LOCALIZATION OF ENZYMES WITHIN MICROBODIES

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ABSTRACT

Microbodies from rat liver and a variety of plant tissues were osmotically shocked and subsequently centrifuged at 40,000 g for 30 min to yield supernatant and pellet fractions. From rat liver microbodies, all of the uricase activity but little glycolate oxidase or catalase activity were recovered in the pellet, which probably contained the crystalline cores as many other reports had shown. All the measured enzymes in spinach leaf microbodies were solubilized. With microbodies from potato tuber, further sucrose gradient centrifugation of the pellet yielded a fraction at density 1.28 g/cm³ which, presumably representing the crystalline cores, contained 7% of the total catalase activity but no uricase or glycolate oxidase activity. Using microbodies from castor bean endosperm (glyoxysomes), 50–60% of the malate dehydrogenase, fatty acyl CoA dehydrogenase, and crotonase and 90% of the malate synthetase and citrate synthetase were recovered in the pellet, which also contained 96% of the radioactivity when lecithin in the glyoxysomal membrane had been labeled by previous treatment of the tissue with [¹⁴C]choline. When the labeled pellet was centrifuged to equilibrium on a sucrose gradient, all the radioactivity, protein, and enzyme activities were recovered together at peak density 1.21–1.22 g/cm³, whereas the original glyoxysomes appeared at density 1.24 g/cm³. Electron microscopy showed that the fraction at 1.21–1.22 g/cm³ was comprised of intact glyoxysomal membranes. All of the membrane-bound enzymes were stripped off with 0.15 M KCl, leaving the "ghosts" still intact as revealed by electron microscopy and sucrose gradient centrifugation. It is concluded that the crystalline cores of plant microbodies contain no uricase and are not particularly enriched with catalase. Some of the enzymes in glyoxysomes are associated with the membranes and this probably has functional significance.

INTRODUCTION

Microbodies from mammalian liver and kidney have been characterized as peroxisomes and studied intensively by de Duve and his colleagues (1, 6, 14, 15). Those from rat liver have received the most attention and their basic enzymatic constitution, including catalase and the H_2O_2 generating oxidases, is common to microbodies isolated from other animal cells (6).

Three classes of microbodies have been distinguished in plants and all three contain the distinctive enzymes of the microbodies from mammalian tissues, namely, catalase, α-hydroxy acid oxidase, and uricase (12). The enzyme constitution of microbodies isolated from many plant tissues seems restricted to these three and we regard them as unspecialized (12). Those from the fatty tissues of germinating seeds contain many additional enzymes including those of the glyoxylate cycle and are known as glyoxysomes (2). The microbodies from green leaves, the leaf peroxisomes (24), have high activities of glycolate oxidase, hydroxypyruvate reductase, and specific transaminases which fit them for a role in the glycolate pathway (24).

The plant microbodies recovered from sucrose density gradients in about 54% (wt/wt) sucrose

are known to be osmotically fragile and dilution results in solubilization of enzyme protein (9, 23). However, not all of the enzymes from glyoxysomes are released readily, since there is evidence that broken organelles may selectively retain malate synthetase (9). In addition, the sedimentation behavior of the enzymes after deliberate breakage in KCl shows that some of the enzymes are not truly solubilized (3).

It is clearly established that in microbodies (peroxisomes) from rat liver, uricase is localized in the crystalline cores (1, 6) which resist solubilization. Microbodies from some plant tissues also have crystalline cores and these have been shown histochemically to be rich in catalase (8, 25) although the staining behavior is interpreted differently by Mollenhauer and Totten (18). Uricase is easily solubilized from the castor bean glyoxysomes (22) suggesting that this enzyme is not localized in the cores.

Apart from these observations little is known about the location of enzymes within microbodies and it seems to be generally assumed that they are dispersed in the amorphous matrix. In this paper we describe experiments with microbodies isolated on sucrose gradients from different sources to which a variety of solubilization treatments have been applied. The results lead to some conclusions about the location of enzymes within the organelles, and particularly that some enzymes are specifically associated with the membranes of glyoxysomes.

MATERIALS AND METHODS

Isolation of Microbodies

FROM PLANT TISSUES: The isolation by equilibrium sucrose density gradient centrifugation from castor bean (*Ricinus communis* L. var. Hale) endosperm, spinach (*Spinacia oleracea* L.) leaves, and potato (*Solanum tuberosum* L.) tubers followed that described previously (12) with one modification. Instead of first preparing a crude particulate preparation, the supernatant solution obtained after centrifuging for 10 min at 270 g was layered directly onto the gradient.

