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Short Communication

Isolation and Purification of Intact Peroxisomes from Green Leaf Tissue[1]

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ABSTRACT

Intact peroxisomes were prepared from green leaves of a number of C_3 species, both monocots and dicots. A protoplast extract from which chloroplasts have been removed by a 1-minute 10,000*g* centrifugation was applied to a step gradient of 5, 15, 30, and 45% Percoll containing 0.5 molar sucrose, 0.1% BSA, and 25 millimolar Hepes-KOH (pH 7.5). After centrifugation, a peroxisomal fraction with low contamination by chloroplastic and mitochondrial markers was recovered from the 30/45% Percoll interface. This fraction was passed through a Sepharose 2B column to remove the Percoll which resulted in a peroxisomal preparation exhibiting high intactness (estimated from enzyme latency) and stability.

Photorespiratory metabolism results in the light-dependent uptake of O_2 into and release of CO_2 from photosynthetic pools. Portions of the pathway are localized in three cellular organelles: the chloroplast, the mitochondrion, and the peroxisome (14). Intact chloroplasts and mitochondria are routinely isolated using a variety of mechanical and enzymic methods (7, 8). To date, leaf peroxisomes have only been isolated using isopycnic sucrose density gradient centrifugation techniques essentially similar to those summarized over a decade ago (13). Peroxisomes isolated in this manner are in a high, nonphysiological osmotic concentration of sucrose. Although useful for localization of enzymic function and isozymic patterns within a leaf cell, these preparations have not been successfully diluted into a physiological osmoticum while retaining organellar intactness. In addition, peroxisomes (bounded by a single membrane) are mechanically fragile, compared to chloroplasts and mitochondria (bounded by double membranes) and thus are highly sensitive to the forces required to disrupt intact leaf cells. As a result, typical mechanical procedures used for isolation of chloroplasts and mitochondria are not as applicable for the isolation of intact leaf peroxisomes.

Studies on regulation of chloroplastic and mitochondrial metabolism have been enhanced by the availability of these organelles in a purified, intact state. In an analogous manner, availability of a method to prepare intact, pure peroxisomes from green leaves may make investigations on the potential regulation of the photorespiratory pathway possible. In this paper, we present such a method for the preparation of pure, stable, intact leaf peroxisomes using physically gentle, isoosmotic techniques. A preliminary report of this paper has been presented (11).

MATERIALS AND METHODS

Seeds of rye (*Secale cereale* L., unstated variety), wheat (*Triticum aestivum* L., var. 'Argee'), and barley (*Hordeum vulgare* L., var. 'Morex') were obtained from Olds Seed Co., Madison, WI. Seedlings (7–10 d old) were grown in an environmentally controlled chamber on a 20/15 thermoperiod. Other growth conditions were as described earlier (10). Spinach (*Spinacia oleracea* L. var. 'Bloomsdale Long Standing'), grown hydroponically on a 21/16 thermoperiod, was provided by S. H. Wettlaufer, Department of Botany, Washington State University.

