ENZYMIC ACTIVITIES OF SUB-CELLULAR PARTICLES FROM LEAVES

III. CENTRIFUGAL FRACTIONATION AND CHARACTERIZATION OF PARTICLES IN HOMOGENATES OF GREEN LEAVES

By R. M. SMILLIE*

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Summary
Homogenates of green pea leaves have been fractionated by differential centrifugation. Six fractions so obtained were examined microscopically and assayed for total nitrogen, deoxyribo-, and ribonucleic acid, starch, and chlorophyll contents, succinic dehydrogenase, succinoxidase, α-ketoglutarate oxidase, cytochrome reductase, cytochrome oxidase, and their oxidative phosphorylation activity, and photolytic capacity. These studies revealed the presence of highly-significant numbers of biochemically-active mitochondria and smaller, non-oxidative particles, containing cytochrome reductase. Both particle types were distinct from chloroplasts, or chloroplast fragments. The enzyme distributions were almost identical with those found for etiolated leaves revealing a basic similarity between dark- and light-grown leaf cells.

I. INTRODUCTION
Homogenates of etiolated pea leaves were previously fractionated by differential centrifugation and the particles in these fractions have been characterized by chemical analysis and enzymic assay (Smillie 1956b). Similar fractionation procedures have now been applied to green pea leaves and the results compared with those for etiolated leaves. It has been shown that green leaves contain not only chloroplasts, which are the site of the photochemical fixation of carbon dioxide (Arnon, Allen, and Whatley 1954), but also particles which contain the mechanism for the respiratory oxidation of tricarboxylic acid cycle substrates to carbon dioxide and water (Smillie 1956a). Other enzymic activities such as cytochrome oxidase (McClendon 1953; Jagendorf and Wildman 1954), oxalic oxidase (Arnon and Whatley 1954), and catalase (Jagendorf and Wildman 1954), have already been associated with particles of mitochondrial size isolated from green leaves.

II. METHODS
(a) Plant Material
Pea seedlings, Pisum sativum (Yates Greenfeast) were grown in a glasshouse and leaves were harvested after 2–3 weeks growth.

(b) Fractionation Procedure
Leaves (40 g) were ground in sucrose (0·5M) containing ethylenediaminetetraacetate (EDTA) (0·005M) at pH 7·2. The mixture was filtered through a double layer

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of muslin and the filtrate made up to 80 ml with the sucrose-EDTA solution. This filtrate was then fractionated according to the procedure in Table 1 to give six fractions. Each fraction was washed once with 80 ml of the sucrose-EDTA solution and finally resuspended in sucrose (0.5M). All operations were performed at 0–5°C.

### Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Relative Centrifugal Force ((\times g))</th>
<th>Time of Centrifugation (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>300</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>1,200</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>2,700</td>
<td>30</td>
</tr>
<tr>
<td>5</td>
<td>6,700</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>17,000</td>
<td>45</td>
</tr>
</tbody>
</table>

(c) Estimations

Starch was estimated as reducing sugar after acid hydrolysis, the sucrose first being removed by repeated washing of the fractions with alcoholic trichloroacetic acid. Chlorophyll was determined by the method of Arnon (1949), and photochemical capacity, using ferricyanide as the oxidant, as described by Arnon and Whatley (1949). Procedures for the other estimations have already been described (Smillie 1956a, 1956b).
III. Results

(a) Distribution of Total Nitrogen

The distribution of total nitrogen is shown in Figure 1. 30–40 per cent. of the total nitrogen was found in fraction 2, and progressively smaller amounts in the following fractions.

![Figure 1](image)

Fig. 1.—The distribution of total nitrogen in particulate fractions from green leaves.