FROM RAT LIVER: Simonson albino female rats were injected with Triton WR-1339 as described (14) 4 days before sacrifice. The livers were homogenized in grinding medium (12) with razor blade and mortar and pestle, and the lambda fraction (14) or the 10,000 g particulate fraction (22) was obtained. The pellet was resuspended in 18% sucrose, layered onto a sucrose gradient (12), and centrifuged at 21,000 rpm for 4–7 h in a Beckman model L2-65B

ultracentrifuge with Spinco Rotor 25.2 (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The microbody fractions were collected from a hole punctured in the bottom of the centrifuge tube.

Subfractionation

A 2-ml microbody fraction in either 50% sucrose (from rat liver) or 54% sucrose (from the rest) was added to 4 ml of 0.05 M Tricine Buffer, pH 7.5 with or without KCl at the concentration indicated, and shaken in a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.). After incubation in the cold for 30 min, the solution was centrifuged at 40,000 g for 30 min in a Beckman Rotor 65 (Beckman Instruments, Inc., Spinco Div.). The pellet was resuspended in 0.05 M Tricine buffer, pH 7.5, 18% sucrose, and as appropriate, KCl at the same concentration as that in the supernate.

Assays

Both the supernatant solution and the pellet were assayed for enzyme activity, protein, and where appropriate, for radioactivity. Percent solubilization was expressed as the amount in the supernate divided by the sum of the amounts recovered in the supernate and the pellet.

The assay of protein, catalase, glycolate oxidase, uricase, and cytochrome c oxidase were as described previously (12). Isocitrate lyase was assayed by the phenylhydrazine method (7). Malate synthetase and citrate synthetase were assayed by the 5,5'-dithiobis-2-nitrobenzoic acid method (11) in the presence of 0.05% Triton X-100. In the assay of citrate synthetase, Mg^{++} was omitted from the reaction mixture.

The following enzymes were assayed by the change in absorbance of $NAD^+/NADH$ at 340 nm. Thiolase was assayed by coupling with citrate synthetase and malate dehydrogenase (4). Crotonase was assayed by coupling with 3-hydroxyacyl CoA dehydrogenase (5). 3-Hydroxyacyl CoA dehydrogenase was assayed in the direction of NADH oxidation (20). Oxalacetate glutamate transaminase was assayed by coupling with malate dehydrogenase (21). Malate dehydrogenase was assayed in 0.1 M potassium phosphate buffer pH 7.4, 0.14 mM NADH, and 1 mM oxalacetate (OAA). Hydroxypyruvate reductase was assayed in 0.1 M potassium phosphate buffer, pH 6.7, and 0.14 mM NADH. The optimal concentration of hydroxypyruvate for the assay was found to be 5 mM for spinach leaf and 1 mM for castor bean.

Labeling of the Glyoxysomal Membranes

Endosperm halves from 4–day old castor beans were treated with [*methyl*-^{14}C]choline and incubated at 25°C for 12 h before isolation of microbodies. The

details of this method and the evidence of the incorporation of the ^{14}C into the lecithin fraction of the glyoxysomes were described by Kagawa et al. (13).

Sucrose Gradient Centrifugation of the Membrane Fraction of Glyoxysomes

The pellet containing the labeled membrane fraction of the glyoxysomes was resuspended in 25% (wt/wt) sucrose and layered onto a linear sucrose gradient composed of 28 ml gradient from 30 to 65% over a cushion of 2 ml 65% sucrose (all percentages are expressed as percent sucrose by weight and all solutions contained 1 mM EDTA, pH 7.5). After centrifugation for 4 h at 21,000 rpm in a Beckman L2-65B ultracentrifuge with Spinco Rotor 27, the gradient was collected in 0.6 ml fractions using an ISCO density gradient fractionator model 640 (Instrumentation Specialities Co., Inc., Lincoln, Nebr.). The sucrose concentration of each fraction was measured in a Bausch and Lomb refractometer (Bausch & Lomb Inc., Scientific Instrument Div., Rochester, N. Y.).

Electron Microscopy

Pellets were fixed for 1 h with 3.5% glutaraldehyde in 18% sucrose, 0.05 M potassium phosphate buffer, pH 7.5. They were postfixed with 1% OsO_4 in 18% sucrose, 0.05 M potassium phosphate buffer, pH 7.5, for 1 h and then overnight with 1% OsO_4 in 0.05 M potassium phosphate buffer, pH 7.5. The material was dehydrated with ethanol and propylene oxide, and embedded in Spurrs plastic. Sections were stained with lead citrate and viewed with a RCA model EMU-3H electron microscope.

