A ZONAL ROTOR METHOD FOR THE PREPARATION OF MICROPEROXISOMES FROM EPITHELIAL CELLS OF GUINEA PIG SMALL INTESTINE

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ABSTRACT

A method is described for the preparation of catalase particles from homogenates made from suspensions of epithelial cells of the small intestine of the guinea pig. Electron microscope examination of the preparations revealed the presence of small diaminobenzidine-positive particles measuring 0.1–0.3 nm in diameter and resembling the microperoxisomes observed by Novikoff and Novikoff (1972. *J. Cell Biol.* **53**:532.). Analytical data upon which the method is based are presented. The technique consisted of a rate zonal separation of microperoxisomes from large particles followed by an isopycnic separation from less dense organelles. Application of the method yielded microperoxisomes purified between 20- and 30-fold.

INTRODUCTION

Although peroxisomes are known to be widespread in nature (1), they were, until recently, thought to be restricted in vertebrates to hepatocytes and renal tubule cells only (2, 3). Peroxisomes from these two cell types have been studied biochemically and found to fulfill the definitive criterion proposed by de Duve (3). This criterion was that catalase together with at least one hydrogen peroxide-producing oxidase be contained within a distinct class of subcellular particles. Organelles meeting this criterion have been found in mammalian (4, 5), avian, and amphibian (6) liver and kidney.

Subsequently, fractionation data have extended this list by including subcellular particles in the goldfish intestine (7). In addition, peroxisome-like catalase particles have been identified in fractions isolated from homogenates of epithelial cells from the guinea pig small intestine (8, 9). Like hepatic peroxisomes, the intestinal particles showed latency of catalase activity and had a relatively high density in sucrose density gradients.

In their cytochemical electron microscope study of mammalian small intestine absorptive cells, Novikoff and Novikoff (10) found huge numbers of catalase particles in all regions of the guinea pig small intestine. These microscopically identified intestinal peroxisomes were notably different in appearance from the peroxisomes of hepatocytes and renal tubule cells. Intestinal peroxisomes were much smaller in size, lacked any core structure, and appeared to represent local dilations of the smooth endoplasmic reticulum (SER). Because of these differences, and because the catalase particles in numerous other mammalian cells conformed to this description, the term microperoxisome was introduced by Novikoff and Novikoff (10).

Recently the diaminobenzidine (DAB) staining technique for catalase (11) has been applied in ultrastructural studies of many other mammalian

tissues. These studies have revealed catalase particles resembling microperoxisomes in a wide variety of cells including: bronchiolar and alveolar epithelial cells (12, 13); adrenal cortical cells (14); interstitial cells (15) and Sertoli cells (16) of the testes; luteal, interstitial, and granulosa cells of the ovary (17); brown adipose cells (18); dorsal root ganglia (19); parenchyma cells of parotid and submandibular glands and the pancreas (20); canine perianal glands (21); and numerous other tissues (10). Whether the catalase particles found in this great variety of cells fulfill the biochemical criteria for peroxisomes is not known; however, it seems possible that all these particles represent a single class of organelle—the microperoxisome.

Whether the difference in morphology between peroxisomes and microperoxisomes represents any functional divergence will best be studied using purified fractions of these organelles. A method for the preparation of hepatic peroxisomes is already available (22), and here we describe a technique for the purification of intestinal microperoxisomes. These preparations offer the opportunity to study the biochemistry of catalase particles from an extrahepatic tissue and will allow comparison to be made between microperoxisomes and "conventional" peroxisomes.

MATERIALS AND METHODS

Cell Preparation

The method of preparation of intestinal absorptive cells was based on that of Evans et al. (23), but employed 1.0 M urea instead of EDTA. The technique was as previously described (7) and briefly was as follows: An adult guinea pig was killed, and its small intestine was removed and irrigated with ice-cold 10% (wt/wt) sucrose to remove food debris. The lumen was then filled with 1.0 M urea, the ends were sealed, and the intestine was incubated in 10% sucrose at 37°C for 20 min. The urea was then drained off and the intestine irrigated with ice-cold 10% sucrose to remove any mucus that had been released from the goblet cells during incubation. The lumen was then filled with cold 10% sucrose and the ends were sealed. The filled intestine was then gently rubbed between the fingers; applied judiciously, this procedure dislodges sheets of absorptive cells from the underlying villus cores. The sucrose, together with suspended epithelial cells, was then drained off and collected. The cell sheets were purified by two centrifugations at 100 g/min.