(b) Distribution of Nucleic Acid

The distributions of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are illustrated in Figure 2. Fraction 1 contained the highest concentration of DNA per mg nitrogen. The small amounts in fractions 3–6 can probably be attributed to the presence of fragments of nuclei disrupted during the grinding. RNA was present in all fractions, the largest concentration being in fraction 2. Results for RNA based on pentose determinations (Drury 1948) gave the same distribution pattern. The ratio of RNA : DNA for fractions 1, 2, and 3 were 0·62, 4·9, and 3·6 respectively. In previous studies with etiolated leaves, similar distributions of the nucleic acids were obtained (Smillie 1956b), values for RNA : DNA ratio of 0·48 and 4·5 being found for fractions corresponding to green leaf fractions 1 and 2 plus 3 combined respectively.

### Table 2

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total starch (%)</td>
<td>89</td>
<td>9</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

These results indicate that the distribution of nucleic acids is influenced by the presence of ruptured nuclei. Further studies are needed to determine the factors controlling this distribution.
Fig. 3.—The distribution of chlorophyll in particulate fractions from green leaves.

Fig. 4.—The distribution of succinic dehydrogenase in green leaf fractions. Reaction mixture contained enzyme (0.1 ml), potassium phosphate at pH 6.8 (0.05M), egg albumin (1 mg), 2,6-dichlorophenolindophenol (0.0001M), and succinate (0.02M). Final volume 6.0 ml.
(c) Distribution of Starch

Eighty-nine per cent. of the starch was sedimented in fraction 1 (Table 2). This would indicate that a high proportion of the chloroplasts are partially disrupted during the extraction procedure. Microscopic examination of the fractions also showed that the majority of starch grains were in fraction 1.

![Graph showing distribution of oxidative and phosphorylative capacities of particulate fractions from green leaves.](image)

Fig. 5.—The distribution of oxidative and phosphorylative capacities of particulate fractions from green leaves. Reaction mixture contained enzyme, sucrose (0·35M), potassium phosphate at pH 7·3 (0·03M), MgSO₄ (0·001M), adenylate (0·0005M), yeast coenzyme concentrate (1 mg), glucose (0·002M), hexokinase (0·2 mg), and succinate (0·02M). Final volume 2·0 ml.

(d) Distribution of Chlorophyll and Photolytic Activity

The distribution of chlorophyll is shown in Figure 3. There was a gradual decrease in the chlorophyll: total nitrogen ratio from fractions 2 to 5. Fractions 2 and 3 contained the highest amount of chloroplastic material. Jagendorf and Wildman (1954) found a chlorophyll:total nitrogen ratio of 1·0 for purified tobacco leaf chloroplasts. No value is available for pea leaf chloroplasts, but assuming it is approximately 1·0, this would indicate that approximately 25 per cent. of fractions 2 and 3 were non-chloroplastic in origin, while in fraction 6 over 50 per cent. was of non-chloroplastic origin.

The photolytic capacity of the particles in the presence of ferricyanide did not follow the chlorophyll distribution precisely, the ratio of mg chlorophyll to photolytic activity (μl O₂ in 25 min × 10⁻²) being 1·52, 1·17, 0·98, 0·83, and 0·72 for fractions
2, 3, 4, 5, and 6 respectively. This result might be expected if it is assumed photolysis is a surface phenomenon (Milner, Koenig, and Lawrence 1950), since the fragments in fraction 6 would contain more of the grana components.

(e) Mitochondrial Activity

The distribution of succinic dehydrogenase activity is shown in Figure 4. The rates of oxygen uptake and phosphorus esterification in the presence of succinate associated with fractions 1–6 are summarized in Figure 5. The rate of oxidation of

\[ \text{a-ketoglutarate} \] was similar to that of succinate oxidation. Results obtained with different preparations were similar in each case and almost identical to those already found for etiolated leaves (Smillie 1956b). The rate of phosphorylation closely followed the oxidative rate, the ratio of phosphorus esterified to oxygen consumed (P:O) for fractions 2, 3, 4, and 5 being 1.52, 1.53, 1.60, and 1.52 respectively.

