Peroxisomes from Spinach Leaves Containing Enzymes Related to Glycolate Metabolism*

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SUMMARY

Microbodies, designated as peroxisomes because of their enzyme complement, have been isolated from spinach leaves. After grinding leaves in 0.5 M sucrose, the peroxisomes were removed with the broken chloroplast fraction by differential centrifugation. During sucrose density gradient centrifugation, the peroxisomes banded in about 1.9 M sucrose and were separated from mitochondria and chloroplasts. The particles, 0.5 to 1.0 μ in diameter, contained a dense granular stroma surrounded by a single membrane.

The leaf peroxisomes contained glycolate oxidase, DPNH-glyoxylate reductase, and catalase. Up to 55% of the activity for these enzymes in spinach leaves have been found in the particulate fractions after the initial centrifugation. The leaf peroxisomes are probably the site of oxygen uptake during photorespiration. No catalase activity was present in chloroplasts after removal of the peroxisomes by density gradient centrifugation. D-Glycolate phosphatase, TPNH-glyoxylate reductase, D-amino acid oxidase, urate oxidase, and peroxidase were not present in leaf peroxisomes.

Glycolate oxidase was first isolated from clarified extracts of tobacco leaves (1, 2). Subsequent investigations (3, 4) supported the concept that the oxidase, as well as DPNH-glyoxylate reductase (5) were soluble cytoplasmic enzymes. Chloroplast (6, 7) and mitochondrial fractions (8, 9) have been reported to contain only a small portion of the total glycolate oxidase activity. Upon examination by sucrose gradient centrifugation, Pierpoint (10) and, most recently, Thompson and Whittingham (11) have concluded that there was no glycolate oxidase in the particulate fractions. A reinvestigation of the intracellular localization of glycolate oxidase in leaves was prompted by our own observations of large amounts of glycolate oxidase in broken chloroplast preparations.

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De Duve and Baudhuin (12) have designated the microbodies from liver and kidney, which contain a-hydroxy acid oxidase and catalase, as peroxisomes. The metabolic function of peroxisomes is unknown. Particles of unknown composition have been reported in plants and designated as cytosomes (13), but there are no reports of particles from leaves with enzymic activity characteristic of peroxisomes. Particles containing enzymes of the glyoxylate cycle have been isolated from germinating castor bean cotyledons (14) and from Tetrahymena (15). A similarity between these microbodies and peroxisomes is suggested by the fact that microbodies from Tetrahymena contain enzymes which are also characteristic of liver and kidney peroxisomes (16).

EXPERIMENTAL PROCEDURE

Preparation of Fractions—Spinach (Spinacia oleracea L.) leaves were purchased locally. Leaves have been stored at 4° for up to 1 month before use. The chilled leaves were washed and deribbed, and then 40 g of tissue were chopped into small segments before grinding at maximum speed for 10 sec in a Waring Blendor with 80 ml of grinding medium. All work was done at about 4°. The standard grinding medium was 0.5 M sucrose in 0.02 M glycylglycine, pH 7.5. The homogenate was hand squeezed through six layers of cheesecloth, and the pH of the sap immediately readjusted to 7.5. Particles from 70 ml of this sap were then precipitated by differential centrifugation at 0° for 20 min at each step. The first pellet (120 × g) contained mostly whole chloroplasts, starch grains, and large cell fragments. The second pellet (3,000 × g) was designated as "broken chloroplasts," although it also contained some mitochondria and a large part of the peroxisomes. The third pellet (35,000 × g), designated as "mitochondria" contained the remaining microbodies and broken chloroplasts. The remaining solution was designated "supernatant." Each pellet was resuspended in grinding medium by stirring, and the final volume of about 4 ml for the first and second pellet and about 2 ml for the third pellet was recorded.

