

# L-Lactate Dehydrogenase A<sub>4</sub>- and A<sub>3</sub>B Isoforms Are Bona Fide Peroxisomal Enzymes in Rat Liver

EVIDENCE FOR INVOLVEMENT IN INTRAPEROXISOMAL NADH REOXIDATION\*

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Eveline Baumgart<sup>‡</sup>, H. Dariush Fahimi, Andrea Stich, and Alfred Völkl<sup>§</sup>

From the Institute for Anatomy and Cell Biology II, University of Heidelberg, Im Neuenheimer Feld 307, 69120 Heidelberg, Federal Republic of Germany

The subcellular localization of L-lactate dehydrogenase (LDH) in rat hepatocytes has been studied by analytical subcellular fractionation combined with the immunodetection of LDH in isolated subcellular fractions and liver sections by immunoblotting and immunoelectron microscopy. The results clearly demonstrate the presence of LDH in the matrix of peroxisomes in addition to the cytosol. Both cytosolic and peroxisomal LDH subunits have the same molecular mass (35.0 kDa) and show comparable cross-reactivity with an anti-cytosolic LDH antibody. As revealed by activity staining or immunoblotting after isoelectric focussing, both intracellular compartments contain the same liver-specific LDH-isoforms (LDH-A<sub>4</sub> > LDH-A<sub>3</sub>B) with the peroxisomes comprising relatively more LDH-A<sub>3</sub>B than the cytosol. Selective KCl extraction as well as resistance to proteinase K and immunoelectron microscopy revealed that at least 80% of the LDH activity measured in highly purified peroxisomal fractions is due to LDH as a bona fide peroxisomal matrix enzyme. In combination with the data of cell fractionation, this implies that at least 0.5% of the total LDH activity in hepatocytes is present in peroxisomes. Since no other enzymes of the glycolytic pathway (such as phosphoglucosomutase, phosphoglucosomerase, and glyceraldehyde-3-phosphate dehydrogenase) were found in highly purified peroxisomal fractions, it does not seem that LDH in peroxisomes participates in glycolysis. Instead, the marked elevation of LDH in peroxisomes of rats treated with the hypolipidemic drug bezafibrate, concomitantly to the induction of the peroxisomal  $\beta$ -oxidation enzymes, strongly suggests that intraperoxisomal LDH may be involved in the reoxidation of NADH generated by the  $\beta$ -oxidation pathway. The interaction of LDH and the peroxisomal palmitoyl-CoA  $\beta$ -oxidation system could be verified in a modified  $\beta$ -oxidation assay by adding increasing amounts of pyruvate to the standard assay mixture and recording the change of NADH production rates. A dose-dependent decrease of NADH produced was simulated with the lowest NADH value found at maximal LDH activity. The addition of oxamic acid, a specific inhibitor of LDH, to the system or inhibition of LDH by high pyruvate levels (up to 20 mM) restored the NADH values to control levels.

A direct effect of pyruvate on palmitoyl-CoA oxidase and enoyl-CoA hydratase was excluded by measuring those enzymes individually in separate assays. An LDH-based shuttle across the peroxisomal membrane should provide an efficient system to regulate intraperoxisomal NAD<sup>+</sup>/NADH levels and maintain the flux of fatty acids through the peroxisomal  $\beta$ -oxidation spiral.

Lactate dehydrogenase (L-lactate:NAD<sup>+</sup> oxidoreductase (LDH);<sup>1</sup> EC 1.1.1.27) is a tetrameric protein catalyzing the reversible conversion of pyruvate to lactate. The enzyme uses NAD<sup>+</sup>/NADH as cofactor and exists in six isoforms, five depending on the combination of the two subunits A (muscle) and B (heart), each with a molecular mass of 35 kDa (1) and an additional homotetrameric LDH-C<sub>4</sub> (testis)-isoform (2, 3). The three different subunits (A, B, and C) are encoded by three structural genes, which most probably originated from a common ancestral gene during evolution. The expression of the LDH genes is developmentally regulated and tissue-specific (4). The liver-specific isoforms are LDH-A<sub>4</sub> and LDH-A<sub>3</sub>B, which are mainly localized to the cytoplasm of hepatocytes. There are several reports, however, on the localization of LDH in other cell compartments. Whereas it is now generally accepted that the tyrosine-phosphorylated LDH-A<sub>4</sub> is localized to the nucleus and functions as a single-stranded DNA-binding protein (5, 6), the debate on the presence of LDH as a bona fide enzyme in other cell organelles has not been settled (7–9).