(f) Distribution of Cytochrome Oxidase and Cytochrome Reductase

Cytochrome oxidase has a similar distribution to the oxidative activities already described (Fig. 6). Cytochrome reductase was located in particles whose average size were smaller than the mitochondria (Fig. 6).
(g) Microscopic Examination of Green Leaf Fractions

The different fractions were examined by phase-contrast microscopy. Fraction 1 contained many starch grains and whole nuclei. Fraction 2 also contained whole nuclei and a smaller number of starch grains, but these were absent from the subsequent fractions. Whole chloroplasts were confined to fractions 2 and 3. Although particles 2 µ in diameter or less were observed in all fractions except fraction 1, there was a decrease in the average size of these particles through fractions 2 to 6. In Table 3 are shown the distribution of particles 0.5-2 µ in diameter and whole chloroplasts or chloroplast fragments. Many of the particles less than 2 µ in diameter were irregular in shape, suggesting that they were also chloroplast fragments. In addition, fractions 2, 3, and 4 contained larger particles, which were obviously fragmented chloroplasts. The particles in fraction 6 were at the limit of resolution of the microscope, and too small for counting.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Whole Chloroplasts</th>
<th>Chloroplast Fragments (&gt;2 µ)</th>
<th>Particles (0.5-2 µ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2.1</td>
<td>1.3</td>
<td>3.0</td>
</tr>
<tr>
<td>3</td>
<td>3.3</td>
<td>4.9</td>
<td>25.4</td>
</tr>
<tr>
<td>4</td>
<td>Trace</td>
<td>3.4</td>
<td>57.0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>Trace</td>
<td>69.0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>Not counted</td>
</tr>
</tbody>
</table>

IV. Discussion

The procedure used in preparing the leaf homogenate resulted in the rupture of many whole chloroplasts. This is in contrast with results obtained with etiolated leaves, where all the plastids were isolated in one fraction. The presence of starch grains in the chloroplasts probably contributed to their ease of rupture during grinding. Although chlorophyll-containing material was present in all fractions except fraction 1, it is obvious from the distribution patterns obtained, that the mitochondrial activities were due to particles which were quite distinct from chloroplasts. The distribution of chlorophyll and the photolytic activity varied in different preparations, but the distribution patterns of the other enzymic activities were reproducible and were strikingly similar to results obtained for etiolated leaves (Smillie 1956b).

Recently Ohmura (1955) has obtained a particulate preparation from spinach leaves which carried out oxidative phosphorylation in the presence of citrate. This activity he attributed to chloroplast fragments. In view of the results described above,
it is more probable that the activity was due entirely to enzymes located on mitochondrial particles which were also present in the leaf preparation.

In both etiolated and green leaves there exist at least four distinct types of particles—nuclei, plastids, mitochondria, and cytochrome reductase-containing particles lacking oxidative activity. Of these particles, both the plastids and the cytochrome reductase-containing particles possess higher amounts of RNA than the mitochondria. It is to be noted that Jagendorf and Wildman (1954) found very little nucleic acid in chloroplasts from tobacco leaves. However, the experiments described above were performed with rapidly growing leaves, whereas those of Jagendorf and Wildman were carried out with mature leaves in which the growth and division of chloroplasts was at a minimum (Strugger 1950). Since RNA has been associated with protein synthesis (Brachet 1950), the RNA level in chloroplasts might be expected to fall as the leaf matures. Similarly the number of non-chloroplastic cytoplasmic particles which are concerned with energy production and cell synthesis may also decrease as the leaf matures.

V. Conclusion

In the growing pea plant, the cellular make-up and the enzyme distribution within the leaf cell is fundamentally similar whether the plant is grown in the presence or absence of light. The leaves contain in addition to the plastids, other cytoplasmic bodies, namely, mitochondria and particles corresponding in size to animal microsomes. The mitochondria and possibly the microsomes function in respiratory processes which lead to the production of energy for cellular synthesis, and which are quite distinct from photosynthetic mechanisms located in the chloroplast. Both the mitochondria and microsomes are present in large numbers in the rapidly growing leaf. These results, which were obtained with young leaves, may not be typical of mature leaves.

VI. Acknowledgments

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VII. References