Centrifugation Procedures

Cell preparations were suspended in ice-cold 10% sucrose to a volume of 35 ml and homogenized by 20 strokes in a Teflon-glass homogenizer with the pestle rotating at low speed. Centrifugations were carried out using an aluminum B XIV zonal rotor. Samples were loaded onto linear sucrose gradients while the rotor was spinning at 2,300 rpm. An overlay of 20 or 35 ml of dilute sucrose (5 or 10% wt/wt) was then added, the rotor sealed, and centrifugation at the required speed carried out. The rotor was then decelerated and unloaded at 2,300 rpm by pumping 60% (wt/wt) sucrose into the peripheral feed.

Estimations

(*a*) Protein was estimated according to Weichselbaum (24) as modified by Hubscher et al. (25). (*b*) Alkaline phosphatase, acid phosphatase, and aryl esterase were estimated using 1-naphthol substrates as previously described (26), except that catalysis was stopped by raising the temperature to 95°C for 3 min, and the released napthhthol was estimated after appropriate pH adjustment by the method of Robinson (27). (*c*) Catalase was estimated according to Baudhuin et al. (4) after treatment with Triton X-100 to a final concentration of 1%. (*d*) Succinic dehydrogenase was assayed by the method of Pennington (28) as described previously (26). (*e*) D-Amino acid oxidase was estimated according to Leighton et al. (22), except that D-alanine was used at 126 mM and incubations were continued for up to 12 h.

Electron Microscopy

Samples of isolated subcellular organelles were diluted 3:2 with glutaraldehyde in cacodylate buffer at pH 7.2 to give a final concentration of 2% glutaraldehyde and 0.1 M cacodylate. The samples were then centrifuged at 16,000 rpm ($3 \times 10^4 g_{max}$) for 3 h and the sediments resuspended in a small volume of 0.1 M cacodylate buffer at pH 7.2. These suspensions were centrifuged at 29,000 rpm ($1.2 \times 10^5 g_{max}$) for 2 h in a 3 × 10-ml swing-out rotor. The sediments were washed in four changes of cacodylate buffer for a total of 12 h. Some sediments were then incubated in DAB media for the demonstration of catalase (29). The medium contained 2 mg/ml of DAB, 0.3% hydrogen peroxide, and 0.05 M 2-amino-2-methyl-1,3-propanediol buffer to give a final pH of 9.7. Incubation was carried out at 37°C for 90 min. Controls were incubated in the complete medium minus peroxide. All sediments were post-fixed in osmium tetroxide, embedded in Epon, and thin sections cut for electron microscopy. (Sections were not stained with uranyl acetate and lead citrate.)

RESULTS

Preliminary experiments and previous work (8, 9) showed that intestinal catalase particles are denser than other subcellular particles of their approximate size, but smaller than those organelles approaching their density. It can be expected, therefore, that separation on the basis of size differences combined with a second separation based

