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Peroxisome-Associated Aldehyde Dehydrogenase in Normal Rat Liver

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SMITH, H. E. AND R. LINDAHL. *Peroxisome-associated aldehyde dehydrogenase in normal rat liver.* PHARMAC. BIOCHEM. BEHAV. 13: Suppl. 1, 111–118, 1980.—We have combined subcellular fractionation and cytochemical staining techniques to study the distribution of aldehyde dehydrogenase in rat liver. In addition to confirming the mitochondrial and microsomal localization of aldehyde dehydrogenase, this combined approach has allowed us to demonstrate that peroxisome-like organelles possess significant aldehyde dehydrogenase. When peroxisomal fractions are cytochemically stained for aldehyde dehydrogenase, activity is observed along membranes of structures resembling peroxisomal ghosts. These bodies lack a matrix but many appear to enclose peroxisomal cores. Moderate to dense reaction product is also located in single membrane-limited structures present in fractions containing morphologically recognizable peroxisomes. On occasion, the osmiophilic precipitate is also present in the matrix of intact peroxisomes. The aldehyde dehydrogenase activity in these peroxisome-like organelles prefers aliphatic aldehydes, including acetaldehyde in both millimolar and micromolar concentrations, and NAD. Aromatic aldehydes and NADP are also metabolized, but to a lesser extent. These results indicate that peroxisome-like organelles contain an aldehyde dehydrogenase activity possessing properties compatible with a role in ethanol metabolism.

Aldehyde dehydrogenase Cytochemistry Peroxisomes Mitochondria

IN normal rat liver, at least three aldehyde dehydrogenase isozymes (aldehyde: NAD(P) oxidoreductase, E. C. 1.2.1.3 and 1.2.1.5, AlDH) can be identified [3, 5, 8, 14, 16]. These isozymes are differentially distributed among the mitochondria, microsomes and cytosol. Two mitochondrial aldehyde dehydrogenases can be distinguished due to differences in their affinity for acetaldehyde as substrate (K_m of approx. 1 μM vs. approx. 1 mM) [3, 5, 8, 14, 16]. Both isozymes prefer aliphatic aldehyde substrates and NAD as coenzyme. An additional isozyme, preferring aromatic aldehydes and NADP may also be present in mitochondria [8,14]. Two aldehyde dehydrogenases with substrate K_ms in the millimolar range are demonstrable in microsomes [8,16]. One isozyme prefers aliphatic substrates and NAD; the other prefers aromatic aldehydes and NADP. Microsomes contain virtually no aldehyde dehydrogenase activity with micromolar K_m for substrate. Occasionally, small amounts of aldehyde dehydrogenase with properties identical to those of mitochondria and/or microsomes can be demonstrated in rat liver cytosol [3, 5, 8, 16]. As only mitochondria possess significant aldehyde dehydrogenase activity with the appropriate substrate affinity (μM K_m for acetaldehyde), it has generally been accepted that the mitochondria are responsible for the oxidation of acetaldehyde during ethanol metabolism [4, 12, 14]. The physiological role(s) of the remaining mitochondrial and the two microsomal aldehyde dehydrogenases remain to be elucidated.

We wished to verify cytochemically the subcellular distribution of aldehyde dehydrogenase indicated by subcellular fractionation studies. In addition to confirming the mitochondrial and microsomal localization of the enzyme, our initial studies indicated that single membrane bound, peroxisome-like organelles possessed significant activity. To determine if these organelles are peroxisomes, we have isolated peroxisomes by differential centrifugation. We report here that organelles morphologically and biochemically identifiable as peroxisomes possess significant aldehyde dehydrogenase activity.

METHOD

Animals

Livers were obtained from adult male or female Sprague-Dawley rats starved for 24 hr prior to sacrifice by cervical dislocation. Three days prior to sacrifice, animals were injected IP with 1.0 ml/100 gm body weight 8.5% Triton WR-1339 in 0.9% saline. Except where noted, all subsequent procedures were performed at 0–4°C.