Protoplasts were prepared by incubating transverse leaf sections in a digestion medium consisting of 3% (w/v) Onozuka 4S cellulase (Yakult Biochemical Co., Ltd., Nishinomiyo, Japan), 0.1% (w/v) Rohament-P (Rohm GmbH Chemische Fabrik, Darmstadt, West Germany), 0.1% (w/v) BSA, 0.5 M sorbitol, 1 mM $CaCl_2$ (pH 5.5). BSA and other biochemicals not otherwise specified were obtained from Sigma Chemical Co. After a 3- to 4-h incubation in a 30°C growth chamber, the protoplasts were filtered through 500 and 211 μm nylon nets and collected by a 3-min 300*g* centrifugation. The pellet was resuspended in 0.5 M sucrose, 0.1% BSA, 25 mM Hepes-KOH (pH 7.5; medium A) containing 5% (w/v) Dextran (mol wt, 20,000; US Biochemicals Corp., Cleveland, OH) and overlayered with 0.5 M sorbitol, 25 mM Hepes-KOH, 0.1% BSA (pH 7.5; medium B). After a 7-min centrifugation at 300*g* the protoplasts banding at the sucrose/sorbitol interface were collected with a Pasteur pipet, washed once, and resuspended in a minimal volume of medium B. Protoplasts were gently lysed by passage through a 20-μm nylon net. The protoplast extract was centrifuged for 1 min at 10,000*g* to remove Chl. The cloudy supernatant containing peroxisomes, mitochondria, and minimal Chl was applied to a Percoll step gradient consisting of 5, 15, 30, and 45% Percoll in medium A. The gradient was centrifuged to an $\omega^2 t$ of 2.35×10^9 rad^2/s (approximately 20 min at 15,000 rpm) at 2°C in a Beckman type 70Ti rotor. Gradients were fractionated from the top into 20 or 60 drop fractions. Percoll was removed from the peroxisomal fraction by passage at 4°C through a 2.5 × 15 cm Sepharose 2B column equilibrated with medium A.

Enzyme assays used were essentially those of Kohn and Warren (5) for hydroxypyruvate reductase, Hatch (3) for fumarase, Latzko and Gibbs (6) for triose-P dehydrogenase, and Kerr and Groves (4) for glycolate oxidase. For the (serine + glyoxylate)-dependent hydroxypyruvate reductase assay, 10 mM serine and 1 mM glyox-

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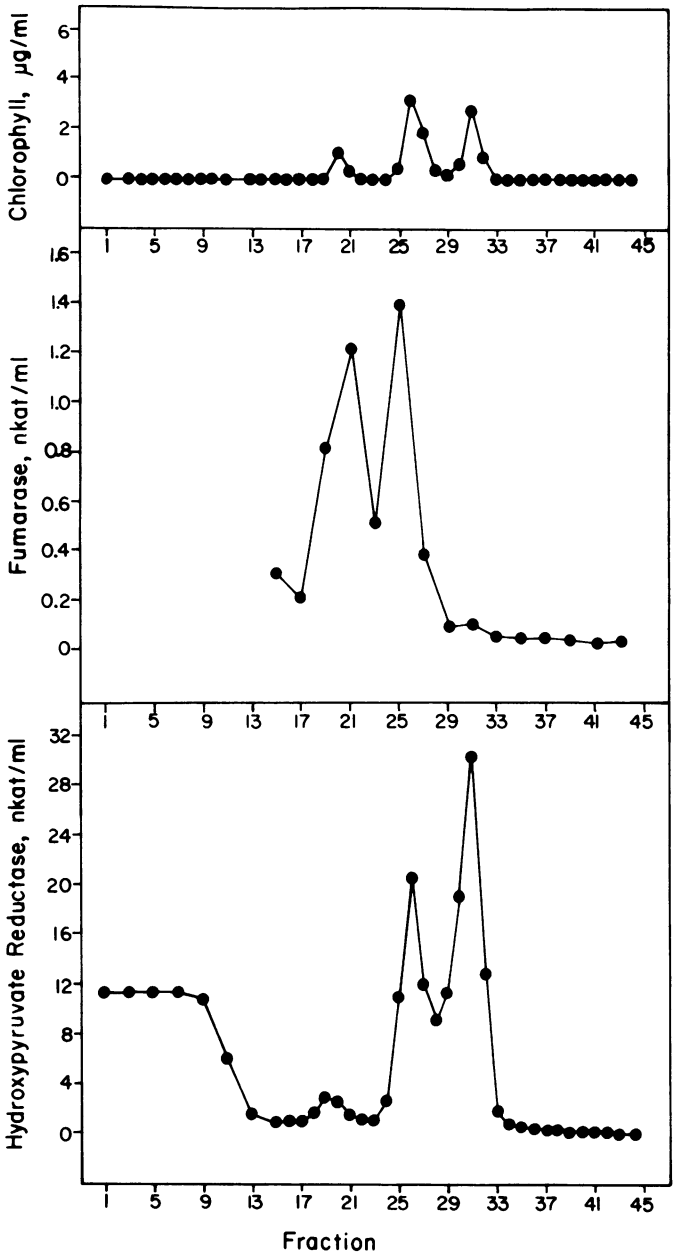

