution (60 g naphthalene, 4 g PPO, 0.2 g POPP, 100 ml absolute methanol, made to 1 liter with p-dioxane) for counting in a Beckman scintillation counter. The sedimented radioactivity in the polycarbonate tubes was extracted for at least 1 hr with methanol which was then counted.

Activities of glucan synthetases I and II were determined following Ray (27). Incorporation of C4-UDPG into ethanol-insoluble material was the assay. Twenty μl of freshly prepared reaction mixture were added to 50 μl of gradient sample (about 180 μg protein), and incubated for 15 min at 25 °C. The reaction mixture contained 230 μl of 80 mm tris-acetate (pH 8), 15 μl C4-UDPG (22 Ci/ml), and either 65 μl of 1 M MgCl2 (glucan synthetase I, low UDPG) or 65 μl of 8 M UDPG (glucan synthetase II, high UDPG). The reaction was terminated by adding 2 ml 70% ethanol and 80 μl of a boiled mitochonial suspension (carrier for the precipitated glucan) and then by heating for 1 min in boiling water. Then 50 μl of 0.1 M MgCl2 (glucan synthetase I) or 100 μl of 0.1 M MgCl2 (glucan synthetase II) were added to the samples. Insoluble material was washed four times with 2 ml of 70% ethanol and centrifuged at 1,000g for 5 min. The washed pellets were then transferred to vials with Bray solution for counting.

Cytochrome c oxidase was measured following Appleman et al. (1). Horse heart Cyta (0.53 mg/ml) in 50 mm tris-acetate buffer (pH 7.4) containing 0.05% Triton-X 100 was reduced with 1 μl of 1 M dithionite solution (sufficient to reduce 80–90% of the Cyta) prepared with distilled H2O immediately before addition. To 0.45 ml of this solution in a 1-ml cuvette was added 50 μl of test suspension. After rapid mixing, the rate of oxidation of Cyta was linear and was followed at 550 nm with a Hitachi Perkin-Elmer 356 recording spectrophotometer.

NADH-dependent Cytc reductase was measured by a slight modification of the methods of Lord et al. (22). A 0.45-ml aliquot of horse heart Cytc (0.53 mg/ml) in 50 mm tris-acetate buffer (pH 7.4) containing 1 mm KCN was pipetted into a 1-ml cuvette, and 20 μl of 50 mm NADH was then added. The reaction was initiated by addition of 2 to 10 μl of test solution. The reduction of Cytc was followed at 550 nm in the Perkin-Elmer spectrophotometer.

Total flavin content was determined by a method adapted from Yagi (31). One ml of 1 M NaOH was added to 0.5 ml of each sample and alkaline solutions were then irradiated with 30 min white light from 2 w fluorescent tubes placed about 10 cm above them. One ml of 20% acetic acid was then added to reacidify the mixture and the lumiflavin formed by irradiation was then extracted with 4 ml of analytical grade chloroform. The height of the fluorescence excitation peak at 460 nm was determined in a Hitachi Perkin-Elmer recording spectrofluorometer. The emission was measured at 540 nm, and both excitation and emission bandwidths were set at 16 nm. The system was calibrated with riboflavin standards.

Cytochrome content was estimated by measuring reduced-minus-oxidized difference spectra across the region of the Soret band. These spectra were measured either with 0.1 mm NADH alone, or with NADH plus 20 μM antimycin A, or with NADH plus antimycin A plus 0.5 mm KCN, or with all of these plus a few crystals of dithionite. Low temperature spectra were obtained as described by Muñoz and Butler (24).

Carotenoids were assayed by a slight modification of Goodwin's technique (10). Fractions were pelleted and the pellets (maximum 4.5 mg protein) were extracted for 1 hr with 5 ml of a 3:1 mixture (v/v) of chloroform and methanol to which 0.3 ml of acetone had been added. The pellets were colorless after extraction. Relative carotenoid content was determined by measuring absorption at 445 nm from absorption spectra taken between 370 and 520 nm. The absorption spectra showed characteristic carotenoid fine structure in the blue and minimal A in the UV.

Protein content was determined following the method of Lowry et al. on 5% trichloroacetic acid precipitates from various fractions (23). BSA was used as the standard.