Tissue Preparation

A 5 to 6 g portion of liver was homogenized 6 to 8 strokes at 1,100 rpm in 20 ml of 20 mM glycylglycine buffer, pH 7.5, containing 0.25 M sucrose (hereafter referred to as buffered sucrose) in a loose-fitting Potter-Elvehjem tissue homogenizer. The homogenate was then made to 10% (w/v) by adding buffered sucrose. Nuclei and debris were removed by centrifugation at 400 g_{max} for 10 min, the supernatant

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drawn off and the pellet washed in 20 ml of buffered sucrose. The pooled supernatant, the cytoplasmic extract, was centrifuged at 19,000 g_{max} for 10 min. The supernatant was discarded and the pellet, containing mitochondria, lysosomes and peroxisomes, was washed with 2×20 ml of buffered sucrose. The final washed pellet was resuspended at 0.4 g liver/ml in 8% sucrose in 20 mM glycylglycine, pH 7.5. This preparation was further fractionated by sucrose-density gradient centrifugation.

Sucrose-Density Gradient Centrifugation

Discontinuous sucrose gradients were prepared using the methods of Vandor and Tolbert [17], modified as described below. Thirty-three milliliter, 7 layer gradients were prepared in 20 mM glycylglycine, pH 7.5, containing the following concentrations (w/w) and volumes of sucrose solutions: 3 ml of 55%, 6 ml of 53%, 6 ml of 50%, 6 ml of 47%, 3 ml of 40% and 3 ml of 33%. The gradients were overlayered with 1.5 ml of resuspended 19,000 g_{max} pellet and the gradients centrifuged at 24,000 rpm (48,500 to 103,900 g) for 5 hr in an SW 27 rotor. Fractions of 2.5 ml each were collected from the bottom of the gradients. Occasionally, corresponding fractions from 2 or more gradients were pooled to provide sufficient material for further analysis. For cytochemistry, pooled fractions from two gradients were diluted to 10 ml with 0.25 M buffered sucrose and centrifuged at 40,000 g_{max} to produce pellets which were then treated as tissue pieces during subsequent operations. Fractionated gradients for enzymatic characterization were frozen at −70°C until needed.

Cytochemistry

For localization of aldehyde dehydrogenase, pellets were incubated for 60 min at 37°C in a medium containing 100 mM propionaldehyde, 7.5 mM NAD, 250 mM sucrose, 4% polyvinylpyrrolidone (PVP) and 0.05% thiocarbamyl-nitroblue tetrazolium (TC-NBT) in 50 mM phosphate buffer, pH 8.0. Pellets used as controls were incubated in substrate-free medium. After incubation the pellets were washed twice in cold 50 mM phosphate buffer containing sucrose and PVP for a total of 10 min and fixed in 2% unbuffered osmium tetroxide at 60°C for 30 or 60 min [1,13]. The samples were then washed, dehydrated in a graded ethanol series and embedded in Spurr's low viscosity resin [15]. Sections were cut with a diamond knife on an LKB-Ultratome and examined in a Zeiss EM10A electron microscope without post-staining.

Catalase was localized by the method of LeHir *et al.* [6]. Pellets were fixed for 15 min at 4°C in 1% glutaraldehyde in 100 mM phosphate buffer, pH 7.4. Samples were washed for 40 min in 3 changes of phosphate buffer and 10 min in 100 mM Tris buffer, pH 8.5. Pellets were incubated for 30 min at 37°C in a medium containing 0.15% H_2O_2, and 0.2% 3,3'-diaminobenzidine (DAB) in 100 mM Tris buffer, pH 8.5. After incubation, the pellets were washed in phosphate buffer, post-fixed in phosphate buffered 1% osmium tetroxide for 30 min and processed for electron microscopy by the methods previously described for aldehyde dehydrogenase.

Enzyme Assays

For enzyme assays, fractions were thawed and made to 0.25% with Triton X-100, incubated for 30 min and centrifuged at 48,000 g_{max} for 15 min to remove any debris. The resulting supernatants were used as enzyme source. All assays were performed at 23–25°C unless otherwise required. Aldehyde dehydrogenase activity was determined with a variety of substrates as previously described [8]. Monoamine oxidase, acid phosphatase, NADPH-cytochrome c reductase and alcohol dehydrogenase were assayed as described by Tottmar *et al.* [16]. Catalase was determined by measuring the disappearance of H_2O_2 spectrophotometrically at 240 nm [10]. Urate oxidase was determined by monitoring the oxidation of uric acid at 292 nm [7]. Succinate dehydrogenase was determined by the reduction of dichlorophenol-indophenol at 600 nm in the presence of sodium succinate [11]. Activities are expressed in munits (1 unit=1 μmole/min)/mg protein. Protein concentrations were determined by the method of Lowry *et al.* [19], using bovine serum albumin as standard.