Chemicals

CoA, acetoacetyl CoA, and crotonyl CoA were obtained from P-L Biochemicals Inc., Milwaukee, Wis. Acetyl CoA was generated from CoA and acetic anhydride (19). Citrate synthetase and 3-hydroxyacyl CoA dehydrogenase were obtained from Boehringer Mannheim Corp., New York.

RESULTS

Comparison of Enzyme Activities in Plant and Animal Microbodies

In isopycnic sucrose gradient centrifugation of the particulate fraction from rat liver, the major protein peak occurred together with cytochrome oxidase, a marker enzyme of mitochondria, at a density of 1.19 g/cm^3. Most of the activity of microbody enzymes appeared at a density of 1.22–

1.23 g/cm^3 as expected (14, 15). The microbody enzymes and the cytochrome oxidase overlapped slightly in the gradient and this cross contamination diminished when the "lambda fraction" (14), instead of the total particulate fraction, was applied to the gradient. The protein in the microbody region of the gradient represented at best a few percent of the total particulate protein. Acid phosphatase, a lysosomal marker enzyme, occurred exclusively at density 1.11 g/cm^3. The separation of microbodies from various plant tissues in sucrose gradient centrifugation has been described previously (12). The specific activities of the various enzymes in the microbody region in the gradient are shown in Table I. The activities of the microbody enzymes measured under standard conditions in the isolated rat liver microbodies are similar to those previously reported (15) when the temperature of the assay is taken into account. Catalase activity in rat liver microbodies is only slightly lower than that in castor bean glyoxysomes, which have the highest catalase activity among plant microbodies (12). The amount of glycolate oxidase activity in rat liver microbodies is comparable to that found in potato tuber microbodies and other unspecialized plant microbodies (12) and in glyoxysomes, and is only one-tenth of that in spinach leaf microbodies, where it has a clearly defined and important role in photorespiration (24). The uricase activity in rat liver microbodies is considerably higher than that in each of the preparations from plants. Thus, although the microbodies from the various tissues have three enzymes in common, there are quantitative differences which presumably reflect functional differences in vivo.

The Crystalline Cores of Plant and Animal Microbodies

The microbodies isolated in either 50% (wt/wt) sucrose (rat liver) or 54% sucrose (the rest) were subfractionated by osmotic shock as follows. Each sample was diluted twofold with buffer and centrifuged at 40,000 g for 30 min to give supernatant and pellet fractions. The data are shown in Table II. All the measured enzymes in spinach leaf microbodies appeared in the supernatant fraction. With rat liver microbodies, while catalase and glycolate oxidase were solubilized, all the uricase was recovered in the pellet and it was not solubilized by treatment with 0.2 M KCl. This finding is consistent with many other reports that

TABLE I

Enzyme Activities in Microbodies Isolated from Plant and Animal Tissues

	Castor bean endosperm	Spinach leaves	Potato tuber	Rat liver
	nmol/min/mg protein			
Catalase	13×10^6	2.2×10^6	0.6×10^6	11.4×10^6
Glycolate oxidase	95	1,130	34	32
Uricase	16	10	19	133
Hydroxypyruvate reductase	36	6,500	—	—
Malate dehydrogenase	38,000	21,600	—	—
Glutamate oxalacetate trans-aminase	4,200	250	—	—
Thiolase	280	—	—	—
Crotonase	19,800	—	—	—
Fatty acyl CoA dehydro-genase	15,900	—	—	—
Citrate synthetase	840	—	—	—
Malate synthetase	2,100	—	—	—
Isocitrate lyase	930	—	—	—

—, denotes activities not tested.

TABLE II

Percent Solubilization of Microbody Enzyme Activities after Osmotic Breakage of Microbodies Isolated from Various Tissues

	Spinach leaves	Potato tuber	Castor bean endosperm	Rat liver
Catalase	94	70	70	80
Glycolate oxidase	91	96	89	63
Hydroxypyruvate reductase	98	—	93	—
Uricase	—	88	94	0
Glutamate oxalacetate transaminase	86	—	78	—
Malate dehydrogenase	91	—	35	—
Isocitrate lyase	—	—	98	—
Thiolase	—	—	90	—
Crotonase	—	—	41	—
Fatty acyl CoA dehydrogenase	—	—	43	—
Citrate synthetase	—	—	11	—
Malate synthetase	—	—	6	—
Protein	—	72	60	—

Results are expressed as the amount in the supernate divided by the sum of the amounts in the supernate and in the pellet. Recovery of each enzyme activity after treatment was within 100 ± 30%.