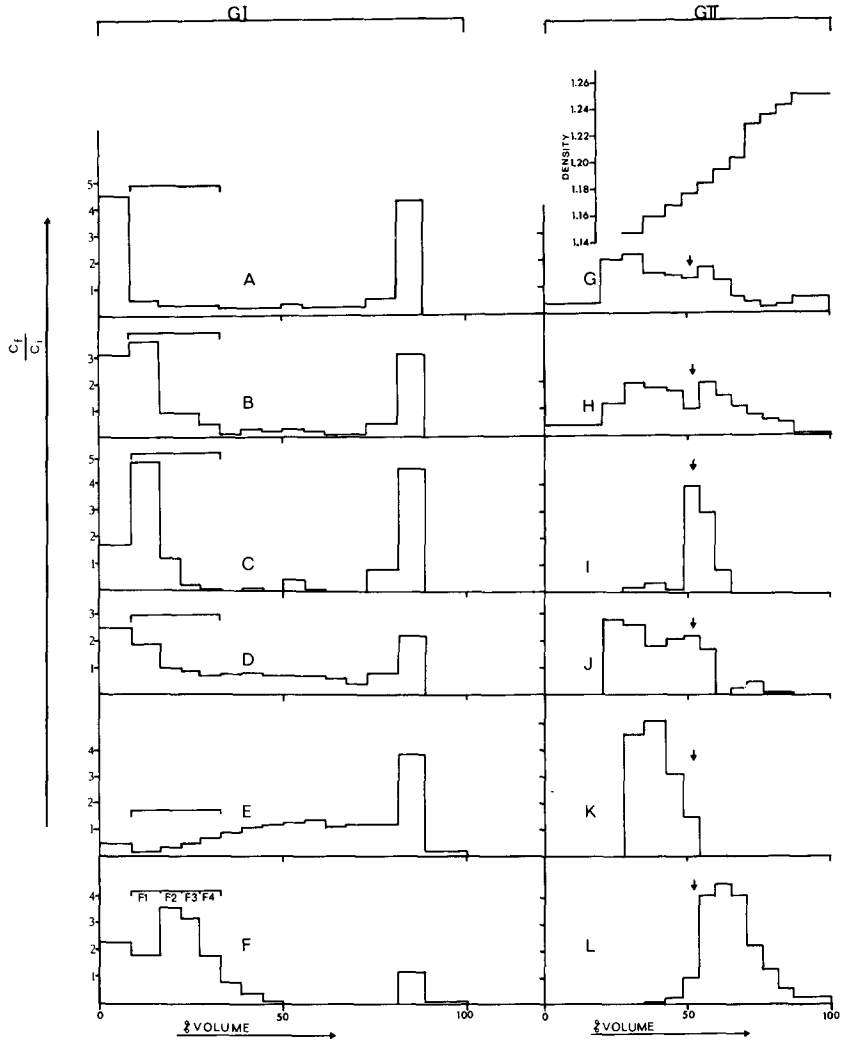


FIGURE 1 Distribution of marker enzymes after rate zonal centrifugation (G I) of homogenate followed by isopycnic centrifugation (G II) of the slow-moving particles (F1, F2, F3, and F4) from G I. 33 ml of homogenate was loaded onto 500 ml of linear gradient G I (20–35% wt/wt) resting on cushions of 20 ml 45% (wt/wt) and of 60% (wt/wt) sucrose. An overlay of 35 ml of 5% (wt/wt) sucrose was added and centrifugation was carried out at 16,000 rpm for 22 min. Aliquots of fractions F1, F2, F3, and F4 collected from G I were combined (total volume 80 ml) and loaded onto 400 ml of linear sucrose gradient G II (30–50% wt/wt) resting on a cushion of 60% (wt/wt) sucrose. An overlay of 35 ml of 5% (wt/wt) sucrose was added and centrifugation carried out at 30,000 rpm for 3 h. A and G = protein (87%), B and H = aryl esterase (118%), C and I = alkaline phosphatase (150%), D and J = acid phosphatase (112%), E and K = succinic dehydrogenase (96%), F and L = catalase (116%). $\frac{C_f}{C_i}$ = the concentration of the marker in the fraction divided by the concentration if it were evenly distributed throughout the gradient. The arrow indicates a density of 1.180 g/cm³ in gradient G II. Percentages refer to the overall recovery of markers relative to the original homogenate.

on density should give considerable resolution between catalase particles and other organelles.

The analysis of such a procedure is depicted in Fig. 1. Homogenate was first submitted to rate zonal centrifugation on a shallow sucrose gradient (G I) and fractions were analyzed for marker enzymes. Markers employed were succinic dehydrogenase for mitochondria, alkaline phosphatase for brush border fragments, acid phosphatase for lysosomes, and aryl esterase for microsomes and catalase. It can be seen that catalase particles have sedimented at a much slower rate than intact brush borders, mitochondria, and the bulk of the lysosomes, but they have sedimented just further into the gradient than microsomes and small brush border fragments. In this experiment fractions F1, F2, F3, and F4 were combined and submitted to isopycnic centrifugation on a second gradient (G II). As can be seen from Fig. 1, there is an efficient separation of catalase particles from the small mitochondria and lysosomes included in the payload. There is also good separation of catalase particles from contaminating microsomes; however, the marker for microsomes (esterase) shows a second peak in activity corresponding to the position of the catalase particles. We think that esterase activity at this density may be due to membranes of the endoplasmic reticulum (ER) which remain attached to the catalase particles, a condition which might be expected from the intimate morphological association of the two structures reported by Novikoff and Novikoff (10) who observed multiple membranous continuities between the SER and the "microperoxisomes." Alkaline phosphatase is also a significant contaminant of the catalase particles in this isopycnic gradient. If the distributions of alkaline phosphatase and catalase from G I are compared, it is evident that the bulk of the alkaline phosphatase in the payload for G II comes from the slower moving catalase-rich fraction (F1). Therefore, if centrifugation of gradient I were continued for a longer time, a more complete separation of catalase particles from brush border fragments might be achieved. That this is the case is shown in Fig. 2. Here the centrifugation was increased from 22 to