TABLE 1

ENZYME ACTIVITIES IN 19,000 g_{max} PELLET OF RAT LIVER

Enzyme	Specific Activity*
Aldehyde Dehydrogenase	
Propionaldehyde-NAD (8)†	58.0 ± 5.4
Benzaldehyde-NADP (7)	12.4 ± 1.2
Acetaldehyde-NAD 5 mM (6)	29.6 ± 2.9
Acetaldehyde-NAD 5 μM (6)	0.9 ± 0.2
Catalase (8)	356.3 ± 45.2
Urate Oxidase (3)	0.3 ± 0.0
Monoamine Oxidase (6)	5.3 ± 1.1
Succinate Dehydrogenase (2)	29.3
Acid Phosphatase (2)	10.2
NADPH-Cytochrome c Reductase (2)	17.8
Alcohol Dehydrogenase (3)	1.2 ± 0.4
Protein (8)	8.7 ± 1.5 mg/ml

*mU/mg protein.
†No. of Determinations.
Values represent mean ± S.E.M.

RESULTS

The 19,000 g_{max} pellet from the initial fractionation of liver homogenates from Triton WR-1339-treated rats is composed primarily of mitochondria, but peroxisomes, peroxisomal cores and some lysosomes are also present (Table 1 and Fig. 1). These observations are consistent with previous studies which showed by marker enzyme distributions that the 19,000 g_{max} pellet from untreated rat liver homogenates is enriched with mitochondria and lysosomes [8]. This fraction is effectively separated into peroxisomes, mitochondria and lysosomes by sucrose-density gradient centrifugation as indicated by both marker enzyme distributions (Fig. 2), and electron microscopy (Figs. 3–10).

Based on marker enzyme distributions, aldehyde dehydrogenase is associated with both mitochondria and peroxisomes (Fig. 2). When expressed as total activity per fraction (mIU/ml), the distribution of aldehyde dehydrogenase throughout the gradient is bimodal for all substrates and coenzymes examined. The major peak (approx. 80%) is associated with mitochondria and a second smaller peak (approx. 12%) is associated with peroxisomes (data not shown). When expressed as specific activity (mIU/mg protein), the

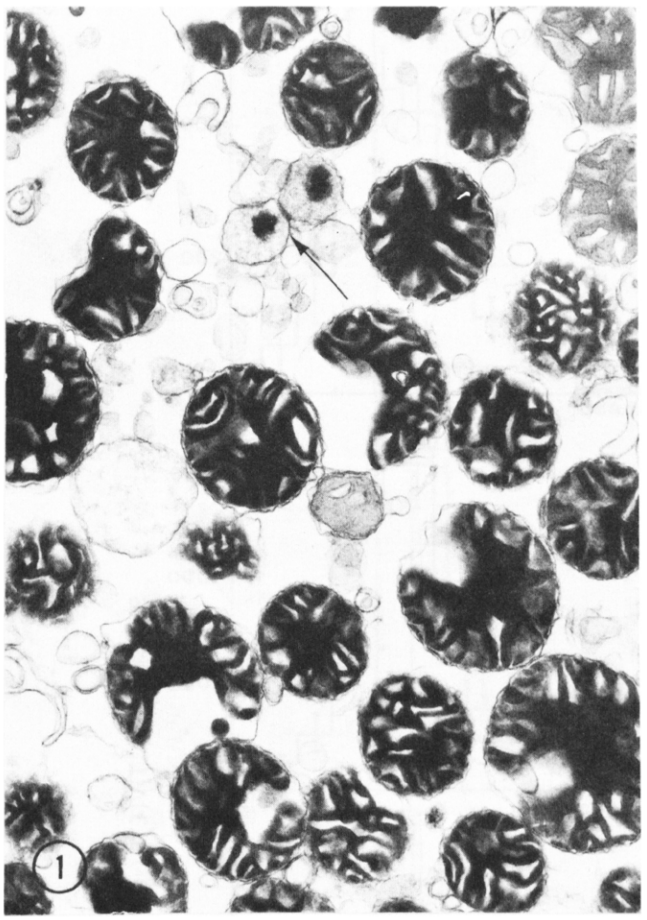

FIG. 1. Electron micrograph of section stained with uranyl acetate and lead citrate illustrating the composition of the 19,000 g_{max} pellet. Mitochondria make up the largest portion of the fraction but peroxisomes (arrow) are also present. The pellet was fixed in 2.5% buffered glutaraldehyde and 2% buffered osmium tetroxide. ×17,700.