the crystalline cores of rat liver microbodies contain all the uricase and have a sedimentation coefficient high enough to be centrifuged down at moderate centrifugal force (1, 10). In contrast, most of the uricase activity in potato tuber microbodies and castor bean glyoxysomes was solubilized under similar treatment. With potato tuber microbodies, a small fraction of the catalase activity, but no uricase or glycolate oxidase activity, appeared in the pellet. When this pellet was resuspended in 25% sucrose and centrifuged at 64,000 g (on the average) for 4 h on a linear sucrose gradient composed of 30–65% sucrose, the catalase, corresponding to 7% of the total microbody catalase activity, appeared at a density of 1.28 g/cm³. No uricase or glycolate oxidase was de-
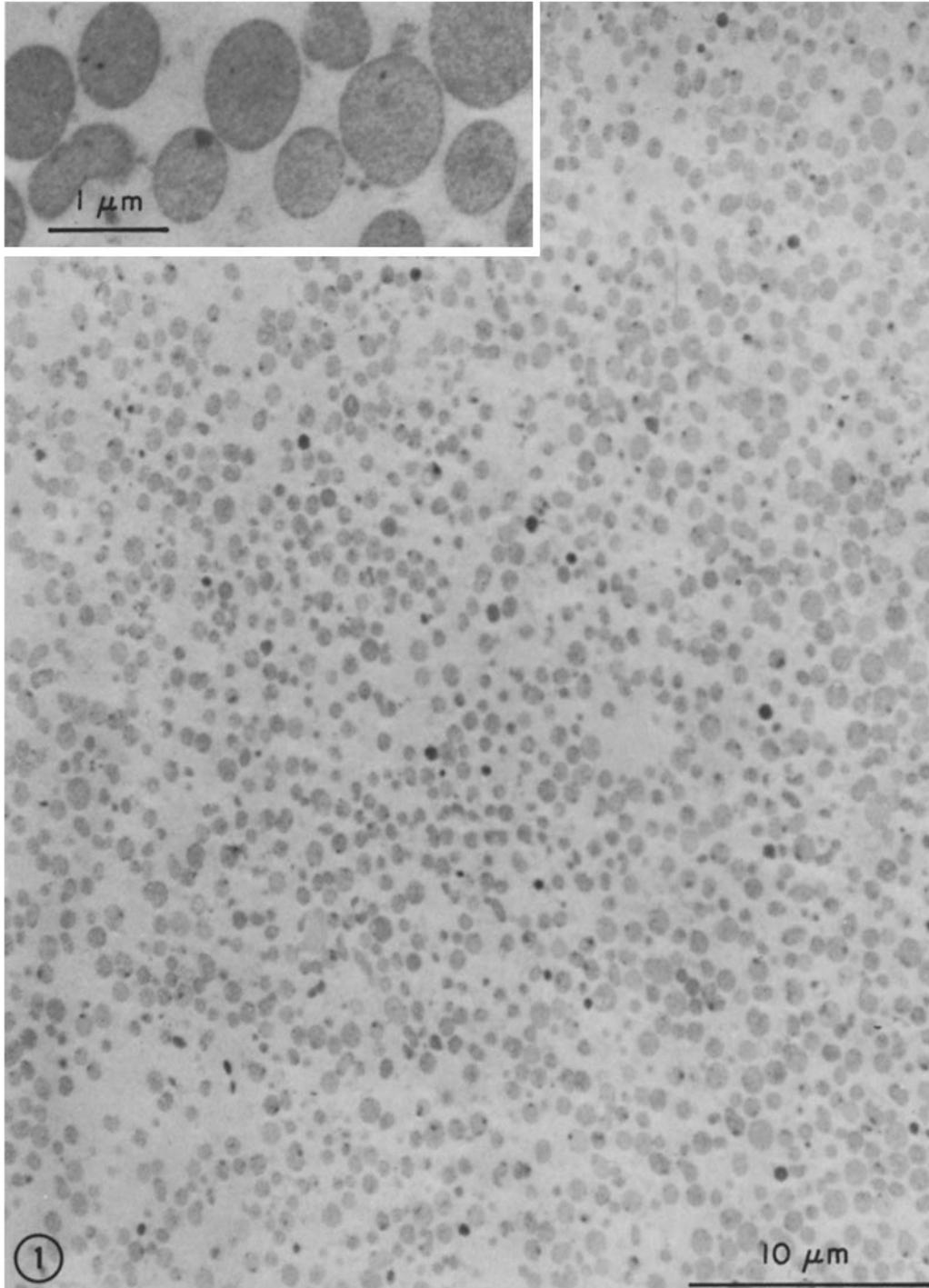
FIGURE 1 Electron micrograph of the glyoxysomal fraction isolated by sucrose gradient centrifugation. The fraction was prepared and fixed as described (16) (courtesy of Dr. T. Moore). Bar represents 10 μm. Section was stained with lead citrate. $\times$ 3,700. *Inset* shows several glyoxysomes at higher magnification. $\times$ 18,000.

tected at this region. Since the crystalline cores of rat liver microbodies come to an equilibrium density at 1.26–1.28 g/cm³ under similar conditions of sucrose gradient centrifugation (1, 10), this fraction from potato tuber microbodies may well represent the crystalline cores.