Sucrose Density Gradient Centrifugation—A noncontinuous sucrose density gradient of five layers was prepared at 4° by pipetting 4 ml of 2.5 M sucrose (85.5%), 10 ml of 2.0 M sucrose (68.4%), 10 ml of 1.8 M sucrose (61.6%), 10 ml of 1.5 M sucrose (51.3%), and 20 ml of 1.3 M sucrose (44.5%). All sucrose fractions contained 0.02 M glycylglycine at pH 7.5. After
Sucrose density gradient centrifugation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Sucrose</th>
<th>Volume</th>
<th>Type of particles in band</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.5</td>
<td>4.3</td>
<td>Supernatant</td>
</tr>
<tr>
<td>8</td>
<td>1.3</td>
<td>8.6</td>
<td>Broken chloroplasts</td>
</tr>
<tr>
<td>7</td>
<td>1.3-1.5</td>
<td>11.9</td>
<td>Broken chloroplasts</td>
</tr>
<tr>
<td>6</td>
<td>1.5</td>
<td>5.4</td>
<td>None</td>
</tr>
<tr>
<td>5</td>
<td>1.5-1.8</td>
<td>3.8</td>
<td>Whole chloroplasts; mitochonidria</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>6.4</td>
<td>None</td>
</tr>
<tr>
<td>3</td>
<td>1.8-2.0</td>
<td>4.0</td>
<td>Peroxisomes</td>
</tr>
<tr>
<td>2</td>
<td>2.0</td>
<td>7.6</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>2.5</td>
<td>3.6</td>
<td>None</td>
</tr>
</tbody>
</table>

The particles were pelleted. The sediment was once in 0.5 M sucrose buffered at pH 7.5 with 10 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid, and then postfixed for 2 hours with 1% OsO4. All fixation steps were carried out in the cold. The pellets were then dehydrated in a graded series of ethanol and embedded in Epon. Sections were stained with uranyl acetate and lead citrate.

**Electron Microscopy**—The peak fraction containing the peroxisomes was withdrawn from the gradient and fixed for 1 hour in 3.0% glutaraldehyde. The sucrose concentration was then lowered to 1 M and the particles were pelleted. The sediment was rinsed in 0.5 M sucrose buffered at pH 7.5 with 10 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid, and then postfixed for 2 hours with 1% OsO4. All fixation steps were carried out in the cold. The pellets were then dehydrated in a graded series of ethanol and embedded in Epon. Sections were stained with uranyl acetate and lead citrate.

**Glycolate Oxidase**—Glycolate-O2 oxidoreductase (EC 1.1.3.1) was assayed anaerobically by 2,6-dichloroindophenol reduction (4). Additions were made to a 3-ml Thunberg Beckman cuvette (10 mm in diameter) in the following order: 2 ml of 0.5 M pyrophosphate, pH 8.3, containing 1.5 × 10-4 M dichloroindophenol; 0.05 ml of 0.1 M KCN in 0.01 M NH4OH (final concentration of KCN, 2 × 10-6 M); 0.05 ml of 2 ml X 10-6 M FMN (final concentration, 0.8 × 10-4 M); water so that the final volume with enzyme would be 2.5 ml; in the side arm, 0.1 ml of 0.125 M sodium glycolate (final concentration, 5 × 10-4 M). Between 0.05 to 0.2 ml of enzyme was also placed in the cuvette before it was evacuated and flushed three times with N2 which had passed through Fieser's solution to remove traces of O2. Dye reduction at 25° was measured at 600 nm by an automatic recording Gilford spectrophotometer. A unit of activity was expressed as 1 O.D. change per min, which was equivalent to 4.78 nmoles of reduced dye. Pyrophosphate buffer with dichloroindophenol should be prepared fresh weekly to avoid an initial lag in the assay.

Since the soluble glycolate oxidase is a FMN protein, FMN was used in the assays. Aerobic assays were unreliable since the H2O2 generated by the glycolate oxidase could be used by contaminating peroxidases to oxidize any reduced dichloroindophenol which was generated. Competing reactions then occurred: H2O2 production, dichloroindophenol reduction, and peroxidase oxidation of the reduced dye. Inhibition of peroxidase by high concentrations of KCN, as originally recommended (4) for an aerobic assay, did not give maximum rates. Peroxidases were so active in sap and supernatant that no aerobic reduction of dichloroindophenol could be detected if KCN were omitted. Consequently, anaerobic assays were the only reliable method, and KCN was left in the assay to ensure against peroxidase activity in case of incomplete anaerobiosis.