distribution of aldehyde dehydrogenase changes and a single peak of activity is associated with the peroxisomal marker enzymes catalase and urate oxidase (Fig. 2A). This is especially apparent when acetaldehyde at either millimolar or micromolar concentrations is substrate. With propionaldehyde and NAD or benzaldehyde and NADP, aldehyde dehydrogenase activity is also detectable in regions of the gradient not clearly associated with any marker enzyme (Fig. 2).

In addition to its affinity for acetaldehyde as substrate, peroxisome-associated aldehyde dehydrogenase is more sensitive to disulfiram *in vitro* than the activity associated with mitochondria. At millimolar substrate concentrations and with NAD as coenzyme, mitochondrial aldehyde dehydrogenase activity is reduced nearly 50% by 100 μM disulfiram. Under the same conditions, peroxisomal aldehyde dehydrogenase activity is reduced almost 80%. At μM substrate concentrations, both mitochondria and peroxisomal aldehyde dehydrogenase activities are reduced approx. 90% by 100 μM disulfiram.

When pellets from peak peroxisomal fractions are cytochemically stained for aldehyde dehydrogenase, the activity is localized within structures limited by a single membrane (Fig. 3–4). Occasionally, reaction product is also present in the matrix of intact peroxisomes with recognizable cores (Fig. 3). The reaction product is also observed along the membranes of structures which lack a matrix but which appear to possess peroxisomal cores (Fig. 4). Control pellets incubated without propionaldehyde contain little, if any osmiophilic precipitate (Fig. 5). In order to compare the structures which show aldehyde dehydrogenase activity with peroxisomes, fractions were cytochemically stained for catalase. Both peroxisomes with cores and single membrane-bound structures without visible cores, but which show catalase activity, are present (Fig. 6).

Mitochondrial fractions cytochemically stained for aldehyde dehydrogenase contain activity in mitochondria and in vesicles bounded by a single membrane (Fig. 7–8). The mitochondrial staining obtained with this method occurs in localized regions of the mitochondrial matrix between the inner membrane and the adjacent cristae. Some generalized staining does occur in control pellets incubated without propionaldehyde, but the dense localized reaction product is absent from the mitochondria (Fig. 9). Sections from pellets cytochemically stained for catalase show that this fraction is almost exclusively mitochondria. No catalase-containing structures are observed (Fig. 10).

DISCUSSION

By combining subcellular fractionation and enzyme cytochemical techniques, we have demonstrated that organelles in rat liver biochemically and morphologically identified as peroxisomes possess significant aldehyde dehydrogenase activity. The substrate and coenzyme preferences and disulfiram-sensitivity studies suggest that peroxisomal aldehyde dehydrogenase possesses characteristics compatible with a role in ethanol metabolism.

To date, studies on the subcellular distribution of liver aldehyde dehydrogenase have employed tissues fractionated according to the principles of de Duve *et al.* [2]. Using these methods, it is possible to achieve good separation of cytosol and microsomes, but the mitochondrial and lysosomal fractions are often contaminated with each other and peroxisomes co-sediment in both fractions. Thus most, if not all, previous studies of the subcellular distribution of mammalian aldehyde dehydrogenase have examined very heterogeneous organelle populations. One effective way to isolate lysosomes, mitochondria and peroxisomes in suitably pure form is to pretreat animals with Triton WR-1339 to alter the density of lysosomes and then separate the organelles using sucrose gradients [7,17]. Using this method, we have achieved an effective separation of these organelles with less than 3% peroxisomes contaminating the mitochondria, less than 5% contamination of peroxisomes with mitochondria and less than 4% contamination of mitochondria and peroxisomes with lysosomes. These observations are confirmed by electron microscopic examination of the appropriate peak fractions.