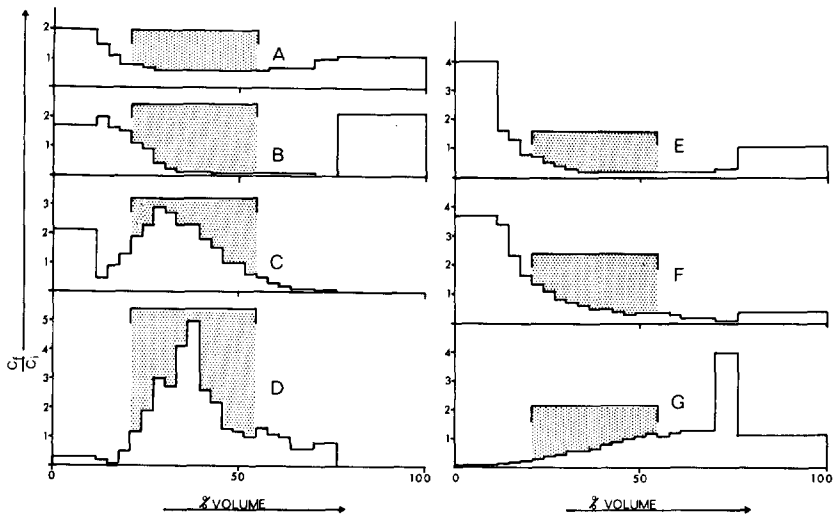


FIG. 2 Distribution of marker enzymes after rate zonal centrifugation of homogenate. 33 ml of homogenate was loaded onto 500 ml of linear sucrose gradient (20–35%) resting on a cushion of 60% sucrose. An overlay of 34 ml of 5% sucrose was added. Centrifugation was done at 16,000 rpm for 36 min. A = acid phosphatase (95%), B = alkaline phosphatase (84%), C = catalase (111%), D = D-amino acid oxidase (186%), E = protein (75%), F = aryl esterase (124%), G = succinic dehydrogenase (82%). $\frac{C_f}{C_i}$ as in Fig. 1. Percentages refer to the recovery of enzymes relative to the original homogenate. The fractions bracketed represent a suitable sample for subsequent isopycnic centrifugation; these fractions contain the following percentages of the total recovered activity of each marker: catalase 63%, D-amino acid oxidase 83%, alkaline phosphatase 10%, acid phosphatase 22%, aryl esterase 22%, succinic dehydrogenase 24%, and protein 10%.

36 min. If the catalase-rich fractions indicated in Fig. 2 were loaded onto the isopycnic gradient (G II), much of the brush border contamination of catalase particles occurring in G II would be avoided. Prolonging centrifugation to 36 min resulted in considerable impaction of fast-moving material against the cushion and rims of the rotor and made efficient unloading of the last fractions of the gradient impossible.

These results allowed the formulation of the purification scheme outlined in Fig. 3. Only the first 440 ml of the first gradient are unloaded. The rotor is then dismantled and its contents are collected; the inside of the two halves of the rotor are then thoroughly washed with ice-cold 10% sucrose so as to remove impacted material from the walls of the rotor, and these washings are also collected.

The results obtained with this purification procedure are given in Table I. The relative specific activity for catalase indicates a purification of 20- to 30-fold for the organelles carrying this enzyme. Table I shows that the major contaminants of the preparation are membrane fragments derived from the brush borders and the ER. In terms of protein, lysosomes (relative specific activity 2.0) would be a minor contaminant because of their relatively low numbers in these cells.

Electron microscopy of the catalase-rich fraction confirmed the biochemical findings. Fig. 4 illustrates that the major constituents of the preparation are small DAB-positive particles of a size corresponding to that reported by Novikoff and Novikoff (10) for microperoxisomes (0.15–0.25 nm). Occasional lysosome-like bodies and rare, extremely small mitochondria were also observed in the electron micrographs. Fig. 5 illustrates the lack of staining by DAB of these small mito-