The association of LDH with rat liver peroxisomes (PO) was first suggested by the group of Tolbert and co-workers (10). However, the methodology used by those authors did not allow an unequivocal conclusion, so that in recent years it has generally been assumed that the LDH activity in peroxisomal fractions is due to the adsorption of the cytosolic enzyme to the outer surface of the peroxisomal membrane (11). Whereas PO in rat liver contain several dehydrogenases utilizing NAD<sup>+</sup> as cofactor such as (a) 3-hydroxyacyl-CoA dehydrogenases (12), (b)  $\alpha$ -glycerol phosphate dehydrogenase (13), and (c) alcohol dehydrogenase (14), a peroxisomal enzyme system for the reoxidation of NADH has not been described. Since the peroxisomal membrane seems to be permeable to NAD<sup>+</sup> *in vitro* (15), it has been suggested that NADH, generated in PO is reoxidized in the cytosol after passage across the peroxisomal membrane (11). Most recently, however, van Roermund *et al.* (16) have shown that *in vivo* the peroxisomal membrane in the yeast *Saccharomyces cerevisiae* is impermeable to NAD<sup>+</sup>/NADH (16).

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<sup>‡</sup> Present address: Dept. of Pharmacology, Catholic University of Leuven, Campus Gasthuisberg, Herestraat 49, 3000 Leuven, Belgium.

<sup>§</sup> To whom correspondence should be addressed. Tel.: 49-6221-563956; Fax: 49-6221-594952.

<sup>1</sup> The abbreviations used are: LDH, L-lactate dehydrogenase; PO, peroxisome(s); PAGE, SDS-polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PTS, peroxisomal targeting signal; PMP, peroxisomal membrane protein.

Moreover, Osmundsen *et al.* (17) noted that the addition of pyruvate to an *in vitro* peroxisomal  $\beta$ -oxidation assay stimulates the  $\beta$ -oxidation of palmitoyl-CoA while addition of exogenous LDH had no effect. Since the exact mechanism of stimulation of  $\beta$ -oxidation by pyruvate remained ambiguous, we speculated that it could be due to the presence of LDH inside the PO, since in our earlier studies this enzyme was consistently found in the highly purified (98%) peroxisomal fractions isolated by metrizamide density gradient centrifugation (18).

In the present study, we have raised a monospecific antibody against cytosolic rat liver LDH and demonstrate the presence of the enzyme in the matrix of rat liver PO by a combination of biochemical and ultrastructural immunocytochemical techniques. Additionally, the involvement of peroxisomal LDH in the reoxidation of NADH produced by the  $\beta$ -oxidation system of this organelle is demonstrated.

#### EXPERIMENTAL PROCEDURES

**Materials**—Blue dextran A coupled to agarose for the isolation of LDH protein was obtained from Amicon (Witten, Germany). Metrizamide for density gradients and nitrocellulose membranes were purchased from Nycomed (Oslo, Norway) and Schleicher and Schüll (Datteln, Germany), respectively. Proteinase K and protein A were from Boehringer Mannheim, and BSA fraction V was from Serva (Heidelberg, FRG). Constituents of enzyme assays (*p*-nitro blue tetrazolium, NAD,  $\alpha$ -ketoacids, glycolate, palmitoyl-CoA, fructose 6-phosphate, glucose-6-phosphate dehydrogenase, glucose 1-phosphate, glyceralate 3-phosphate, phosphoglycerate kinase, LDH-test Kit) were bought from Sigma and Boehringer Mannheim. Titaniumoxisulfate was provided by Riedel-de-Haen (Seelze, Germany), and glutaraldehyde was from Serva. All other chemicals used were obtained from Merck (Darmstadt, Germany) and were of the purest grade available.