FIG. 1. Percoll step gradient for purification of leaf peroxisomes. Steps consisted of 5, 15, 30, and 45% Percoll in 0.5 M sucrose, 0.1% BSA, 25 mM Hepes-KOH (pH 7.5). The 1-min 10,000*g* supernatant of a rye protoplast extract was applied to the gradient and centrifuged to a $\omega^2 t$ of 2.35×10^9 rad^2/s (approximately 20 min at 15,000 rpm) at 2°C in a Beckman type 70Ti rotor. P = peroxisomal fraction at the 30/45% Percoll interface.

ylate (final concentrations) were used in place of hydroxypyruvate. Osmotic support for intact peroxisomes was provided by inclusion of 0.5 M sucrose in the assay. Chl was determined in 80% acetone using the constants of Arnon (1).

RESULTS

When the 1-min 10,000*g* supernatant of a rye protoplast extract is separated on a Percoll step gradient as described above, a fraction can be isolated which is essentially devoid of Chl and is highly enriched in the peroxisomal marker hydroxypyruvate reductase with respect to the mitochondrial marker enzyme fumarase (Figs. 1 and 2). Table I which summarizes the peroxisomal

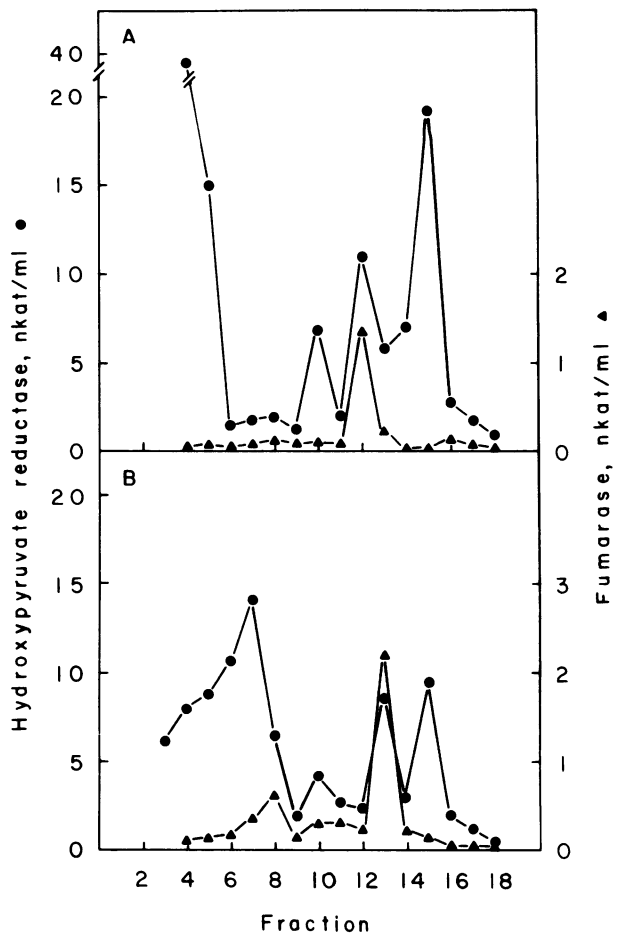

FIG. 2. Purification of peroxisomes from a rye protoplast extract on a Percoll step gradient as in Figure 1. Peroxisomes (fraction 31) indicated by hydroxypyruvate reductase activity were well separated from chloroplasts (marker = Chl) and mitochondria (marker = fumarase). Fraction 1 was at the top of the gradient. nkat^3 = 10^{-9} mol/s.

Table I. *Purification of Peroxisomes from a Protoplast Extract of Rye Leaves by Differential and Percoll Gradient Centrifugation*

Fresh weight of leaves used was 65 g.