Subfractionation of Castor Bean Glyoxysomes

When castor bean glyoxysomes were subjected to the same subfractionation procedure, many enzymes were solubilized, but 50% of the crotonase and 3-hydroxyacyl CoA dehydrogenase, 50–80% of the malate dehydrogenase, and 90% of the citrate synthetase and malate synthetase appeared in the pellet (Table II). When the lecithin in the glyoxysomal membranes had been labeled by previous treatment of the tissue with [¹⁴C]choline, 96% of the radioactivity was also recovered in the pellet. Thus, the above-mentioned enzymes sedimented together with the membrane fraction. The appearance of the glyoxysomes

before and after dilution is shown in Figs. 1 and 2. The membranes of the glyoxysomes after osmotic breakage (Fig. 2) appear intact and the shape has been maintained, but much of the amorphous material from within, including the enzymes shown to be solubilized, has been lost. The crystalline cores, where present, appeared inside the "ghosts." A few percent of the glyoxoysomes apparently still retained their protein since they are quite similar to those undiluted (Fig. 1).

When the membrane-labeled pellet was subjected to sucrose gradient centrifugation, each of the enzyme activities, protein, and radioactivity were recovered at a mean density of 1.21–1.22 g/cm³ (Fig. 3) and thus at a significantly lower density than intact glyoxysomes (1.24 g/cm³) before extraction of soluble enzymes. The membrane fraction did not move further into the gradient on prolonged centrifugation (17 h) and thus 1.21–1.22 g/cm³ is the equilibrium density.