NADH dehydrogenase was measured by adding 50 μl of 2 mm NADH to 0.85 ml of sample. The rate of NADH oxidation was measured by following the A change at 340 nm on the Hitachi Perkin-Elmer spectrophotometer. Up to 10-fold dilution of sample with gradient medium (pH 7) was frequently required to obtain oxidation rates sufficiently slow for measurement. Antimycin A and KCN, when used, were in the same concentrations as given above for the Cyt measurements.

RESULTS

For characterization of the various subcellular fractions, two methods were used: differential centrifugation and equilibrium density gradient centrifugation. In the first experiments, particulate fractions were separated according to the protocol shown in Figure 1. Table I, from a single experiment, shows the distribution of various enzyme markers in the different fractions, all expressed on a per mg protein basis. There is clearly enrichment for the mitochondrial marker, Cyt c oxidase, in the 9,000g pellet (9KP) and for the ER marker, NADH-dependent Cyt c reductase, in the 186KP. The so-called "PM" marker, NPA binding, shows a different pattern with activity in all three pellets, but somewhat more in the 21KP and 186KP. The percentage of recovered activity for each marker showed that approximately 70% of the mitochondria are in the 9KP and 40% of the ER in the 186KP. NPA binding was about equal in the 21KP and 186KP with these two fractions accounting for about 70% of the total recovered. Each of these pellets contains some bound flavin with 50% of that bound in the mitochondrial fraction, and about 25% in each of the other two particulate fractions. The concentration of flavin in the 21KP is equivalent to 0.2 to 0.3 μM in the tissue. Bound flavin accounts for 11% of the total flavin measured; that in the 21KP about 2.5%.

Three distinct classes of Cyt were investigated (Table I): those
reducible by NADH alone (in the presence of excess reductant), those reducible by NADH in the presence of antimycin A, and those reducible only by dithionite. An enrichment for the first class is found in the 186KP, and is probably Cyt b5, a known constituent of the ER (22, 30). Its room temperature oxidized-minus-reduced difference spectrum also matches that for Cyt b5 found in the literature (29) (see below for discussion of low temperature spectra). As might be expected (19), Cyt reducible in the presence of antimycin A are found largely in the 9KP where the bulk of the mitochondria are found. Cyt reducible only in the presence of dithionite are present in highest amount in the 21KP and 186KP. This class will be considered further below.

Table II shows the results of a similar experiment with roughly the same distribution of marker enzymes and activities as in Table I. The yields and distribution show some variation between the two experiments, but this variation is most likely related to different final extraction volumes (leading to different sedimentation path lengths) and unavoidable in seedling growth from one experiment to the next. For the experiment shown in Table II, NADH dehydrogenase activity was measured instead of Cyt content. The majority of the NADH dehydrogenase activity is antimycin A-sensitive and mitochondrial in origin. Not surprisingly, 87% of that found in the 9KP is sensitive, while in the other two pellets, only about 50% of the total is sensitive. Sensitivity of the dehydrogenase to KCN, not shown in the table, has the same distribution as antimycin A sensitivity. In the 186KP, there appeared the majority of that KCN- and antimycin A-insensitive dehydrogenase activity which could be stimulated by addition of boiled supernatant. These three particulate fractions therefore showed a mixture of three different types of NADH dehydrogenase activity, with enrichment for antimycin-sensitive, antimycin-insensitive, and supernatant-stimulated antimycin-insensitive activity in the 9KP, 21KP, and 186KP, respectively.

More complete separation of the various particulate components results if the 9KS (supernatant), which contains 7.5%

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**Table I. Distribution of protein, membrane markers, flavin, and cytochromes following differential centrifugation scheme shown in Figure 1. All activities are expressed on a per mg protein basis.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>NPA binding (mg/ml)</th>
<th>Cyt. c oxidase (cpm x 10^-3)</th>
<th>Cyt. c reductase (umole/min x 10^2)</th>
<th>Flavin content (moles x 10^-10)</th>
<th>Cytochrome content (A, Soret)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>1.66 (66.5)</td>
<td>3.1</td>
<td>7.4</td>
<td>14</td>
<td>14.0</td>
<td>-</td>
</tr>
<tr>
<td>0.5KP</td>
<td>0.21 (2.5)</td>
<td>0.5</td>
<td>7.7</td>
<td>4</td>
<td>6.7</td>
<td>-</td>
</tr>
<tr>
<td>9KP</td>
<td>0.40 (4.8)</td>
<td>16.4</td>
<td>30.0</td>
<td>15</td>
<td>9.5</td>
<td>23</td>
</tr>
<tr>
<td>21KP</td>
<td>0.22 (2.6)</td>
<td>25.7</td>
<td>8.4</td>
<td>25</td>
<td>5.5</td>
<td>41</td>
</tr>
<tr>
<td>186KP</td>
<td>0.20 (2.4)</td>
<td>24.8</td>
<td>6.3</td>
<td>65</td>
<td>5.5</td>
<td>111</td>
</tr>
<tr>
<td>186KS</td>
<td>1.26 (49.2)</td>
<td>0.05</td>
<td>0.4</td>
<td>7.7</td>
<td>14.7</td>
<td>0</td>
</tr>
</tbody>
</table>