**Animals and Drug Treatment**—Normal Sprague-Dawley rats of both sexes weighing  $\sim 250$  g, kept under normal laboratory conditions, were used for all experiments. For the induction of the peroxisomal  $\beta$ -oxidation enzymes, some rats were treated for 7 and 14 days with 75 mg/kg of bezafibrate (obtained through the courtesy of Boehringer Mannheim), a dosage shown previously to be highly effective (19). All rats were fasted for 16 h prior to sacrifice.

**Isolation of LDH and Raising of the Antibody**—LDH was isolated from total rat liver homogenates of untreated control Sprague-Dawley rats according to the protocol described by Thompson *et al.* (20). The purity of the enzyme was assessed by SDS-PAGE. An antibody against purified LDH was raised in rabbits (21), and its specificity was confirmed by Western blotting (22).

**Cell Fractionation and Isolation of Highly Purified Peroxisomes**—Subcellular fractions comprising the heavy mitochondrial (M), light mitochondrial or crude peroxisomal (L), microsomal (P), and cytosolic (S) fractions were prepared by differential centrifugation as described by Völkl and Fahimi (18). Briefly, rat livers were homogenized for 2 min at 1000 rpm in isotonic homogenization buffer (5 ml/g of tissue), 5 mM MOPS, 250 mM sucrose, 1 mM EDTA, 0.1% ethanol, and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM dithiothreitol, and 1 mM  $\epsilon$ -aminocaproic acid) using an ice-cooled Potter-Elvehjem homogenizer. In order to obtain highly purified PO, the L-fraction was subjected to density gradient centrifugation on a continuous Metrizamide gradient ( $\rho = 1.10$ – $1.26$  g/cm<sup>3</sup>) as modified by Lüers *et al.* (23), spun in a vertical rotor (VTi 50, Beckman Instruments) at an integrated force of  $1.252 \times 10^6 \times g \times \text{min}$ . 20 fractions of 2 ml each were collected from bottom to top. Two peroxisomal peaks were obtained with densities of 1.24–1.23 g/cm<sup>3</sup> (fractions 2 and 3  $\equiv$  highly purified "heavy" peroxisomes) and 1.15–1.14 g/cm<sup>3</sup> (fractions 15 and 16  $\equiv$  "light" peroxisomes). The light peroxisomal fraction contained mainly microsomes as well as small PO with high levels of  $\beta$ -oxidation activity. This fraction corresponds to the one recently characterized by Schrader *et al.* (24) in human hepatoblastoma (HepG2) cells, which was also obtained by density gradient centrifugation using an exponential metrizamide gradient.

Enzyme activities were determined according to standard procedures: catalase and  $\alpha$ -hydroxyacid oxidase (25), palmitoyl-CoA oxidase (26), enoyl-CoA hydratase (27), cyanide-insensitive palmitoyl-CoA  $\beta$ -oxidation (28) phosphoglucomutase, phosphoglucoisomerase, and glyceraldehyde-3-phosphate dehydrogenase (29), esterase (30), and cytochrome-c oxidase (31). LDH and acid phosphatase were assayed using commercially available test-kits (53). Protein was measured according

to Lowry *et al.* (32) with bovine serum albumin as standard. Data are presented in histogram form (30).

**Subfractionation of Isolated Peroxisomes**—The peroxisomal matrix proteins were extracted with a hypotonic TVBE buffer (0.01% Triton X-100, 0.1% ethanol, 1 mM NaHCO<sub>3</sub>, 1 mM EDTA, pH 7.6) followed by separation of the core and membranes by centrifugation for 60 min at  $100,000 \times g$ . For the isolation of the peroxisomal integral membrane proteins, PO were treated with an alkaline carbonate buffer, pH 11.5, according to Fujiki *et al.* (33). The cores were purified by metrizamide density gradient centrifugation (34).