Supporting evidence of the membrane-bound nature of the enzymes comes from treatment of the membrane-labeled glyoxysomes with increas-
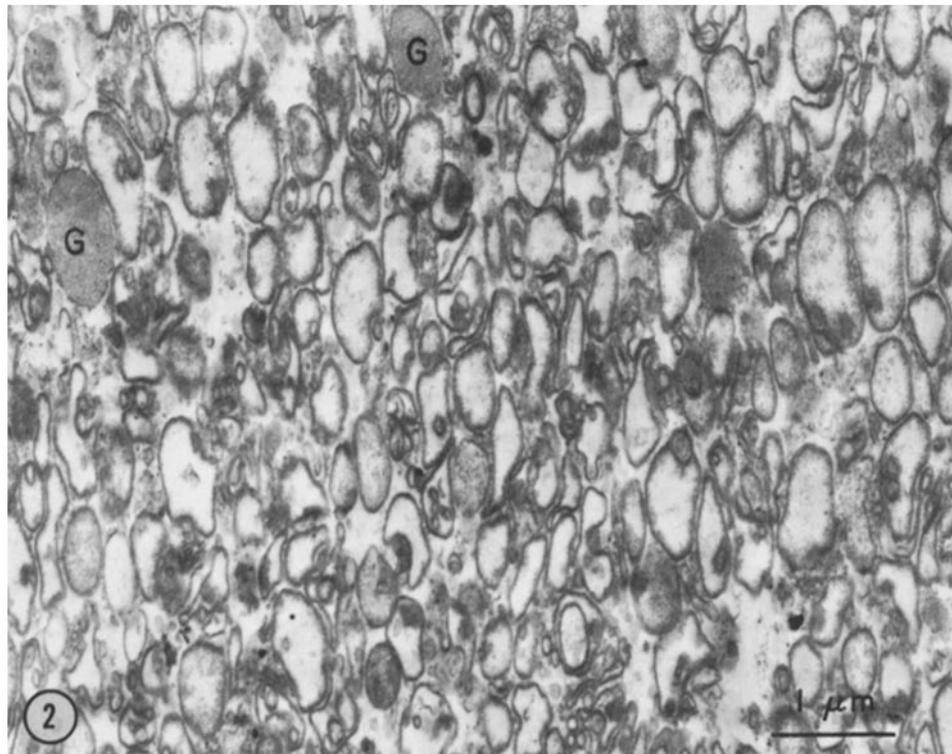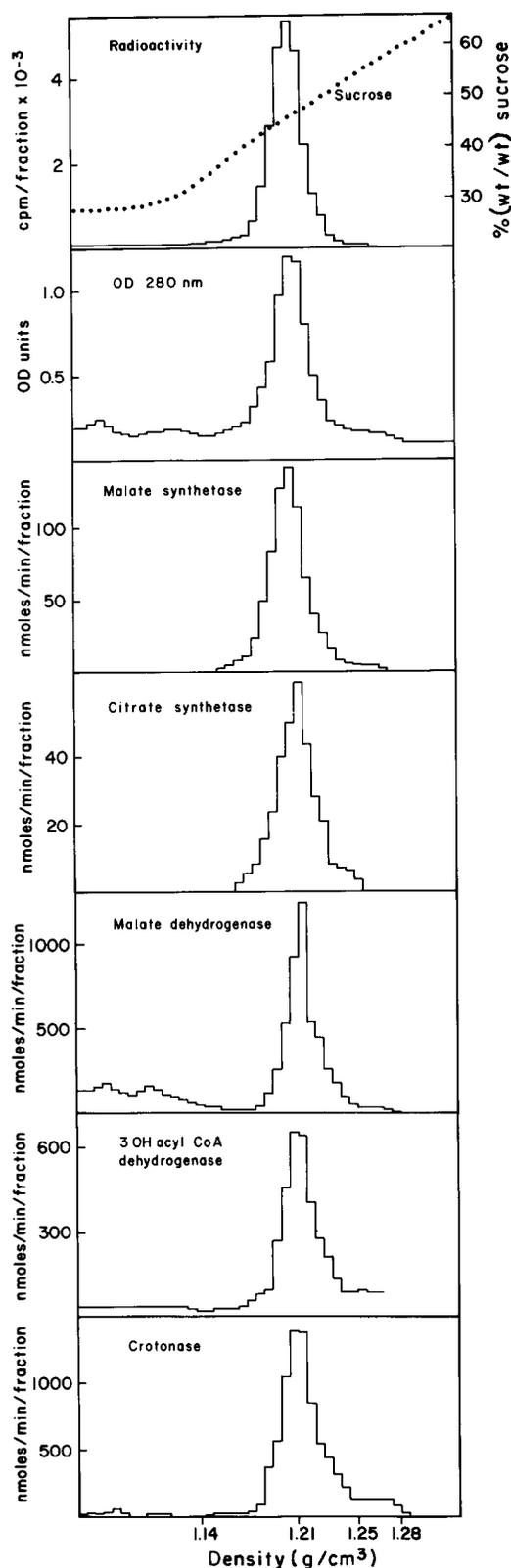

FIGURE 2 Electron micrograph of the glyoxysomal fraction after osmotic shock. The membranes appear intact and the cores, where present, occur inside the ghosts. A few of the glyoxysomes (G) still retained their amorphous matrix. Bar represents 1 μm. Section was stained with lead citrate. × 17,000.

ing concentration of KCl together with dilution treatment (see Materials and Methods). As shown in Fig. 4, malate dehydrogenase, 3-hydroxyacyl CoA dehydrogenase, and crotonase (in a separate experiment) were all solubilized quantitatively at 0.05 M KCl, while at this concentration of KCl, citrate synthetase and malate synthetase were still sedimented with particulate material. Further increasing of the KCl concentration to 0.15 M solubilized the two synthetases. Concomitant with the solubilization of the latter two enzymes, 12% of the radioactivity from lecithin was also released into the supernate. This strongly supports the notion that, while malate dehydrogenase, crotonase, and 3-hydroxyacyl CoA dehydrogenase are only loosely bound to the membrane, malate synthetase and citrate synthetase are comparatively tightly membrane bound. Whether or not the two synthetases are lipoprotein enzymes requires further investigation. The fact that more than 80% of the radioactivity in the membrane was still in the pellet suggests that these two enzymes are not important structural components of the membrane.

When the enzyme-free, lecithin-labeled membrane fraction obtained after treatment with 0.2 M KCl was subjected to sucrose gradient centrifugation, all the protein and radioactivity appeared together at a density of 1.21 g/cm³ (Fig. 5). Electron microscopy of this fraction (Fig. 6) showed that the membrane still appeared intact with occasional crystalline cores inside. As viewed from the electron micrographs, there is no appreciable difference in the membrane before (Fig. 2) and after (Fig. 6) KCl solubilization of the enzymes which were still membrane bound after the dilution treatment.