Parameter	Protoplast Extract	10,000 *g*, 1-Min Supernatant	Peroxisomal Fraction
Volume, ml	14.0	12.4	3.30
Chl, μg	9,390	36.4	1.39
Hydroxypyruvate reductase, nkatal	2,144	346.5	71.52
Fumerase, nkatal	175.4	109.1	0.220
Hydroxypyruvate reductase/Chl	0.228	9.52	51.5
Hydroxypyruvate reductase/fumarase	12.22	3.176	325.1

purification from a protoplast extract achieved by the Percoll gradient shows a greater-than-200-fold purification relative to Chl and a greater-than-25-fold purification relative to fumarase. In a separate experiment, we found a greater-than-800-fold purification of the peroxisomal fraction from the protoplast extract with reference to triose-P dehydrogenase, the marker enzyme for intact chloroplasts (data not shown). The yield of peroxisomes (calculated from hydroxypyruvate reductase activity in protoplast extract and peroxisomal fractions) was about 3% (Table I).

Due to the ease of obtaining large yields of protoplasts from rye

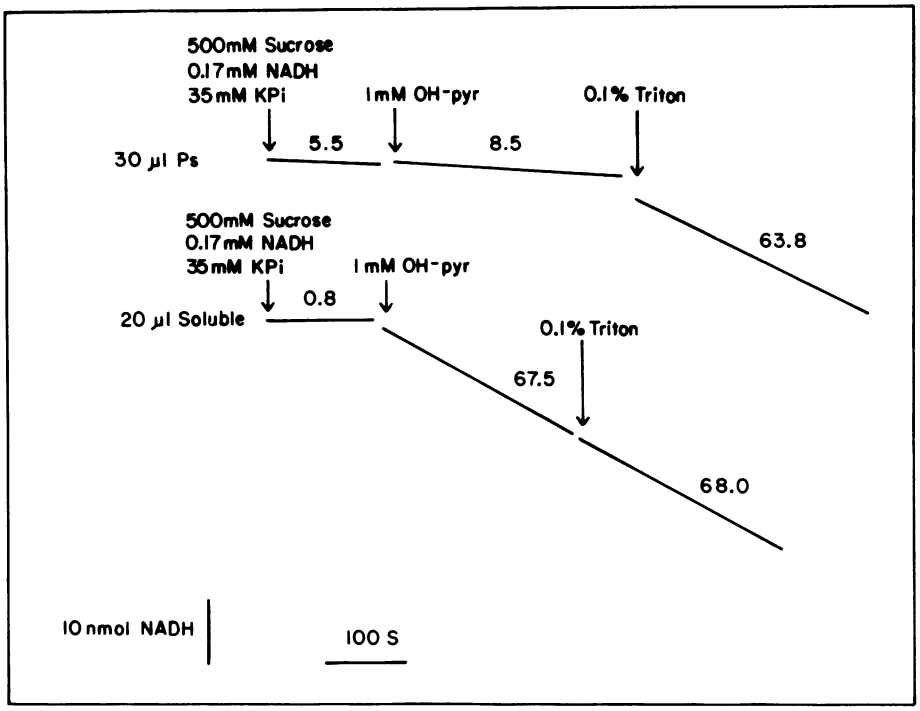

FIG. 3. Peroxisomal purification from mitochondria in spinach (A) and wheat (B) on a Percoll step gradient as in Figure 1. Chl distributions (data not shown) were similar to those in Figures 1 and 2.