Because of their intrinsic fragility and sensitivity to slight changes in osmolarity, the preservation of peroxisomes following cytochemical staining has been less than optimal. Many of these organelles appear to be peroxisomal ghosts resulting from damage during preparation [7]. However, the distribution of the reaction product within intact peroxi-

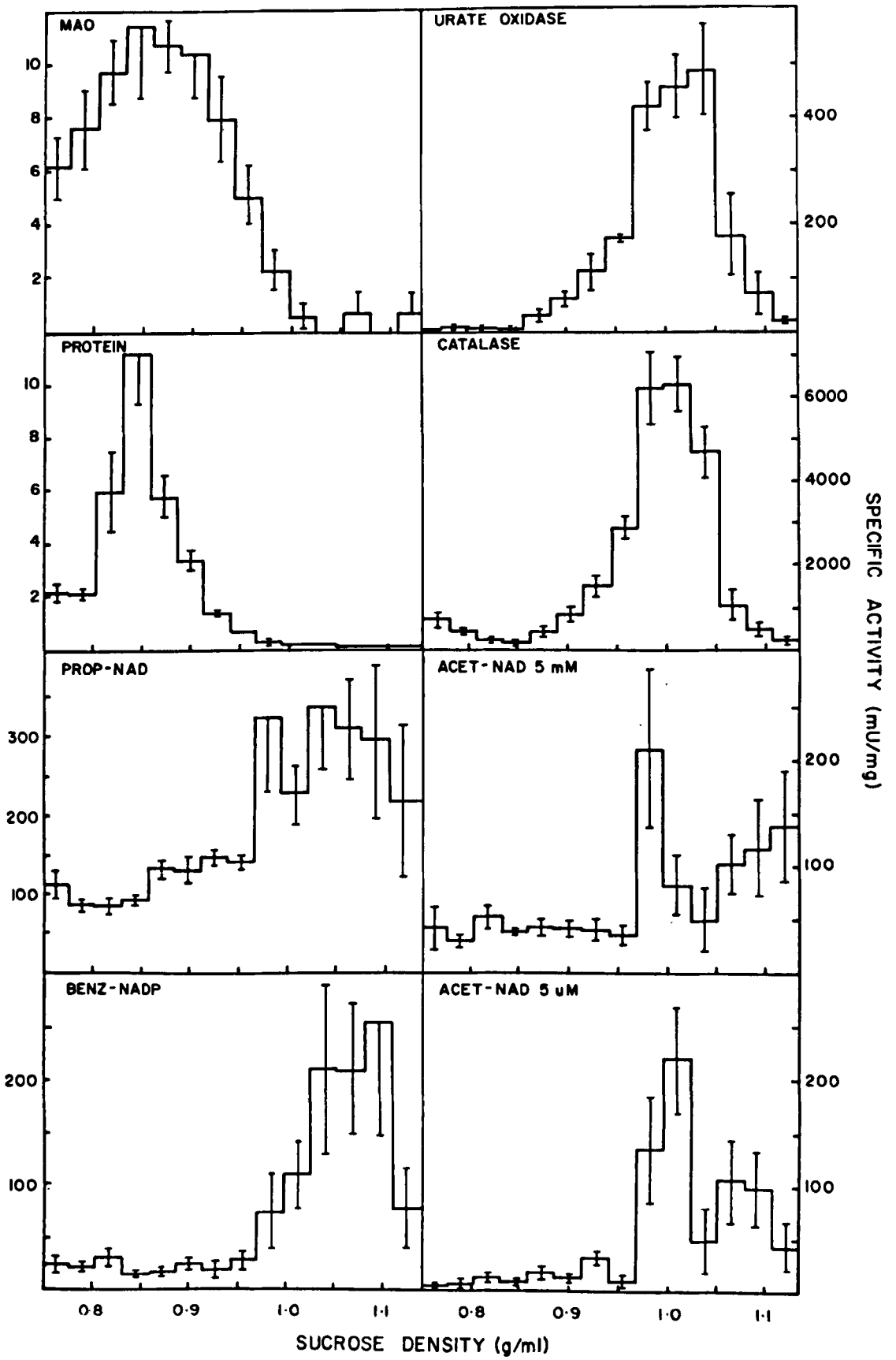

FIG. 2. Enzyme activity determinations after sucrose density gradient separation of peroxisomes, mitochondria, and lysosomes in 19,000 g_{max} pellet. FIG. 2A. Distribution of monamine oxidase (MAO), urate oxidase, protein, catalase, and aldehyde dehydrogenase with propionaldehyde and NAD (PROP-NAD), benzaldehyde and NADP (BENZ-NADP), and acetaldehyde and NAD at both 5 mM (ACET-NAD 5mM) and 5 μM (ACET-NAD 5 μM) final concentrations of substrate.