**Cytochrome c Oxidase**—From 5 to 25 μl of enzyme was pipetted into a bottom corner of a 0.3-ml spectrophotometer cuvette (diameter, 10 mm) and 5 μl of 4.0% digitonin were added, mixed, and allowed to stand for 1 min. Then 200 μl of 0.1 M phosphate buffer (pH 7.0) and 50 μl of 1.5 mM cytochrome c reduced with dithionite were added successively and mixed (17). Readings of optical density at 550 nm were obtained with the Gilford recording spectrophotometer. The first order rate constant for the disappearance of reduced cytochrome c was calculated according to the method of Smith (18).

**Other Enzymes**—Glyoxylate reductases were assayed by the rate of oxidation of either DPNH or TPNH in the presence of 0.005 M substrate (19, 20). Catalase was assayed by the disappearance of H2O2 as measured spectrophotometrically at 240 μM (21). A unit of activity was a change of 1 O.D. in 1 min, and based on the extinction coefficient was equal to 2.76 μmoles of H2O2. Peroxidase was assayed by the method of Gregory which was based upon the length of time to oxidize a standard amount of ascorbic acid (22). The assay for P-glycolate phosphatase has been described (23).

**Protein and Chlorophyll**—Protein was determined by the Lowry procedure. Although the green color in aliquots from the chloroplast fractions interfered, the recovery of protein in the various fractions indicated that the method gave a valid estimate of protein content. Chlorophyll was determined by its absorption at 652 μm (24). Aliquots from 1 ml to 1 ml were diluted to 5 ml with water and acetone to make a final concentration of 80% acetone. They were allowed to stand in the dark at 0°C with occasional stirring for several hours to completely solubilize the chlorophyll. In samples with high sucrose concentration, the acetone extracted so much of the water that the sucrose and chlorophyll formed a second phase. Since sucrose is more soluble in cold acetone, it was necessary to let these samples stand overnight at −18°C in order to obtain effective chlorophyll extraction. Samples were centrifuged or filtered before reading the extinction at room temperatures.

**RESULTS**

**Distribution of Glycolate Oxidase among Particles**—Spinach leaves were ground in the sucrose grinding medium and separated by centrifugation into whole chloroplasts, broken chloroplasts, mitochondria, and supernatant fractions as described under "Experimental Procedure." The distribution of certain enzymes among these fractions is shown in Table II. In this type of experiment, the broken chloroplast fraction contained 29 to 34% of the total oxidase activity and had the highest specific activity. About 40 to 55% of all the oxidase activity was in the total particle fractions. Most of our investigations have been done with the broken chloroplast fraction, because glycolate oxidase from spinach leaves was greatest in this fraction on the basis of amount and specific activity. Removal of the whole chloroplast fraction was beneficial for reducing the load put on the subsequent sucrose gradient.

The broken chloroplast and mitochondrial fractions were subjected separately to sucrose density gradient centrifugation
found in a band (No. 3) sedimenting in about 1.9 m sucrose. Activity, also present at the top of the gradient (No. Q), was shown to illustrate the maximum activity found in the pellet fractions. Specific activity is expressed as micromoles per min per mg of protein.

Fractions were numbered in the order in which they were drained from the bottom of the centrifuge tube. Fraction 1 was in the bottom and 9 is the top supernatant. For approximate sucrose molarity see Fig. 1. The broken chloroplasts were from Experiment A of Table II. Specific activity is expressed as micromoles per min per mg of protein.

### Table II

**Distribution of enzymes among fractions obtained by differential centrifugation**

Data, from a typical experiment, are designated as Experiment A. The percentage of distribution from a second experiment, B, is shown to illustrate the maximum activity found in the pellet fractions. Specific activity is expressed as micromoles per min per mg of protein.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Glycolate oxidase</th>
<th>Catalase</th>
<th>DPNH-glyoxylate reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>µmoles/min</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Whole chloroplasts</td>
<td>360</td>
<td>5.5</td>
<td>0.015</td>
<td>5.5</td>
</tr>
<tr>
<td>Broken chloroplasts</td>
<td>618</td>
<td>28.9</td>
<td>0.047</td>
<td>28.9</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>208</td>
<td>5.2</td>
<td>0.025</td>
<td>5.2</td>
</tr>
<tr>
<td>Supernatant</td>
<td>2,730</td>
<td>60.6</td>
<td>0.022</td>
<td>60.4</td>
</tr>
</tbody>
</table>