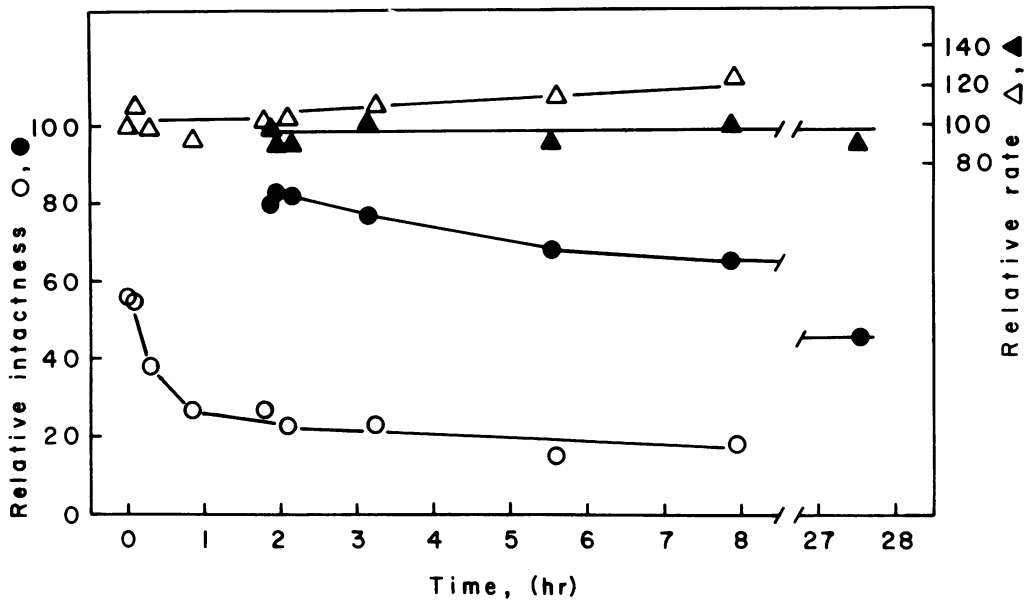

FIG. 4. Triton X-100 stimulation of hydroxypyruvate reductase activity in an osmotically protected peroxisomal preparation (Ps) and in a soluble, membrane-free (150,000*g*, 1 h supernatant) peroxisomal extract. Additions are as shown at arrows. Numbers above the spectrophotometer tracings are hydroxypyruvate reductase rates in nkat/ml. Calculated intactness was 95% for the peroxisomal preparation and 0.7% for the membrane-free extract.

(approximately 10 mg Chl/70 g fresh weight leaf tissue), the technique has been primarily developed with this species in order to maximize the yield of peroxisomes. The procedure may be generally applicable to C_3 monocots and dicots since the Percoll gradient can be used to obtain a peroxisomal fraction of similar purity from protoplast extracts of spinach, wheat (Figs. 3, A and B, respectively), and barley (data not shown). Chl distributions on the gradients for spinach, wheat, and barley were similar to those seen in Figures 1 and 2 for rye (data not shown). Peroxisomes isolated from mesophyll protoplasts of the two C_4 species tested (*Zea mays* and *Digitaria sanguinalis*) were not separated well from mitochondria on the Percoll gradient designed for the C_3 species. No further attempts were made to optimize the gradient for the C_4 species.

The activity of hydroxypyruvate reductase (hydroxypyruvate + NADH-dependent) was assayed in an intact peroxisomal preparation and in a membrane-free (1 h, 10,000*g* supernatant) peroxisomal extract. The activity in the peroxisomal preparation was strongly stimulated by addition of Triton X-100 whereas the detergent had no effect on activity in the peroxisomal extract (Fig. 4). Similar results were obtained in a (serine + glyoxylate)-dependent (intraperoxisomally-generated hydroxypyruvate) hydroxypyruvate assay and in a polarographic glycolate oxidase assay (data not shown).