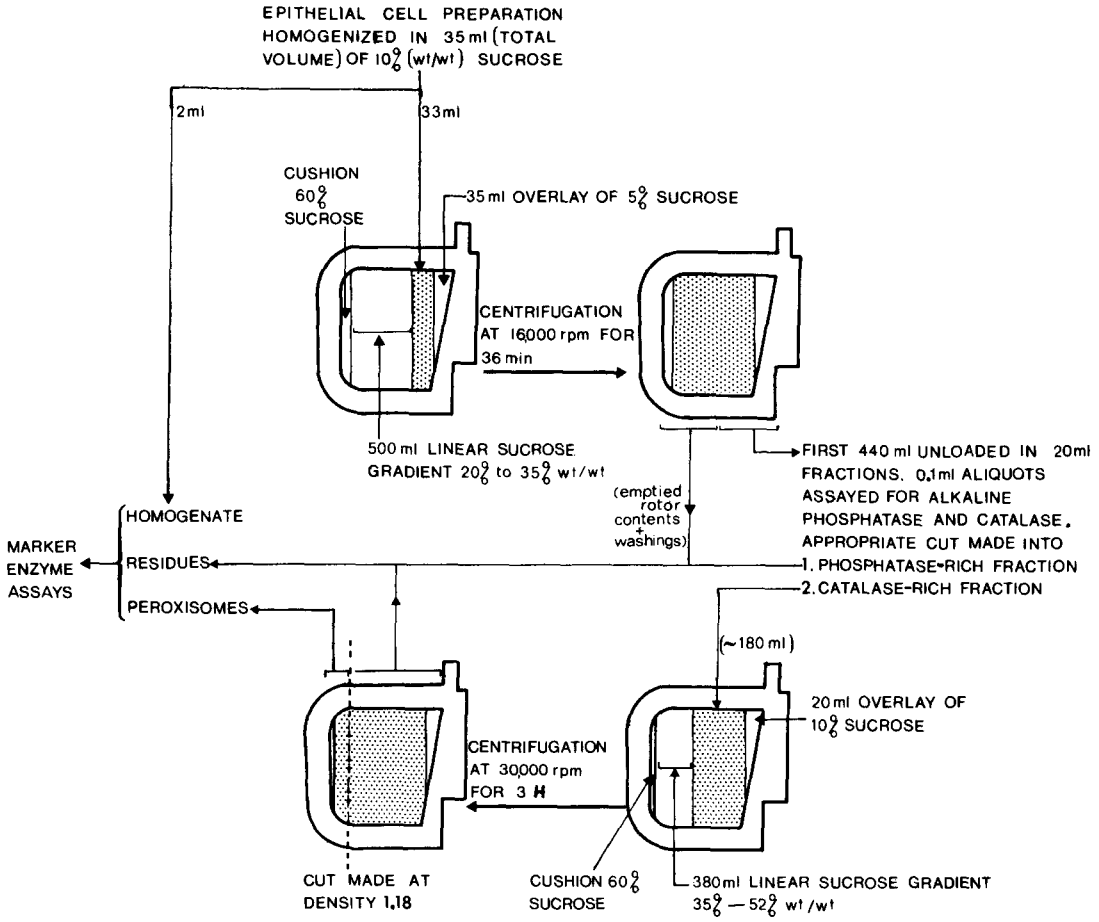


FIG. 3 Flow sheet for the preparation of catalase particles.