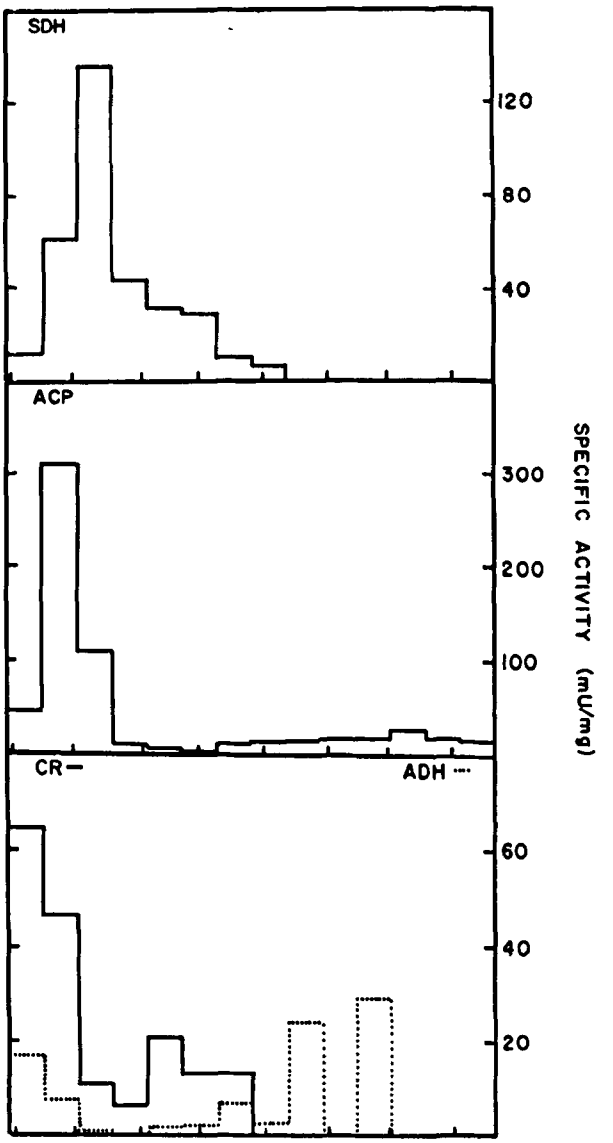

FIG. 2B. Succinate dehydrogenase (SDH), acid phosphatase (ACP), NADH cytochrome C reductase (CR), and alcohol dehydrogenase (ADH).

somes and the dense osmiophilic product throughout the interior of single membrane-limited structures in the peak peroxisomal fractions indicate that aldehyde dehydrogenase activity could be located in the matrix. Comparison of these latter structures with organelles showing catalase activity but which lack cores, the number of such structures present in the peak fractions, and the distribution of marker enzymes suggest that these structures are peroxisomes, although no cores are visible. Currently, we are attempting to achieve better preservation of peroxisomes and to accurately determine the location of the enzyme activity.

At present, we have succeeded in demonstrating aldehyde dehydrogenase activity within the mitochondria to localized regions of the matrix just below the inner mitochondrial membrane. Whether this unusual pattern of staining indicates a restriction of activity to a limited region of the mitochondria or whether this result is due to the technique remains to be determined. However, the presence of reaction product in this region of the mitochondrion confirms the work of Siew *et al.* [14], who postulated from marker enzyme release data that the micromolar K_m mitochondrial aldehyde dehydrogenase is located within the matrix. We have not yet found reaction product in the intermembrane space, the location postulated by Siew *et al.* [14] for the millimolar K_m aldehvde dehydrogenase.