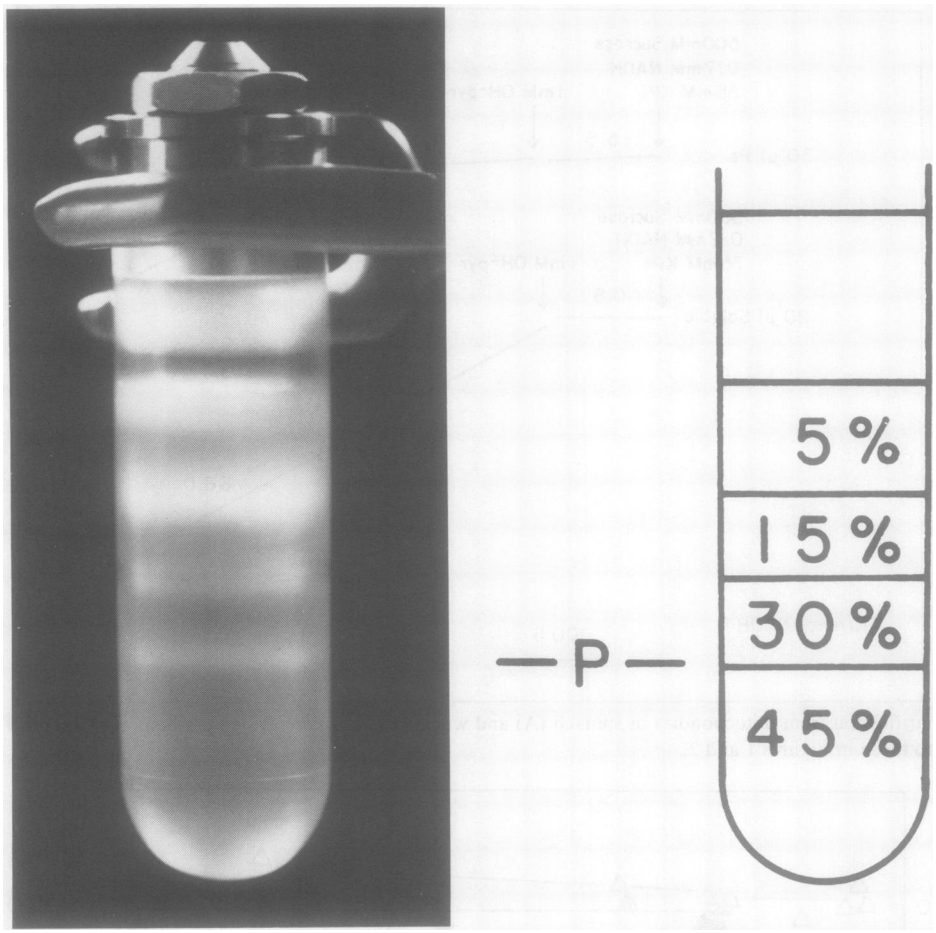

FIG. 5. Stability of hydroxypyruvate reductase activity and Triton X-100 latency in peroxisomal preparations remaining in Percoll (○, △) and with Percoll removed (●, ▲) by passage through a Sepharose 2B column. The peroxisomal fraction was removed from the Percoll gradient at $t = 0$ h and assayed for enzyme latency immediately. A portion of the preparation was applied to the Sepharose column at $t = 0.3$ h and was available for latency determinations at $t = 2$ h. Relative intactness (latency) was calculated by $\frac{(+\text{Triton}) - (=\text{Triton})}{(+\text{Triton})} \times 100$ where (+Triton) is the rate after membrane solubilization by 0.1% Triton X-100 and (−Triton) is the rate in an osmotically protected assay prior to detergent addition. Relative activity was defined as 100% at $t = 0$ h for the untreated and at $t = 2$ for the Sepharose-treated preparations.

Latency of hydroxypyruvate reductase activity in peroxisomes declined rapidly if the peroxisomes remained in Percoll (Fig. 5). However, if the peroxisomal fraction was passed through a Sepharose 2B column, intact peroxisomes were removed from broken peroxisomes and from the Percoll resulting in a stable preparation showing substantial hydroxypyruvate reductase latency. Even after a storage period of over 24 h, Sepharose-treated peroxisomes showed a relative intactness of at least 50%. Based on enzyme latency, intactness of the peroxisomal preparations routinely was 70% or greater.