**SDS-PAGE and Western Blotting**—Polypeptides of highly purified PO, PO subfractions, and cytosolic fractions were separated by SDS-PAGE (10% resolving mini slabgels,  $8 \times 4.5 \times 0.1$  cm) and were either silver-stained or electrotransferred to nitrocellulose membranes (22). For immunocomplexing, the monospecific polyclonal rabbit anti-rat-LDH-antibody was used. The antibodies against rat urate oxidase and catalase were raised in rabbits and characterized previously (34, 35). The antibody to the rat 22-kDa peroxisomal membrane protein (PMP 22) was kindly provided by Professor Hashimoto (Shinshu University, Matsumoto, Japan). The immune complexes were visualized by a modified protein A-gold technique (35).

**Isoelectric Focussing (IEF)**—IEF polyacrylamide gels with a linear gradient, pH 2–11, were run on a Multiphor system according to the instructions of the manufacturer, Pharmacia Biotech, Inc. For crude peroxisomal and cytosolic fractions, amounts of protein corresponding to 5 milliunits of LDH activity were loaded per lane. The isoforms of LDH in the subcellular fractions were visualized by staining of the enzyme activity with the nitro blue tetrazolium method (4), as well as by immunoblotting with a modified protein A-gold procedure (35).

**Differential Salt Extraction of Isolated Peroxisomes**—Freshly isolated PO (0.668 mg/ml) were diluted 10-fold in 5 mM MOPS, pH 7.4, 1 mM EDTA, 0.05% deoxycholate containing different concentrations of KCl (20, 100, and 500 mM). After incubation for 15 min on ice, the mixtures were centrifuged for 60 min at  $100,000 \times g$ , and pellets and supernatants were assayed for protein, LDH, and catalase activities.

**Limited Proteolysis of Isolated Peroxisomes**—Freshly isolated PO were diluted (1:10) either in isotonic homogenization buffer (5 mM MOPS, pH 7.4, 250 mM sucrose, 1 mM EDTA, 0.1% ethanol) or hypotonic TVBE buffer (see above) and subjected to repeated freeze/thaw cycles (4 times each). The appropriate preparations were treated with proteinase K (1 mg/ml stock solution in 50 mM Tris buffer, pH 8; final concentration, 0.33 mg/ml) for different time intervals up to 60 min at room temperature. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (0.2 mM final concentration). As controls, freshly isolated unfrozen PO were diluted in the gradient medium with appropriate banding density and treated in parallel. The enzyme activities of catalase and LDH were determined, and the results were presented as a percentage of the corresponding total activities.

To unravel the alterations of the individual LDH-isoforms, in a separate set of experiments, freshly isolated PO as well as cytosolic fractions exhibiting the same LDH activities (10 mU) were treated with proteinase K (3.3 mg/ml stock solution in metrizamide 1.23 g/cm<sup>3</sup>; final concentration, 0.33 mg/ml) for different time intervals up to 60 min and subjected to IEF, followed by activity staining. For this purpose, (a) the undiluted unfrozen PO were treated with Triton X-100 (0.2 and 1%) before protease digestion and (b) metrizamide was added to the cytosolic fraction in a concentration corresponding to that in PO gradient fractions in order to rule out any influence of the high metrizamide concentration in peroxisomal fractions on proteolysis.

**Peroxisomal Palmitoyl-CoA- $\beta$  Oxidation and LDH Activity**—LDH and cyanide-insensitive  $\beta$ -oxidation of palmitoyl-CoA (28) were measured in freshly isolated hepatic PO of control rats and animals treated for 7 and 14 days with 75 mg/kg bezafibrate. In order to evaluate the impact of LDH activity on peroxisomal  $\beta$ -oxidation, the cyanide-insensitive palmitoyl-CoA  $\beta$ -oxidation was assayed in the presence of (a) different concentrations of pyruvate (0.1–20 mM), (b) increasing concentrations of the LDH-inhibitor oxamic acid (0.05–10 mM) in the presence of 2 mM pyruvate, and (c)  $\alpha$ -ketoacids other than pyruvate, glyoxylate, and oxaloacetate (2 mM). Moreover, the activities of the individual enzymes of the  $\beta$ -oxidation system, *i.e.* palmitoyl-CoA oxidase (26) and enoyl-CoA hydratase (27), were determined in the presence of 0.1–20 mM pyruvate.