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**Table II. Distribution of protein, membrane markers, flavin, and NADH oxidase activities following differential centrifugation scheme shown in Figure 1. All activities are expressed on a per mg protein basis.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>NPA binding (mg/ml)</th>
<th>Cyt. c oxidase (cpm x 10^-3)</th>
<th>Cyt. c reductase (umole/min x 10^2)</th>
<th>Flavin content (moles x 10^-10)</th>
<th>NADH oxidase activity (nmole/min x 10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5KP</td>
<td>0.21 (2.5)</td>
<td>5.5</td>
<td>7.6</td>
<td>5.2</td>
<td>10.5</td>
<td>15</td>
</tr>
<tr>
<td>9KP</td>
<td>0.50 (6.0)</td>
<td>11.5</td>
<td>22</td>
<td>10.0</td>
<td>10.3</td>
<td>44</td>
</tr>
<tr>
<td>21KP</td>
<td>0.25 (3.0)</td>
<td>13</td>
<td>2.1</td>
<td>13.0</td>
<td>4.2</td>
<td>4.0</td>
</tr>
<tr>
<td>186KP</td>
<td>0.23 (2.8)</td>
<td>9.1</td>
<td>2.4</td>
<td>26.0</td>
<td>6.8</td>
<td>6.1</td>
</tr>
<tr>
<td>186KS</td>
<td>1.26 (29.0)</td>
<td>-</td>
<td>-0.7</td>
<td>1.4</td>
<td>22.9</td>
<td>-</td>
</tr>
</tbody>
</table>

1Sup: boiled supernatant, 186KS; 10 μl added per ml enzyme.
sucrose, is layered on a discontinuous sucrose gradient made up of 15%, 30%, and 45% layers. Such a preparation was centrifuged for 2 hr in a Beckman SW 27 rotor in a Sorvall OTD (oil turbine drive) ultracentrifuge at 27,000 rpm (186,000g). The tubes were punctured from below and fractions collected to keep the two turbid zones and the supernatant separate for assay. Table III shows the result of one such experiment. Since the smaller volume in the centrifuge tube than that in the previous experiments led to a shorter sedimentation path length, over 90% of the mitochondria were obtained in the 9KP. There was enrichment for “PM” over the 45% cushion (fraction A), as evidenced by the relative abundance of NPA binding with respect to other markers, and enrichment for ER over the 30% cushion (fraction B) as evidenced by the high NADH-dependent Cyt c reductase activity. The “PM” fraction was also substantially enriched for a Cyt reducible only by dithionite, while the ER fraction was enriched for the presumed Cyt b5, reducible directly with NADH.

Low temperature oxidized-minus-reduced difference spectra were obtained for the 21KP from the experiment shown in Table I (Fig. 2) and from fractions A and B (Fig. 3) and the 9KP (not shown) from the experiment illustrated in Table III. As expected, the 9KP contained Cyt c, a, and a mixture of b-type Cyt. In the two fractions enriched for “PM” (21KP and fraction A), the Cyt showed no reduction by excess NADH in the presence of antimycin A, with fraction A in addition showing no reduction in the presence of KCN (Tables I and III). Hence, these fractions were not seriously contaminated by mitochondrial Cyt. The difference spectrum for the “PM” fraction shown in Figure 2 shows difference maxima at 425 and 557 nm with a suggestion of a shoulder near 552 nm, and a minimum at 412 nm. The difference spectra for ER and “PM” fractions are compared in the a-band region in Figure 3. The major component of the ER fraction has bands at 551 and 558 nm, consistent with those described in the literature for Cyt b5 (29, 30). The “PM” fraction in this figure again shows an a-band near 557 nm with the suggestion of a shoulder near 552.