DISCUSSION

Because of the mechanical and osmotic fragility of leaf peroxisomes, two requirements were paramount in the development of an experimental protocol for their successful isolation. They are that the procedure should be (a) isoosmotic throughout and (b) physically gentle, avoiding osmotic shock in the first case and mechanical rupture in the second. In addition, a gentle procedure for tissue disruption would minimize generation of thylakoid fragments which have caused difficulties in the purification of Chl-free leaf mitochondrial preparations. The first requirement (isotonicity) was met by utilizing the modified silica sol Percoll (which has an insignificant osmotic potential) as a means to separate particles and the second requirement by using cell wall-free protoplasts which can be lysed under much more gentle conditions than those required to disrupt plant cells bounded by a rigid cell wall. In addition, we avoided any pelleting and resuspension of the peroxisomes to minimize further mechanical stresses on the organelle.

Data presented in Figures 1 and 3 and in Table 1 show that a peroxisomal preparation highly purified from intact and broken chloroplasts and mitochondria is obtained using the gentle protoplast/Percoll procedure outlined in this paper. Recently, Nishimura *et al.* (8) used an analogous procedure (protoplasts/Percoll) to obtain Chl-free, functional mitochondria from spinach leaves. By gentle rupture of protoplasts using a Teflon tissue homogenizer, they were able to obtain functional mitochondria of high purity by differential and Percoll gradient centrifugation. In the study by Nishimura *et al.*, an osmoticum of 0.25 M sucrose was used compared to the 0.5 M sucrose used in this study. In contrast to our findings, they found that the peroxisomes banded at a lower density than that of the mitochondria. This agrees with the observations of de Duve (2) that peroxisomes seem to be somewhat permeable to sucrose and will sediment to a greater equilibrium density in higher concentrations of this sugar.

Inasmuch as the ultimate goal of the purification was to obtain intact as well as purified peroxisomes, it was necessary to devise a measure of intactness. A commonly used criterion of intactness

is enzyme latency (stimulation of enzyme activity upon disruption of organellar integrity) such as the stimulation of ferricyanide-dependent O_2 evolution upon osmotic shock of intact chloroplasts. It must be noted, though, that the validity of intactness quantitation based on enzyme latency is dependent on the degree of accessibility of substrates to the enzyme. If the substrates are somewhat permeant in an intact organelle, the measured intactness will be an underestimate of the actual intactness. The low rates of hydroxypyruvate reductase activity seen in intact peroxisomes prior to addition of detergent to solubilize the peroxisomal membrane may be due to either a low permeability of the peroxisomal membrane to the reaction substrates or to breakage of some peroxisomes and resultant solubilization of a portion of the total hydroxypyruvate reductase activity in the assay. The contributions of each of these possible explanations cannot be evaluated at the present time because data on the entrance of photorespiratory intermediates into the peroxisome is lacking.

Although Percoll, by virtue of its coating of PVP, is much less toxic to organelles than the earlier used unmodified silica sols, we observed a rapid loss of peroxisomal intactness (measured as hydroxypyruvate reductase latency) when the organelle was stored in the Percoll gradient medium. This could be remedied by removal of the Percoll with a Sepharose 2B column resulting in a stable, intact preparation. Previously, several other workers have noted difficulty in working with Percoll. Stitt and Heldt (12) noted that chloroplasts isolated in unpurified Percoll showed photosynthetic rates that were strongly inhibited compared to rates of chloroplasts prepared in purified Percoll. Riezman (9) found that glyoxysomes prepared on a Percoll gradient showed no Triton-susceptible protection of glyoxysomal polypeptides from protease degradation. When these glyoxysomes were applied to a sucrose density gradient and thus separated from the Percoll, protease protection was restored. The reasons for these undesirable effects of Percoll are not clear at present. However, these effects can be overcome (with peroxisomes) by subsequent removal of the Percoll and are more than compensated for by the benefits resulting from the ability to separate organelles under isoosmotic conditions with Percoll.

Among the possibilities now open to investigation using intact leaf peroxisomes, transport properties will be of particular interest. We are currently evaluating the proposed malate/aspartate shuttle for providing reducing equivalents to the peroxisomal matrix to drive hydroxypyruvate reduction to glycerate.

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