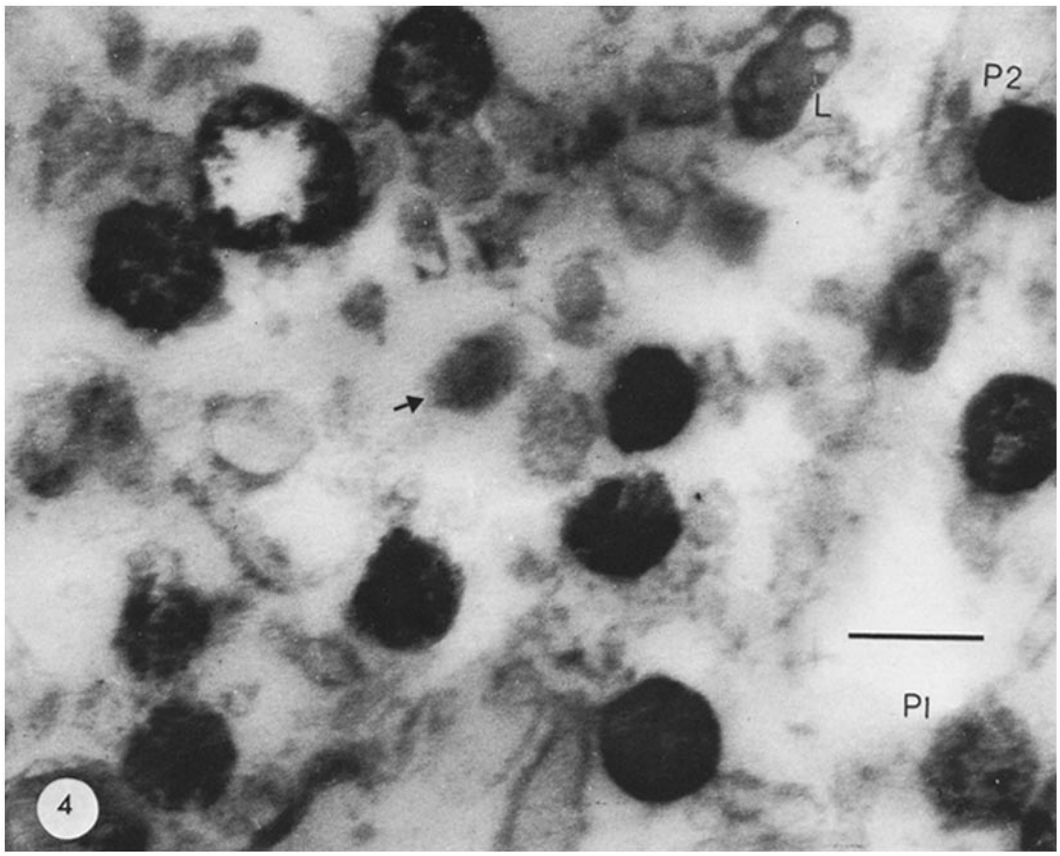


FIG. 4 Electron micrograph of catalase-rich preparation incubated 90 min at 37°C in DAB medium, pH 9.7. Note the presence of small DAB-positive microperoxisome-like particles P. The intensity of staining varies (compare P1 and P2). Note also the vacuolated lysosome-like particle L. Arrow indicates image of what is interpreted as microperoxisome which is weakly stained and/or cut peripherally. Scale = 0.2 μm. × 88,000.

TABLE I

Composition of Catalase Particle Fraction Prepared as Shown in Fig. 3.

Marker	Relative specific activity or percent in peroxisome fraction	Percent recovery
	%	%
Protein	1.16 (1.2–2.2)	91
Alkaline phosphatase	2.6 (2.0–3.3)	89
Acid phosphatase	2.0 (1.5–3.0)	102
Aryl esterase	4.0 (2.4–8.6)	93
Succinic dehydrogenase	0.2 (0–0.6)	101
Catalase	25.0 (19–33)	118

Figures quoted are the results of three preparations; those in parentheses give the range obtained.