Equilibrium density gradient centrifugation was used to achieve a better resolution of the nonmitochondrial Cyt, flavin, and NADH oxidase components. Homogenates were precentrifuged as indicated in figure legends to remove at least 85% of the mitochondrial activity. Although the gradients shown in Figures 4 through 6 were run in three different laboratories following different precentrifuging protocols, specific activities

![Fig. 2. Low temperature oxidized-minus-dithionite-reduced difference spectrum for the resuspended 21KP from the experiment shown in Table I. Resuspension was in 0.5 M K-phosphate buffer (pH 7.4) plus an equal volume of glycerol.](image)

![Fig. 3. Low temperature oxidized-minus-dithionite-reduced difference spectra for fractions A ("PM") and B ("ER") from the experiment shown in Table III. Fractions in gradient buffer at pH 7.0.](image)

| Table III. Distribution of protein, membrane markers, and cytochromes following centrifugation on a step gradient of 15, 30, and 45% sucrose. All activities expressed as a per mg protein basis. |
|----------------------------------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Fraction | Protein | NPA binding | Cyt. c oxidase | Cyt. c reductase | Glucan synthetase | NADH reducible | NADH + AM | NADH + AM + KCN | NADH + AM + KCN + DT |
|----------|---------|-------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|          | mg/ml  | (total mg) | cpm x 10⁻⁴ | μmol/min x 1 | cpm x 10⁻⁴ | A, Soret, x 10³ |
| Homogenate | 1.62 | (88) | 0.85 | 6.7 | 1.37 | 5.9 | - | - | - | - | - | - | - |
| 0.5KP | 0.88 | (10.6) | - | - | - | - | - | - | - | - | - | - | - |
| 9KP | 1.26 | (15.1) | 2.0 | 38.2 | 1.3 | 15.2 | 26.2 | 27.5 | 14.7 | 14.4 | - | - | - |
| A | 0.21 | (2.56) | 2.2 | 0.9 | 2.2 | 13.3 | 14.4 | 0 | 0 | 0 | 20.9 | - | - |
| B | 0.62 | (5.08) | 0.5 | 1.0 | 8.0 | 0.5 | 42.4 | 0 | 0 | 0 | 11.2 | - | - |
| 100KS | 0.68 | (57.6) | 0.1 | - | 0.3 | 0.2 | - | - | - | - | - | - | - |

1. 9KS diluted to 2/3 original protein concentration with gradient media containing 7.5% sucrose.
2. A: Fraction collected at 30-45% sucrose interface, "PM" enriched.
3. B: Fraction collected at 15-30% sucrose interface, ER enriched.
were consistent from one gradient to the next and there was consistency in the positions of the various markers with respect to sucrose concentration. Recoveries were similar to those for the experiment shown in Table I. In Figure 4 are plotted the relative activities of four marker enzymes, plus other components of interest as a function of per cent sucrose. Although presented in three graphs for clarity, all of these results were obtained from a single gradient. In Figure 4A, the positions of the residual mitochondria (38% sucrose), “plasma membrane” (34%), Golgi apparatus (28%), and ER (25%) are shown by their respective marker enzymes. Figure 4B shows the distribution of carotenoids and flavins. The carotenoids, with a peak near 28% sucrose, represent plastids not well separated from G. In other gradients, however, the carotenoid peak is clearly one fraction lower than that for the marker (30% sucrose). Not surprisingly, bound flavin is found in several of the membrane fractions. Figure 4C shows the distribution of two Cyt: one, reducible by NADH, corresponds closely to the distribution of ER, peaking at 25% sucrose, and is undoubtedly Cyt b. As was the case in the differential centrifugation experiments described above, its identity was confirmed by its oxidized-minus-reduced difference spectrum. The other Cyt, reducible only by dithionite, shows a broader peak, with one side underlying the plastids (or G) and the other underlying the “PM” fraction. The mitochondrial content of this gradient was too low for the detection of Cyt reducible by excess NADH in the presence of antimycin A or KCN. Hence the heavier portion of the Cyt reducible only by dithionite is most likely not mitochondrial, but rather associated with the “PM” fraction.