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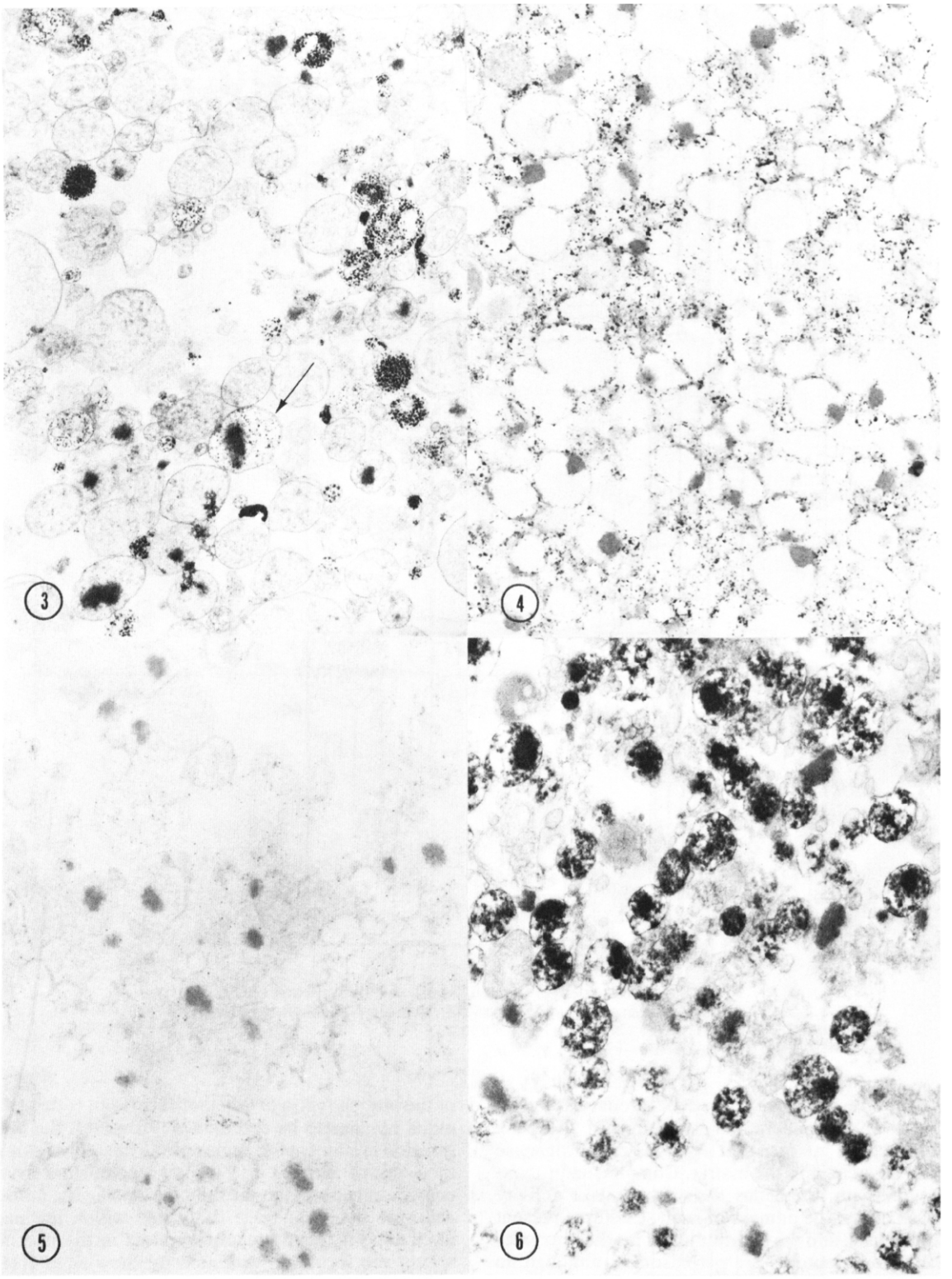
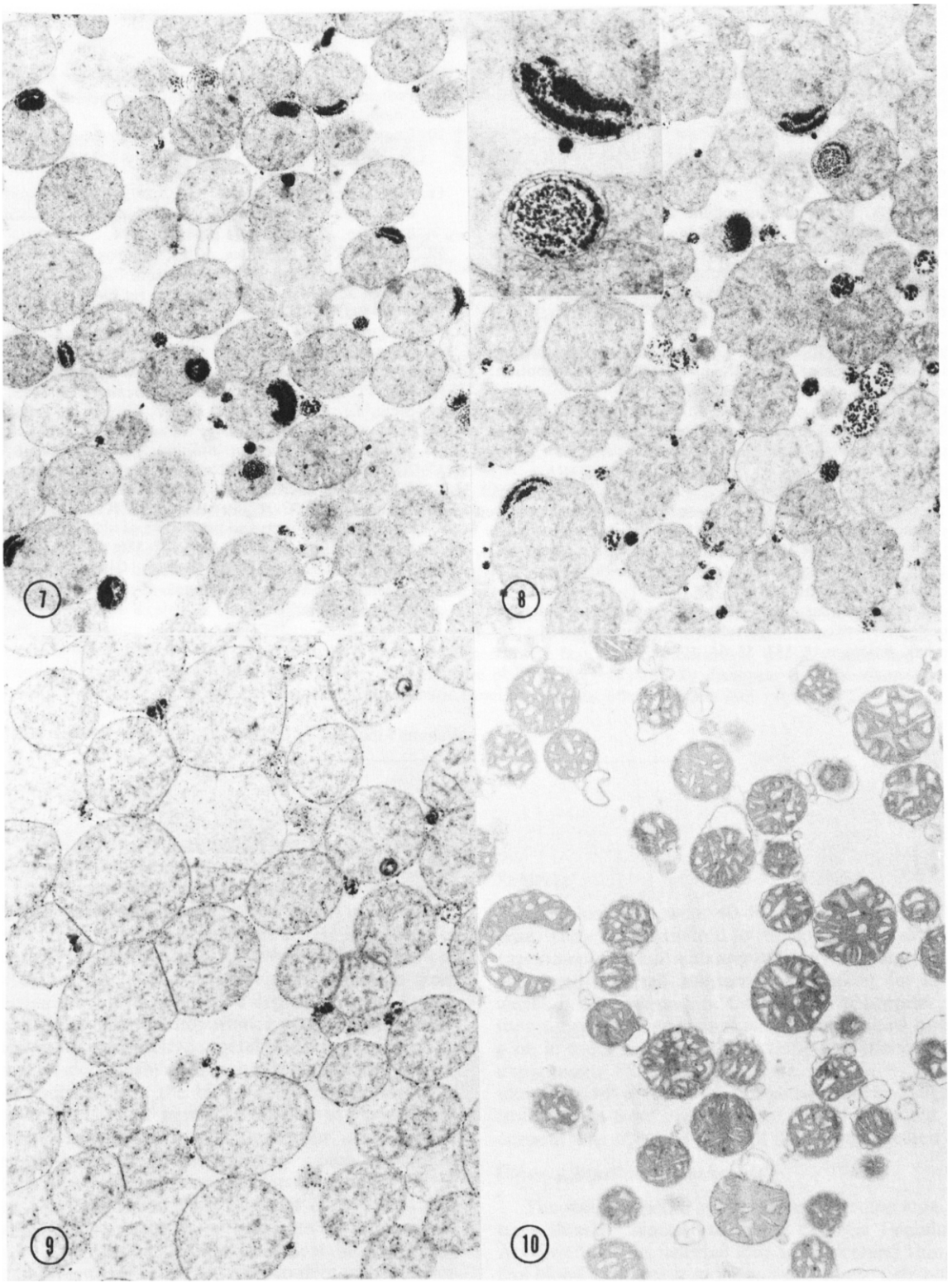