All of the membrane-bound enzymes were readily solubilized by 0.03% Triton X-100 without losing their activities. Malate synthetase, the most tightly membrane-bound enzyme, was not solubilized by repeated washing and centrifugation with diluted buffer. Its activity was increased two- to threefold when 0.05 Triton X-100 was included in the assay mixture.

It seems clear that when microbodies which have been recovered in 50–54% sucrose after

FIGURE 3 Sucrose gradient centrifugation of the glyoxysomal fraction after osmotic shock. The radioactivity represents the membrane in which the lecithin had been labeled by previous treatment of the tissue with [¹⁴C]choline.

gradient centrifugation are subsequently diluted to 18% sucrose, many enzymes are solubilized. The release of these enzymes, which remain associated with the organelles during preparation in even lower sucrose concentrations is presumably due to the fact that sucrose enters the organelle matrix while they pass through the gradient (6) and, as a consequence they are, at least temporarily, disrupted on sudden dilution. This behavior of the microbodies is different from that of mitochondria which are not permeated by sucrose. Thus when the mitochondrial fraction in
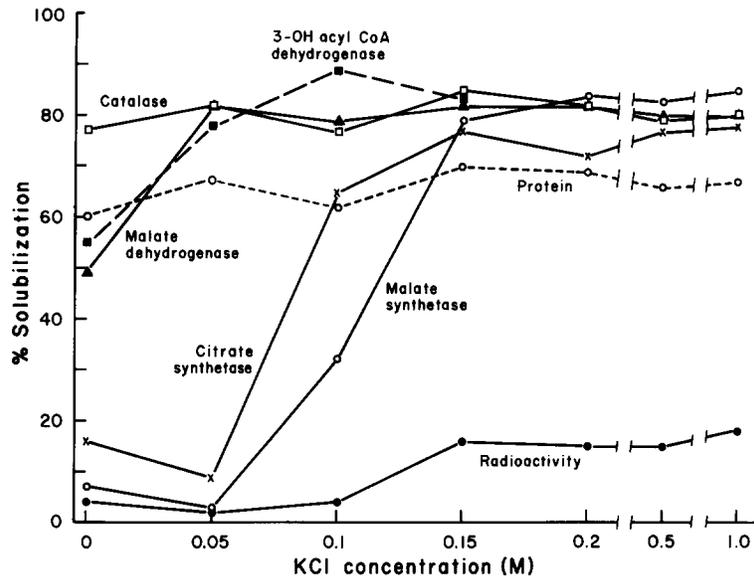
FIGURE 4 Solubilization of the glyoxysomal enzymes with increasing concentration of KCl. The concentration of KCl shown is the final concentration after mixing. The radioactivity represents the membrane in which the lecithin had been labeled by previous treatment of the tissue with [^{14}C]choline.
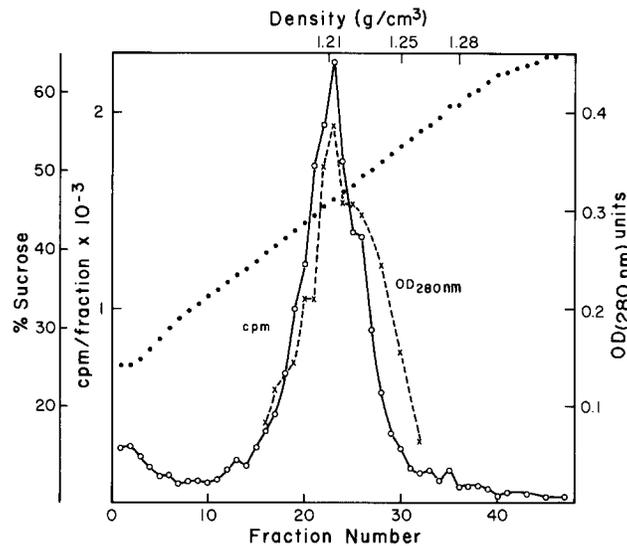