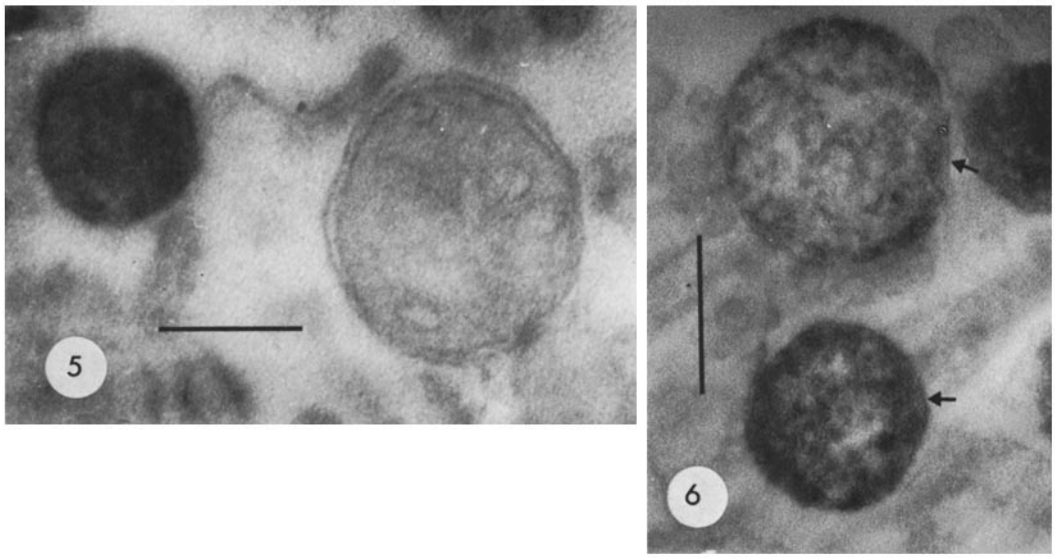


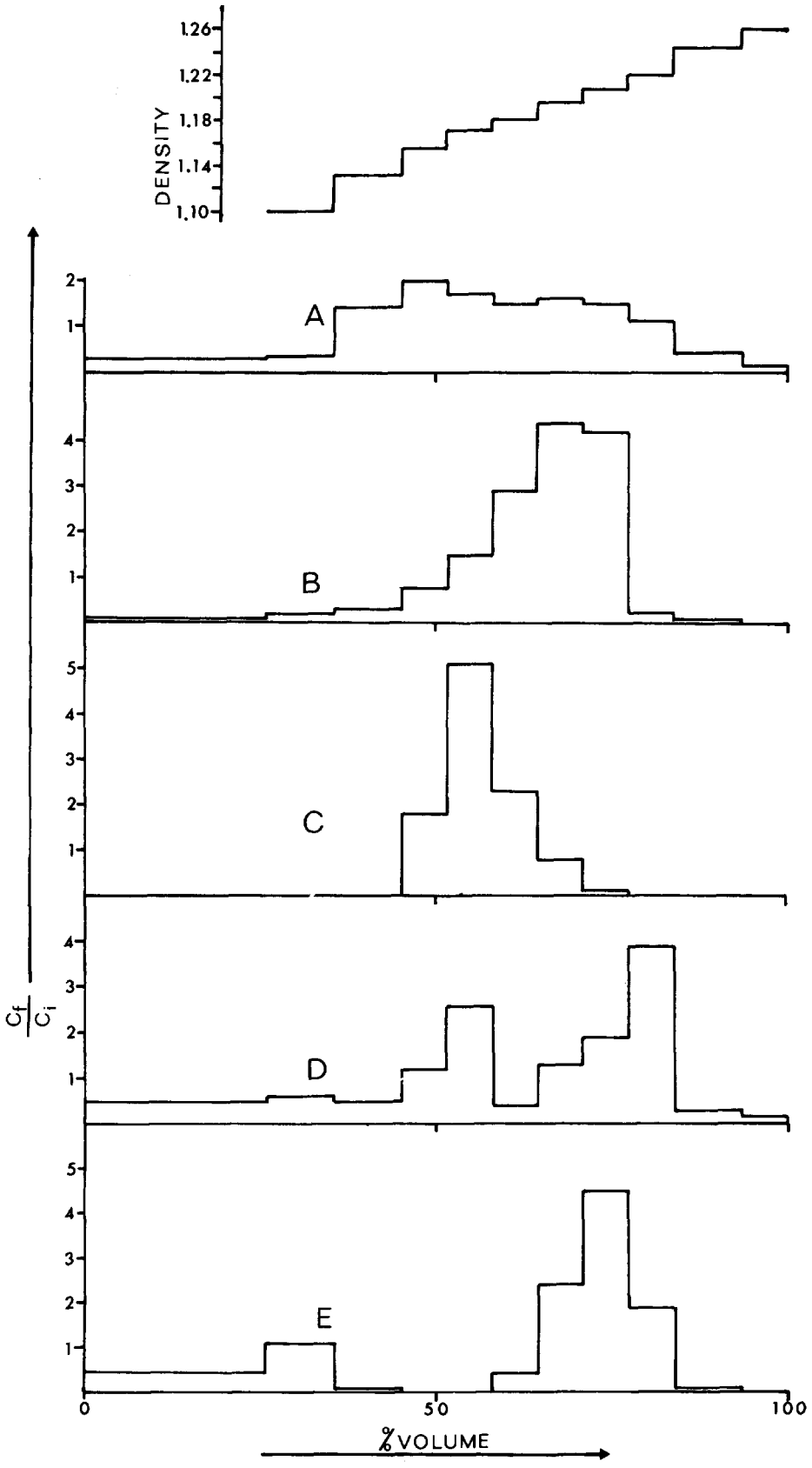
FIG. 5 As Fig. 4, showing an unstained small mitochondrion near a strongly stained microperoxisome. Scale = 0.2 μm. × 95,000.

FIG. 6 Catalase particles, incubated in DAB medium, showing regions (arrowed) with some indication of a surrounding membrane. Scale = 0.1 μm. × 210,000.

chondria. The optimum pH for staining mitochondria with DAB is reported to be around pH 7.4 (30), a much lower value than that employed with this preparation. Also seen in the DAB-stained material are images of what appear to be weakly stained and/or poorly resolved microperoxisomes.

With material incubated in the DAB medium there was only rarely any sign of a membrane surrounding the catalase particles (Fig. 6). Novikoff and Novikoff (10) have pointed out that the membranes associated with microperoxisomes are extremely tortuous and that this condition contributes to the frequent inability to resolve the tripartite nature of the membrane, and explains the apparent discontinuities in the membrane. The inability to find frequent catalase particle membranes in DAB-stained material is possibly due to the diffusion of oxidized DAB within the particle to positions very close to the membrane. Both oxidized DAB and the membrane are osmiophilic, so that after fixation it might be difficult to resolve the DAB deposit from the closely associated membrane. It has been shown by Novikoff et al. (31) that diffusion of DAB from highly reactive sites can indeed occur.

From Fig. 2 it can be seen that D-amino acid oxidase, like catalase, is bound to particles which sediment faster than the microsomes and small brush border fragments, but more slowly than the mitochondria and the bulk of the lysosomes. The basic similarity between this distribution and that of catalase indicates a possible peroxisomal nature for intestinal catalase particles. The distribution of catalase and D-amino acid oxidase after isopycnic centrifugation of postnuclear supernate is shown in Fig. 7. This supernate contains the bulk of all markers except alkaline phosphatase, about two-thirds of which is sedimented with the nuclei (8). It can be seen that both catalase and D-amino acid oxidase are bound to particles distributed at densities greater than 1.20 g/cm^3; however, particles carrying the oxidase have a greater modal density than those with catalase activity. In addition, a component with oxidase activity has a distribution corresponding to that for mitochondria. The interpretation of oxidase distributions is complicated by the fact that very long incubations were necessary for measuring this enzyme and by the high recoveries obtained relative to the activity of the original homogenate. However, catalase and oxidase particles do have a


DENSITY
1.26
1.22
1.18
1.14
1.10
A
B
C
D
E
$\frac{C_f}{C_i}$
0
50
100
% VOLUME

similar sedimentation rate and both have a density greater than 1.20 g/cm³ indicating that they may belong to a single class of particles which contains within it some heterogeneity.