### Table III

**Distribution of enzymes upon sucrose density gradient centrifugation of broken chloroplast fraction**

Fractions were numbered in the order in which they were drained from the bottom of the centrifuge tube. Fraction 1 was in the bottom and 9 is the top supernatant. For approximate sucrose molarity see Fig. 1. The broken chloroplasts were from Experiment A of Table II. Specific activity is expressed as micromoles per min per mg of protein.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Glycolate oxidase</th>
<th>Catalase</th>
<th>DPNH-glyoxylate reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>µmoles/min</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>9</td>
<td>247.2</td>
<td>3.39</td>
<td>0.014</td>
<td>23.9</td>
</tr>
<tr>
<td>8</td>
<td>109.0</td>
<td>0.77</td>
<td>0.007</td>
<td>5.4</td>
</tr>
<tr>
<td>7</td>
<td>35.8</td>
<td>0.47</td>
<td>0.013</td>
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<tr>
<td>6</td>
<td>14.5</td>
<td>0.31</td>
<td>0.021</td>
<td>2.2</td>
</tr>
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<td>5</td>
<td>22.5</td>
<td>0.78</td>
<td>0.035</td>
<td>5.5</td>
</tr>
<tr>
<td>4</td>
<td>9.6</td>
<td>1.80</td>
<td>0.190</td>
<td>12.7</td>
</tr>
<tr>
<td>3</td>
<td>5.5</td>
<td>5.07</td>
<td>0.920</td>
<td>35.8</td>
</tr>
<tr>
<td>2</td>
<td>4.1</td>
<td>1.53</td>
<td>0.370</td>
<td>10.8</td>
</tr>
<tr>
<td>1</td>
<td>2.0</td>
<td>0.07</td>
<td>0.030</td>
<td>0.5</td>
</tr>
</tbody>
</table>

(Table III and Fig. 1). The glycolate oxidase activity was found in a band (No. 3) sedimenting in about 1.9 m sucrose. Activity, also present at the top of the gradient (No. 9), was attributed to soluble protein from the supernatant and from broken particles. The specific activity of the oxidase in the particles was about 92-fold greater than the specific activity in the top fraction. The oxidase activity in the 1.9 m sucrose band was distinctly separated from both the chloroplast bands, as indicated by chlorophyll analyses, and from the mitochondrial fraction, as indicated by cytochrome c oxidase activity (Fig. 1).

The particulate fraction containing the plant glycolase oxidase has been designated as peroxisomes after the terminology of De Duve and Baudhuin (12). These plant peroxisomes sedimented similarly in the sucrose gradient and contained similar types of enzymes as those found in peroxisomes from liver or kidney.

The α-hydroxy acid oxidase activity of the particles has been designated glycolase oxidase because it was more active with glycolate than lactate or cY-hydroxybutyrate. With sufficient substrate for maximum activity, the relative rates of glycolate, lactate, and cY-hydroxybutyrate oxidation at pH 8.3 by the peroxisomes were about 100:40:30. Since this activity ratio with the three substrates was the same in each fraction from the sucrose gradient, it is assumed that their oxidation was catalyzed by the same enzyme. This ratio of activity is similar to that reported earlier for the soluble spinach glycolate oxidase (25), but the oxidase from tobacco leaves is much more specific for glycolate than for cY-hydroxybutyrate (1). Peroxisomes from rat liver have a similar specificity for glycolate, the shortest carbon chain of the α-hydroxy acid series, while peroxisomes from rat kidney contained an α-hydroxy acid oxidase which oxidizes the longer chain substrates (α-hydroxybutyrate) faster than glycolate (12). The reason for these substrate differences is not apparent.

The prosthetic group of glycolate oxidase, as prepared from soluble extracts of spinach leaves, is FMN (3, 4). Addition of excess (10−4 m) of FMN generally increases the activity of glycolate oxidase in crude extracts or a fraction precipitated by ammonium sulfate. Similarly, the oxidation of glycolate by peroxisomes generally was stimulated as much as 50% by added FMN.