FIGURE 5 Sucrose gradient centrifugation of the glyoxysomal fraction after treatment with 0.2 M KCl. The radioactivity represents the membrane in which the lecithin had been labeled by previous treatment of the tissues with [^{14}C]choline.
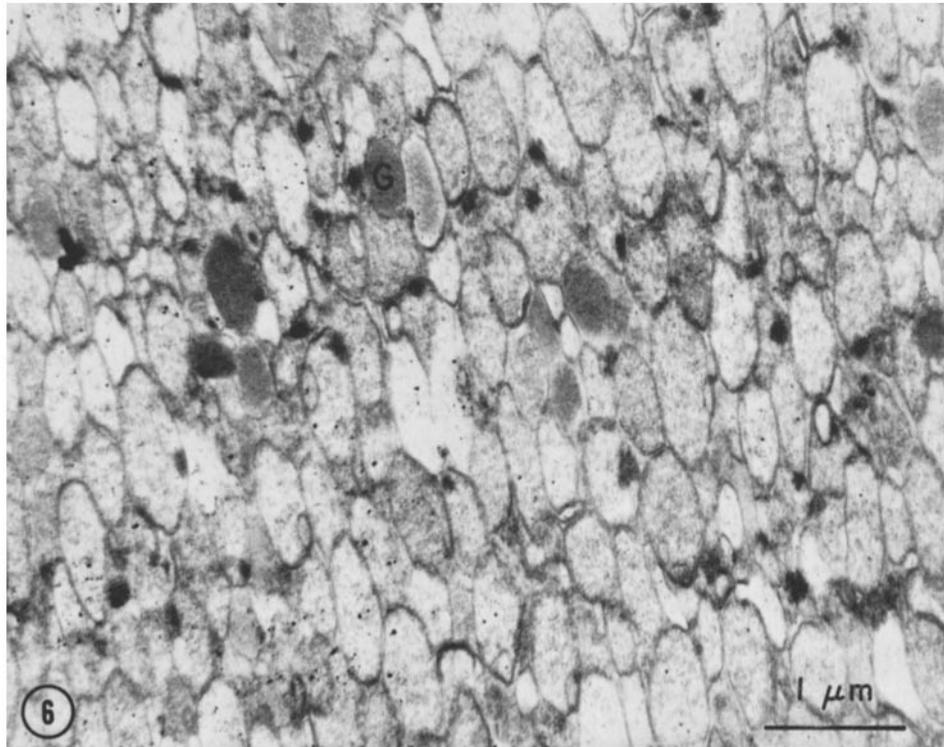
FIGURE 6 Electron micrograph of the glyoxysomal fraction after treatment with 0.2 M KCl. Bar represents 1 μm. Section was stained with lead citrate. G, glyoxysome. × 19,500.

42% sucrose obtained by sucrose gradient centrifugation of the castor bean endosperm extract was similarly diluted twofold with or without 0.2 M KCl and then centrifuged, more than 90% of the activities of fumarase, malate dehydrogenase, and citrate synthetase were recovered in the pellet.

DISCUSSION

The present work indicates that rat liver microbodies are different in at least two aspects from those of plant tissues. First, rat liver microbodies have some ten times greater activity of uricase than any of the plant microbodies (Table I). Second, while uricase is localized in the cores of rat liver microbodies, it is present in the matrix rather than in the cores of plant microbodies.

It has been suggested that in plant microbodies, catalase is concentrated in the crystalline cores (8, 25). The present work shows that in potato tuber microbodies, 7% of the total catalase can be recovered in a fraction which presumably represents the crystalline cores. Electron microscopy

of the isolated potato tuber microbodies (not shown) shows that roughly half of the organelles have a crystalline core which usually occupies most of the cross-sectional area. This rough estimate suggests that even though catalase is present in the cores of potato tuber microbodies, it is not particularly enriched there. In castor bean glyoxysomes, solubilization of most of the measured enzyme activities including catalase by osmotic shock yielded a fraction composed of glyoxysomal ghosts that still retained the crystalline cores. Thus, also in castor bean glyoxysomes, the crystalline cores are not a particularly enriched site of catalase.

It has been shown that in castor bean glyoxysomes, at least five enzymes remain associated with the membranes after dilution. Three of these are released simply by increasing the salt concentration of 0.05 M and two, citrate synthetase and malate synthetase, are released in 0.15 M KCl. While this behavior does not suggest a rigorous association with the membrane, the following considerations indicate that it is

not simply a nonspecific binding and without physiological significance.

(a) That portion of the enzyme activities that was solubilized during preparation of the glyoxysomes did not bind to the membranes of other organelle fractions such as endoplasmic reticulum or mitochondria; it was recovered either in the soluble fraction or on the ghosts.

(b) Malate synthetase and citrate synthetase activities in the glyoxysomes or ghosts can be activated by detergent treatment.

(c) Once released, the enzymes do not bind back to the ghosts when the KCl is removed.

One possible physiological significance of the association of these enzymes to the membrane may be the oxidation and reduction of NADH/NAD at the glyoxysomal surface. Isolated glyoxysomes cannot oxidize NADH generated in the β-oxidation of fatty acids and the glyoxylate cycle (4) and no electron shuttle system in the organelles has been found to reoxidize the NADH (17). The fact that the two NADH-producing dehydrogenase are present on the membrane may thus facilitate the NADH oxidation by enzymes outside the glyoxysome.

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