**Immunoelectron Microscopy**—The livers of all of the animals were fixed for 5 min by perfusion via the portal vein with a fixative containing 0.25% glutaraldehyde, 2% sucrose, and 0.1 M PIPES buffer, pH 7.4. The tissue was processed for immunoelectron microscopy as described previously (36). Embedding of isolated peroxisomal fractions and postembedding protein A-gold immunocytochemistry was done accord-

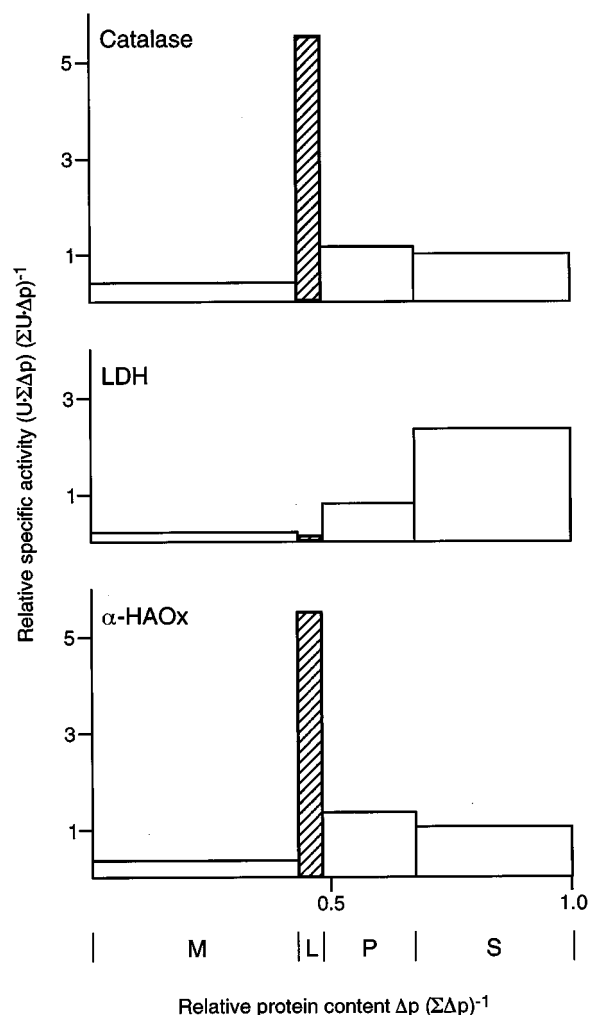


FIG. 1. Distribution of marker enzyme activities (catalase, glycolate oxidase ( $\alpha$ -HAOx), and LDH) after subcellular fractionation by differential centrifugation according to Völkl and Fahimi (18). The rates of recovery of marker enzymes were as follows: catalase: M, 20%; L, 23%; P, 17%; S, 39%; glycolate oxidase ( $\alpha$ -HAOx): M, 15%; L, 23%; P, 17%; S, 43%; LDH: M, 9%; L, 0.5%; P, 17%; S, 73%. Abbreviations used are as follows: M, heavy mitochondria; L, light mitochondria  $\equiv$  crude PO; P, microsomes; S, cytosol; U, total units of an enzyme found in a single fraction;  $\Sigma U$ , total units found in all fractions;  $\Delta p$  = total protein content of a single fraction,  $\Sigma \Delta p$  = total protein content of all fractions.

ing to Baumgart (37). Silver-intensification of the gold particles was accomplished in a light-tight box using a slight modification of the method described by Danscher *et al.* (38).