**Catalase**—Catalase activity had nearly the identical distribution among the particulate fractions as did glycolate oxidase (Table II and Fig. 1). About 40 to 50% of the catalase activity was in the particulate fractions. Upon subsequent sucrose density gradient centrifugation of the broken chloroplast or mitochondria fraction, about 70% of the activity in these fractions was present in the peroxisome particles. The presence of glycolate oxidase and catalase together in leaf peroxisomes is consistent with data on liver peroxisomes and with the concept...
FIG. 1. Distribution of enzymic activities from broken chloroplast fraction of spinach leaves after sucrose gradient centrifugation. The range of sucrose molarity is proportional to the volume of each molarity used to prepare the gradient. The width of the columns is proportional to the volume of the fraction removed from the gradient for assay.

that the catalase is present to destroy the H$_2$O$_2$ formed by the FMN oxidase. Total catalase activity was at least 10$^6$ times greater than the activity of glycolate oxidase or glyoxylate reductase (Table III).

Catalase has often been recorded as present in chloroplasts and in cytoplasm. Little catalase activity was present in the chloroplast fractions from the sucrose gradient, but rather catalase was associated with the peroxisome fraction (Fig. 1). Since the bulk of the peroxisomes was removed initially from the sap in the broken chloroplast fraction, previous reports on chloroplast catalase could be due to peroxisome particles in the chloroplast preparations. If so, the unknown role for catalase in the photosynthetic apparatus need not be of concern. While isolating plant mitochondria by sucrose density gradient centrifugation, Plesnicar, Bonner, and Storey observed, but did not comment on, catalase activity at a location on the gradient, which would be characteristic of peroxisomes (see Table III of Reference 26).

**DPNH-Glyoxylate Reductase**—This enzyme was located in the peroxisomes similarly to glycolate oxidase and catalase (Tables II, III, and Fig. 1). On the basis of total or specific activity in the peroxisomes (Table III), glyoxylate reductase activity was the same order of magnitude as glycolate oxidase. Glyoxylate reductase has not been reported to be a constituent of liver peroxisomes (12), and Zelitch, when isolating this enzyme from plants, believed that it was a cytoplasmic component (5).

**Enzymes Not Detected in Peroxisomes**—The leaf peroxisomes were tested for other enzymes associated with the glycolate pathway (27), or with liver peroxisomes (12), d-Amino acid oxidase and urate oxidase, which have been found in liver peroxisomes, were absent in leaf peroxisomes. P-Glycolate phosphatase and TPNH-glyoxylate reductase were absent in the peroxisomes, but some activity for each was associated with the whole chloroplasts. The activity of P-glycolate phosphatase was found on the sucrose gradient in the same area as cytochrome c oxidase. This area contained the mitochondria as well as a small band of whole chloroplasts. The broken chloroplast bands containing the bulk of the chlorophyll did not contain P-glycolate phosphatase.

**Peroxidases** are extremely active and abundant in leaves. Both chloroplast and mitochondria fractions contained some peroxidase activity; however, the bulk of this activity was found in the supernatant. When the particulate fractions were further separated on sucrose gradient, no peroxidase activity by the ascorbate assay was detected in the peroxisome fraction. The catalase activity of the peroxisomes did not show peroxidase activity by the assay employed. Recently, Plesnicar et al. (26) with the use of sucrose gradient centrifugation, also concluded that peroxidase from mung bean hypocotyls was mostly soluble, although some activity was apparently located in microbodies.

**Peroxisome Morphology**—An electron micrograph of spinach leaf peroxisomes is shown in Fig. 2. The particles are characterized as containing a dense granular stroma surrounded by a single membrane. In some cases, a denser area was visible within the particles. The shape of the peroxisomes varied, but most often they appeared spherical and about 0.5 to 1.0 $\mu$m in diameter. The fact that the membranes of the particles were often broken is attributed to shearing forces as they passed through the discontinuous sucrose gradient. Thus, it was not surprising that a portion of the enzymic activity attributed to the peroxisomes was found at the top of the gradients. The preparation also contained chromatin material, broken chloroplasts, and other particles, presumably mitochondria. The amount of chlorophyll and cytochrome c oxidase (Fig. 1) paralleled these visual observations.