Figure 5A shows results from another gradient with four markers for M, “PM,” G, and ER showing the same distribution as that shown in Figure 4A. Figure 5B, from the same gradient, shows carotenoid distribution (PP), and also the distribution of two of the three classes of NADH dehydrogenase shown in Table II: antimycin A-insensitive, and supernatant-stimulable antimycin A-insensitive (the latter plotted on one-third the vertical scale of the former). Clearly, most of the supernatant-stimulable activity coincides with the ER marker (Fig. 5A) while the majority of the nonstimulable activity underlies the “PM” marker, glucan synthetase II.

Figure 6 shows the distribution of ER and M markers, plus NPA binding and glucan synthetase II, for a third gradient. The NPA binding is almost coincident with the glucan synthetase II. In some gradients (those with the very lowest mitochondrial activity), NPA binding showed a distinct peak over the mitochondrial marker. The reason for this occasional discrepancy is unknown, since NPA binding is readily separated from Cyt c oxidase activity by differential centrifugation (Tables I–III).

**DISCUSSION**

In the present work we have been able to resolve and identify ER, plastids, G, and mitochondria on the basis of known markers. There is clearly an additional component which forms a broad band in sucrose gradients, centered near 35%. It contains the following: NPA-binding sites; glucan synthetase II activity; a Cyt not reducible by NADH even in the presence of antimycin A or KCN; but reducible by dithionite; and a NADH dehydrogenase which is not stimulated by supernatant, and which is both KCN- and antimycin A-insensitive. Recently, Cross and Briggs (6) have shown that one K+-activated ATPase from the particu-
late fraction of corn coleoptiles may coincide with the other "PM" markers, coming to equilibrium at about 35% on sucrose gradients, although there is more than one such ATPase. Finally, Quail (26) has shown a small amount of phycocyanobilin-binding activity from corn coleoptiles in this region of his gradients. One should be cautious in assuming that it is the same fraction, since his conditions were slightly different from ours.

As mentioned in the introductory section, a number of workers have used glucan synthetase II or NPA binding as diagnostic for plasma membrane, which they have identified in purified vesicle preparations by phosphotungstic acid-chromic acid staining and electron microscopy. The present authors do not find this evidence entirely convincing, since there is no assurance that a staining procedure in vitro will yield results identical with those obtained in vivo. Modification of a membrane fraction during extraction could allow staining of a membrane which does not normally react to stain in thin sections of tissue.

The "PM" fraction in the present work could be one of at least four different things: tonoplast (for which there is date no specific marker in corn), plasma membrane, a mixture of both, or an unidentified nonmembranous pelletable component. It seems highly unlikely that the latter would carry with it all of the activities characterizing the "PM" fraction. Because the tonoplast shows minimal staining with phosphotungstic acid-chromic acid in vivo, it seems unlikely that the fraction in question is entirely tonoplast. Hence, circumstantial evidence favors the notion that it is at least partially plasma membrane.

Of particular interest in the present study is the finding of a Cyt which is neither mitochondrial, proplastid, nor from the ER. It clearly differs spectrally from Cyt b2 and also differs in that it is not reducible by NADH even in the presence of KCN or antimycin A. Lambowitz and Bonner (18) have reported on one or more b-type Cyt in gradient-purified mitochondria which are not reducible in the presence of antimycin A except by dithionite. It is unlikely that the Cyt described in the present work is the same as that of Lambowitz and Bonner, since their preparation showed band-splitting at liquid N2 temperature with bands at 554 and 560 nm.

The "PM" fraction from corn coleoptiles contains the necessary components for the scheme that Poff and Butler (25) and Munoz and Butler (24) have proposed as potentially diagnostic for the blue light photoreceptor. There is a b-type Cyt, bound flavin, and the components of an electron transport chain. The bound flavin has an absorption spectrum in the blue which shows fine structure compatible with the action spectra (5). As shown elsewhere (3), a comparable "PM" fraction from Neurospora contains the blue light-inducible Cyt reduction system described by Munoz and Butler (24). This fraction has a sedimentation behavior intermediate between that of ER and mitochondria, and is distributed in differential centrifugation experiments in precisely the same way as in an Na+-dependent ATPase. Finally, a Cyt b-deficient Neurospora mutant is far less light-sensitive than wild type, suggesting that b-type Cyt may be involved in photoreception (2). Similar though smaller light-induced Cyt reduction could be obtained in vitro in the resuspended 21KP from corn (3). The distribution of the light-induced Cyt reduction among the various particular fractions from corn could not be studied because methods for keeping the light-sensitive system in corn active long enough to do the appropriate centrifugations were not achieved, but the system shows promise for further study.

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