#### RESULTS

**Association of LDH with Highly Purified Peroxisomes**—After the differential centrifugation, the marker enzymes of PO (particulate catalase and  $\alpha$ -hydroxyacid oxidase) were associated mainly (>23%) with the L fraction (Fig. 1), mitochondrial cytochrome-c oxidase was associated with the M fraction (>80%), and microsomal esterase (>73%) was associated with the P fraction (data not shown). The bulk of LDH activity was recovered in the cytosolic fraction (S), with only about 1% of the total activity being present in the crude peroxisomal fraction (Fig. 1). After density gradient centrifugation for the isolation of highly purified PO, however, LDH consistently colocalized with peroxisomal marker enzymes (Fig. 2). As further shown in Fig. 2, the main portion of particle-bound LDH activity was associated with the highly purified heavy peroxisomal fractions 2 and 3, ( $\rho = 1.23$ – $1.24$  g/cm<sup>3</sup>) as well as with the fractions 15 and 16 with lower densities ( $\rho = 1.13$ – $1.14$  g/cm<sup>3</sup>). The latter contained in

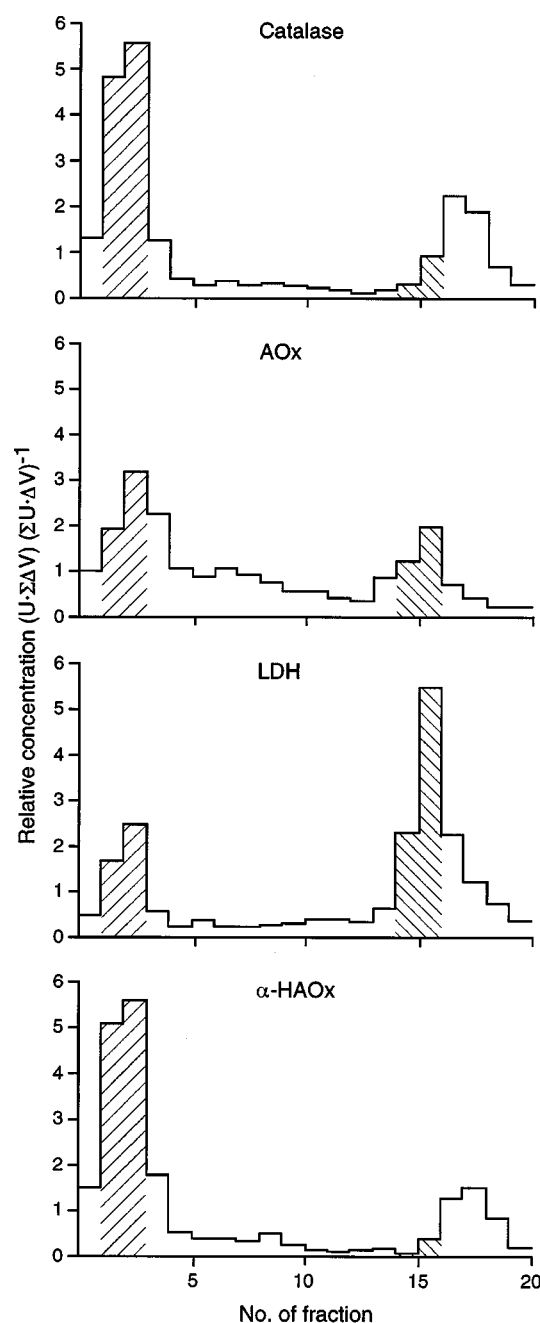


FIG. 2. Distribution of marker enzyme activities (catalase, palmitoyl-CoA oxidase (AOx), LDH, and glycolate oxidase ( $\alpha$ -HAOX)) after further purification of the L-fraction by Metrizamide density gradient centrifugation. In addition to the marker enzymes presented on the graph, the ones for other cell organelles were enriched in the following fractions: cytochrome-c oxidase (mitochondria), fractions 13 and 14; esterase (microsomes), fractions 15 and 16; acid phosphatase (lysosomes), fractions 17 and 18. U, total units of an enzyme found in a single fraction;  $\Sigma U$ , total units found in all fractions;  $\Delta V$ , total volume of a single fraction;  $\Sigma \Delta V$ , total volume of all fractions; //, heavy PO (fractions 2 and 3) banding at  $\rho = 1.23$ – $1.24$  g/cm<sup>3</sup>; \\\, light PO (fractions 15 and 16) banding at  $\rho = 1.13$ – $1.14$  g/cm<sup>3</sup>.