**Grinding Medium and Particle Stability**—The grinding medium of 0.5 M sucrose in 0.02 M glycylglycine at pH 7.5 has been changed, but so far no variation has altered the primary conclusions or improved significantly the yield of peroxisomes. Without sucrose to prevent osmotic shock, much more of the
glycolate oxidase activity was in the supernatant fraction, suggesting that the particles were labile. This also provides an explanation for previous literature citations that glycolate oxidase was a soluble enzyme. Substitution of NaCl or mannitol in the grinding medium for sucrose resulted in poorer recovery of peroxisomes, and shifted their location on the sucrose gradient to 1.8 M sucrose as if they were somewhat lighter in weight. Substitution of Carbowax 4000 for sucrose or addition of polyvinylpyrrolidone with sucrose was not beneficial.

The final concentration of sucrose in the leaf homogenate after grinding was lowered by the dilution from the leaf sap, and an unknown amount of peroxisome destruction may have occurred. Best results were obtained when the particles were removed as rapidly as possible by centrifugation and further separated on the sucrose gradient. If the leaf homogenate or the resuspended broken chloroplasts stood at 0°C for several hours before the sucrose gradient centrifugation, a large part of the enzyme activity was found in the supernatant or top fraction. Stability of the particles was estimated by resuspending them in media with different concentrations of sucrose, and then removing them again at different time periods (Fig. 3). If resuspended in buffer without sucrose, 60% of the activity was immediately found in the supernatant, and it is assumed that the osmotic shock ruptured the particles. When resuspended in 0.5 M sucrose, the peroxisomes appear stable for 2 hours and in 1.0 M sucrose, most of the glycolate oxidase activity remained with the microbody pellet for about 8 hours. Enzyme activity lost from the pellet fraction was found in the supernatant or suspending medium. We do not understand why a constant level of about 40% of the glycolate oxidase activity remained in the microbody fraction even after 21 hours in buffer without sucrose.

Mitochondria Fraction—The designation, mitochondria or broken chloroplasts fraction, was arbitrary, as the initial centrifugal technique did not clearly separate these fractions. In the examples shown in Table II, 5 to 15% of the total enzymic activity associated with the peroxisomes was initially separated with the mitochondria fraction. The mitochondria fraction also contained broken chloroplasts (about 20% of the total chlorophyll). The fraction designated as broken chloroplast also contained cytochrome c oxidase activity, which is attributed to mitochondria. Thus, the initial separation of the particles into fractions, designated as whole chloroplasts, broken chloroplasts, and mitochondria, as has generally been done, is arbitrary and for convenience. When using sucrose gradient centrifugation, the initial particle separation would not be necessary except to prevent overloading of the gradient.

When the mitochondria fraction was sedimented in the sucrose gradient, the peroxisomes also banded in 1.9 M sucrose as they did from the broken chloroplast fraction. There was no glycolate oxidase, catalase, or DPNH-glyoxylate reductase in the 1.5 M sucrose region of the gradient where mitochondria were located, as indicated by cytochrome c oxidase activity. Previous reports have indicated that a particulate fraction typical of plant mitochondria contained part of the leaf glycolate oxidase activity (8, 28). However, the investigators were unable to couple glycolate oxidation to phosphorylation. The presence of peroxisomes in these mitochondria preparations undoubtedly accounted for the glycolate oxidase activity.

**DISCUSSION**

The significance and function of peroxisomes in liver and kidney are unknown, but have been carefully considered by De Duve and Baudhuin (12). One possibility has been that these microbodies function as a site to dispose of cell excesses by the

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**Fig. 2.** Electron micrograph of a peroxisome-rich fraction. X 20,000.