FIGS. 3–4. Electron micrographs of sectioned pellets from the two peak peroxisomal fractions (Fig. 2A, catalase) cytochemically stained for aldehyde dehydrogenase. The activity is present in structures limited by a single membrane, in some morphologically recognizable peroxisomes (Fig. 3, arrow) and along membranes of structures which lack a matrix but which often appear to contain peroxisomal cores (Fig. 4), ×15,045.

FIG. 5. Section of pellet from peak peroxisomal fraction incubated without propionaldehyde. The control pellets contain very little osmiophilic precipitate. ×15,045.

FIG. 6. Section of pellet from peak peroxisomal fraction cytochemically stained for catalase. This pellet contains both peroxisomes with cores and structures without visible cores which show catalase activity. ×15,045.



FIGS. 7–8. Electron micrographs from the two peak mitochondrial fractions (Fig. 2A, monoamine oxidase) cytochemically stained for aldehyde dehydrogenase. The activity is restricted to localized regions of mitochondria and in vesicles bounded by a single membrane. The reaction product in the mitochondria is located in the matrix between the inner membrane and adjacent cristae (Fig. 8, insert). ×17,700. Insert×35,700.

FIG. 9. Section of control pellet from peak mitochondrial fraction incubated without propionaldehyde. Some generalized staining does occur, but the dense, localized reaction product is absent from the mitochondria. ×15,045.

FIG. 10. Section of pellet from peak mitochondrial fraction cytochemically stained for catalase. This fraction is composed almost exclusively of mitochondria. No structures with catalase activity are observed. ×15,045.

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