DISCUSSION

Sedimentation and electron microscope data presented here indicate that biochemically studied catalase particles and electron microscopically defined microperoxisomes observed by Novikoff and Novikoff (10) have a common identity. The distributions of catalase and D-amino acid oxidase in rate zonal and isopycnic centrifugation experiments lend some support to the idea that these particles are peroxisomal in nature; however, further study of direct oxidases is required before this can be established unequivocally.

To date, there has been no published method for the purification of microperoxisomes, although Reddy and Svoboda (32) have suggested that Leydig cell tumors of rat testes would be a suitable source material. The method described here yields particles of sufficient purity and yield (about 5 mg protein) for biochemical studies to be carried out. It should be pointed out, however, that in comparison with the method of Leighton et al. (22) for the purification of rat hepatocyte peroxisomes, the yield of microperoxisome protein is many times smaller and contamination is significantly greater with the technique reported here. There are several factors, however, that warrant some application of the present method; first, intestinal microperoxisomes are isolated from normal animals, whereas the method of Leighton et al. (22) involves injecting the animal with the detergent Triton WR-1339, a chemical which is known to affect metabolism in ways which may be undesirable for particular biochemical studies. Second, the study of intestinal microperoxisomes offers the opportunity for observations on an extrahepatic tissue, a necessity in determining any tissue differences in the function of mammalian peroxisomes. It is noteworthy that, so far, the study of peroxisomes from a wide variety of plant and animal sources has indicated diversity rather than conformity in enzymic equipment. Third, to determine whether the morphological variations between microperoxisomes and the large peroxisomes of liver and kidney reflect any diversity in function, preparations of microperoxisomes suitable for biochemical studies will be necessary. It is relevant here to point out that up to 25% of hepatocyte peroxisomal protein may be derived from NADP-linked isocitrate dehydrogenase (33); in preliminary studies, we have been unable to detect this enzyme in intestinal microperoxisomes; all the activity of the homogenate is accounted for by the mitochondria with little or no activity in the supernate.

In the present series of experiments, we have observed that between 35 and 45% of the catalase activity of the homogenate is not sedimentable. This is significantly more than that observed in previous experiments where only about 10% of the catalase was nonparticulate. Peters (34) found that in his homogenate of guinea pig small intestine more than 50% of the recovered catalase activity was soluble and appeared in the postmicrosomal supernate. We think that these results may indicate differences between strains of guinea pig or, more probably, the sensitivity of microperoxisomes to damage during homogenization (the present experiments employed a different homogenizer from that used previously). In common with many other cell suspensions, epithelial cells are more difficult to homogenize than the solid tissue. A possible improvement in technique could be the application of the homogenization procedure suggested by Dingle (35). In this procedure the cell suspension is embedded in gelatin which allows greater binding between the pestle and mortar of the homogenizer, thus permitting

FIG. 7 Distribution of marker enzymes after isopycnic centrifugation of postnuclear supernate (PNS). Homogenate was centrifuged at 10^4 *g*/min in an 8 × 50 angled rotor. The sediment was resuspended and centrifugation at 10^4 *g*/min was repeated. Supernates from the two spins were combined and labeled PNS. 60 ml of PNS was loaded onto 200 ml of linear sucrose gradient (30–50% wt/wt) resting on a cushion of 60% sucrose. An overlay of 250 ml of 5% sucrose was added and centrifugation was carried out at 30,000 rpm for 4 h. A = aryl esterase (98%), B = alkaline phosphatase (104%), C = succinic dehydrogenase (66%), D = D-amino acid oxidase (179%), E = catalase (120%). $\frac{C_f}{C_i}$ = as Fig. 1. Percentages refer to the recovery of enzymes relative to the PNS.

gentler forces to be employed for a given degree of cell disruption. Although the use of epithelial cell preparations makes homogenization difficult, we think that the benefits of such preparations greatly outweigh the disadvantages of contamination inevitable when intestinal scrapes are employed.

The choice of species for the preparation of intestinal microperoxisomes may be important. Fractionation of small intestinal homogenates of the rat is complicated by the presence of mucus (25) which binds subcellular particles together. In addition, the catalase particles from this tissue were found by Peters (36) to have a relatively low equilibrium density in sucrose density gradients, and were inseparable from both lysosomes and mitochondria. These factors make it unlikely that microperoxisomes could be purified from the rat enterocyte using the method reported here.

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