**Fig. 3.** Stability of peroxisomes in grinding media of different sucrose concentration. Equal aliquots of the chloroplast fraction were sedimented by centrifugation from sap prepared in the standard grinding medium of 0.5 M sucrose and buffer. The broken chloroplast fractions were resuspended and stored in different grinding media which contained 0, 0.5, and 1.0 M sucrose and buffer. At designated times, aliquots of these suspensions were recentrifuged at 39,000 x g for 20 min before measuring glycolate oxidase activity in the pellet or peroxisomes and in the soluble supernatant grading media. For assay purposes, the particles were resuspended in the corresponding grinding medium in which they had been stored. Both the supernatant and the resuspended microbodies were assayed for glycolate oxidase with added FMN and KCN. There was little decrease in total activity during the experiment; decrease in activity in the microbody fraction was balanced by a corresponding increase in activity of the supernatant.
combined action of oxidases and catalase. This description of peroxisomes from spinach leaves establishes a wider distribution for these microbodies. A current survey indicated that peroxisomes are present in many different plants, although spinach has so far yielded the highest percentage of the glycolate oxidase activity in peroxisomes.1

In considering the function of leaf peroxisomes, two facets of the photosynthetic system provide clues which could not be studied with the liver and kidney. Glycolate is a major and universal product of CO2 photosynthesis (for review see Reference 27). In the light, many leaves have an enhanced rate of respiration, which is called photorespiration and is attributed to glycolate oxidation (29–32). Thus, in contrast to the liver and kidney, in the leaf a major substrate for the peroxisomes is known. Further, in the leaf, photorespiration may be attributable to the peroxisomes, and photorespiration can be differentiated in vivo from mitochondria respiration. The present state of knowledge does not assign a clear function to the glycolate pathway and the peroxisomes. A working hypothesis is that during CO2 photosynthesis, P-glycolate is synthesized by the chloroplasts and excreted as glycolate (27) by the aid of the specific P-glycolate phosphatase (25). Excess glycolate would be oxidized by the peroxisomes and account for photorespiration.

When glycolate-14C has been fed to leaves, it is rapidly converted into sugars via glycine, serine, and glyceric acid, i.e., via the glycolate pathway (33). The glycolate-14C was not converted into CO2 and respired photosynthetically. If all the initial oxidation of added glycolate-14C were occurring in the peroxisomes, then the peroxisome function is not to destroy the glycolate by complete oxidation to CO2. H2O2 production from glycolate oxidase has been considered as a potential source of H2O2 for peroxidase metabolism. However, the absence of peroxidases in the peroxisomes suggest that glycolate oxidase and the peroxisomes do not function for synthesis or metabolism via peroxidases. The presence of 7000-fold more catalase activity in the peroxisomes than glycolate oxidase activity indicates that the H2O2 produced there is not used.

Zelitch has emphasized that the glycolate system could serve as a terminal oxidase for any pyridine nucleotide-linked dehydrogenase (5). Glyoxylate reductase would catalyze the oxidation of DPNH and the resynthesis of glycolate, which, in turn, would be reoxidized by oxygen as catalyzed by glycolate oxidase. The packaging of the DPNH-specific glyoxylate reductase in the peroxisomes, along with glycolate oxidase and catalase, is certainly convincing evidence that such a terminal oxidase system could function to dispose of excess DPNH. Oxidation of excess DPNH, then, is a possible function for peroxisomes.

In this paper, glycolate oxidase, glyoxylate reductase, and catalase are designated as constituents of the peroxisomes. Previously, these enzymes have been considered to be associated with the soluble fraction of the leaf homogenate. This error seems to have been caused by the fragility of the peroxisomes. Investigators isolating enzymes from leaves did not grind in 0.5 M sucrose, and even if they did, when isolating chloroplasts, the peroxisomes were stable for only a few hours and were broken if the solutions were diluted. By use of techniques described herein, it has been shown that 50% of the total glycolate oxidase in spinach leaves can be isolated in particulate fractions. We do not know whether the other half of the oxidase activity in the supernatant represents a soluble glycolate oxidase or whether it arises from fragmentation of the labile peroxisomes during their isolation.

Pierpoint (10) and Thompson and Whittingham (11), with the use of sucrose density gradient centrifugation to separate particles, concluded that glycolate oxidase activity was only in the soluble fraction from tobacco leaves. Pierpoint’s data indicate that there was activity which moved into the sucrose gradient, but it was ignored. Thompson and Whittingham probably did not detect the peroxisomes because they ground the leaves in too dilute a solution of NaCl or sucrose and used only the whole chloroplast fraction. On the basis of the present work, glycolate oxidase can be found in peroxisome particles when a simple isolation procedure is followed.

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