addition to microsomal proteins as measured by esterase activity (data not presented), the "light" PO, as shown by peroxisomal palmitoyl-CoA oxidase distribution. Interestingly, the PO in the heavy and light fractions differed substantially in their enzyme composition, with the light fractions containing significantly higher ratios of palmitoyl-CoA oxidase/catalase (~factor 5). These results are consistent with the data reported previously by Schrader *et al.* (24) from our laboratory and

TABLE I  
Percentage distribution (PD) and relative specific activities (RSA) of glycolytic enzymes

LDH, PGI, PGM, and GAPDH were measured in total liver homogenate and subcellular fractions of untreated rats. Values are expressed as means for two experiments. PGI, phosphoglucose isomerase; PGM, phosphoglucose mutase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PO, peroxisomal fraction; ND, not detected.

Enzyme	Homogenate		Crude PO		Highly purified PO		Cytosol	
	Units/g liver	Units/mg	PD	RSA	Units/mg	RSA	PD	RSA
LDH	452.5	2.06	0.47	0.147	0.426	0.207	77.08	1.66
PGI	89.8	0.41	0.40	0.12	ND	ND	77.14	1.66
PGM	56.4	0.25	0.41	0.07	ND	ND	93.00	2.00
GAPDH	75.4	0.34	1.34	0.41	ND	ND	75.32	1.62

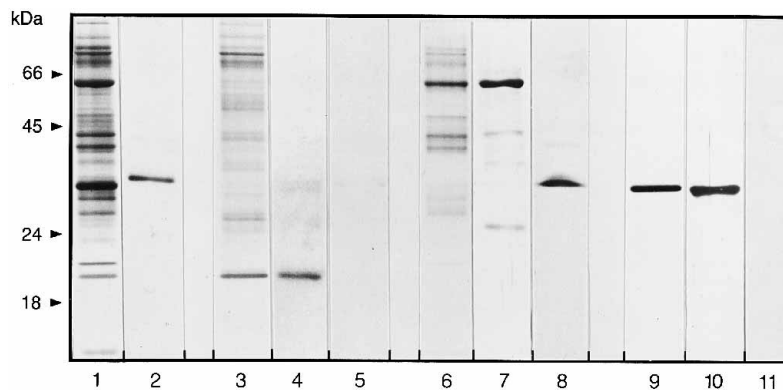


FIG. 3. Distribution of LDH in subfractions of highly purified peroxisomes obtained by Metrizamide density gradient centrifugation. Lanes 1 and 2, untreated total peroxisomes. Lane 1, polypeptide pattern after SDS-PAGE and silver staining (5  $\mu$ g protein); lane 2, immunoblot using anti-LDH antibody (2  $\mu$ g of protein). Lanes 3–5, integral membrane protein fraction. Lane 3, polypeptide pattern after SDS-PAGE and silver staining (4.8  $\mu$ g of protein); lane 4, immunoblot using anti-PMP 22 antibody (2  $\mu$ g of protein); lane 5, immunoblot using anti-LDH antibody (2  $\mu$ g of protein). Lanes 6–8, matrix fraction. Lane 6, polypeptide pattern after SDS-PAGE and silver staining (5  $\mu$ g of protein); lane 7, immunoblot using anti-catalase antibody (0.5  $\mu$ g of protein); lane 8, immunoblot using anti-LDH antibody (2  $\mu$ g of protein). Lanes 9–11, core fraction. Lane 9, polypeptide pattern after SDS-PAGE and silver staining (1  $\mu$ g of protein); lane 10, immunoblot using anti-urate oxidase antibody (0.5  $\mu$ g of protein); lane 11, immunoblot using anti-LDH antibody (2  $\mu$ g of protein). Molecular mass standards in kDa are as follows: 66, bovine serum albumin; 45, ovalbumin; 24, trypsinogen; 18.5,  $\beta$ -lactoglobulin.

confirmed recently by Wilcke *et al.* (39). In contrast, other cytosolic enzymes involved in glycolysis, phosphoglucose mutase, phosphoglucose isomerase, and glyceraldehyde-3-phosphate dehydrogenase were not detectable in the highly purified peroxisomal fractions (Table I). This would argue against a nonspecific uptake of cytosolic LDH into peroxisomes. Subfractionation of purified peroxisomal fractions followed by Western blotting using an anti-LDH antibody revealed the association of LDH with the soluble peroxisomal matrix proteins (Fig. 3), whereas the core and membrane fractions were negative.

**Peroxisomal and Cytosolic LDHs Are Closely Related Proteins**—Since the total activities of LDH in cytosolic and peroxisomal fractions differed markedly, aliquots of each fraction exhibiting equal LDH-activities were used for Western blot and IEF analysis. As shown in Fig. 4A, both polypeptides have the same molecular weight ( $M_r$  35,000). Additionally, IEF revealed that both LDH-A<sub>4</sub>- and LDH-A<sub>3</sub>B-isoforms are present in PO (Fig. 4B). Interestingly, the proportion of LDH-A<sub>3</sub>B is higher in PO than in the cytosol.

**LDH Is a Bona Fide Peroxisomal Protein**—To assess the extent of peripheral association of cytosolic LDH to the surface of PO, freshly isolated peroxisomal fractions were subjected to salt extraction with increasing KCl concentrations (Fig. 5) and to limited proteolysis (Figs. 6 and 7). As shown in Fig. 5, the extraction patterns of LDH and catalase were comparable, confirming the intraperoxisomal localization of LDH. The release of about 40% of peroxisomal catalase in the absence of KCl is due to the dilution of the peroxisomal fraction in the hypotonic buffer and subsequent centrifugation at  $100,000 \times g$  in this experiment. In Fig. 6, the rates of degradation of LDH and catalase in differently pretreated PO are compared. Whereas in repeatedly frozen and thawed peroxisomes (diluted

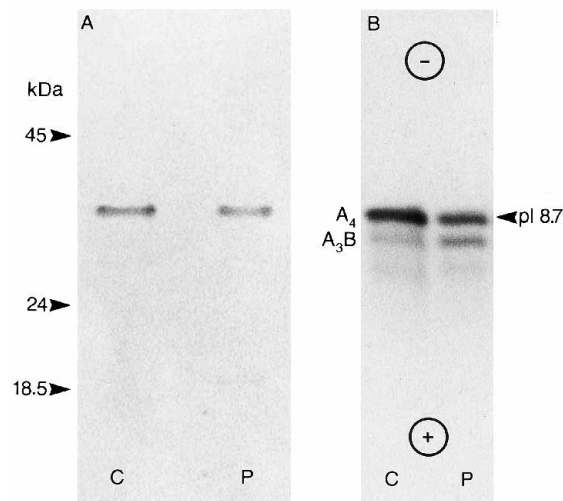


FIG. 4. A, Western blot showing the presence of LDH polypeptides with identical molecular masses in PO and the cytosol. The following protein amounts were loaded per lane: cytosol (C), 0.25  $\mu$ g; highly purified peroxisomes (P), 2  $\mu$ g. B, isoelectric focussing gel stained for LDH activity with the NBT-method, depicting LDH-A<sub>4</sub> and LDH-A<sub>3</sub>B isoforms in both subcellular compartments. Protein amounts corresponding to 5 milliunits of LDH activity were loaded per lane: cytosol (C), 1.5  $\mu$ g; crude peroxisomes (P), 38  $\mu$ g. Molecular mass standards in kDa are as follows: 45, ovalbumin; 24, trypsinogen; 18.5,  $\beta$ -lactoglobulin.

in an isotonic homogenization buffer) the degradation rates of both enzymes were comparable, in intact PO, major differences were observed. The initial increase of catalase activity after 15 min of proteolysis under the latter conditions is most probably

FIG. 5. Influence of increasing concentrations of KCl on the extraction of LDH, catalase, and protein from highly purified PO. Freshly isolated PO (0.668 mg/ml) were diluted 10-fold in 5 mM MOPS, pH 7.4, 1 mM EDTA, 0.05% deoxycholate containing different concentrations of KCl (20, 100, and 500 mM). After incubation for 15 min on ice, the mixtures were centrifuged for 60 min at  $100,000 \times g$ , and pellets and supernatants were assayed for protein, LDH, and catalase activities. The results are expressed as the release in percent of total activity or protein.

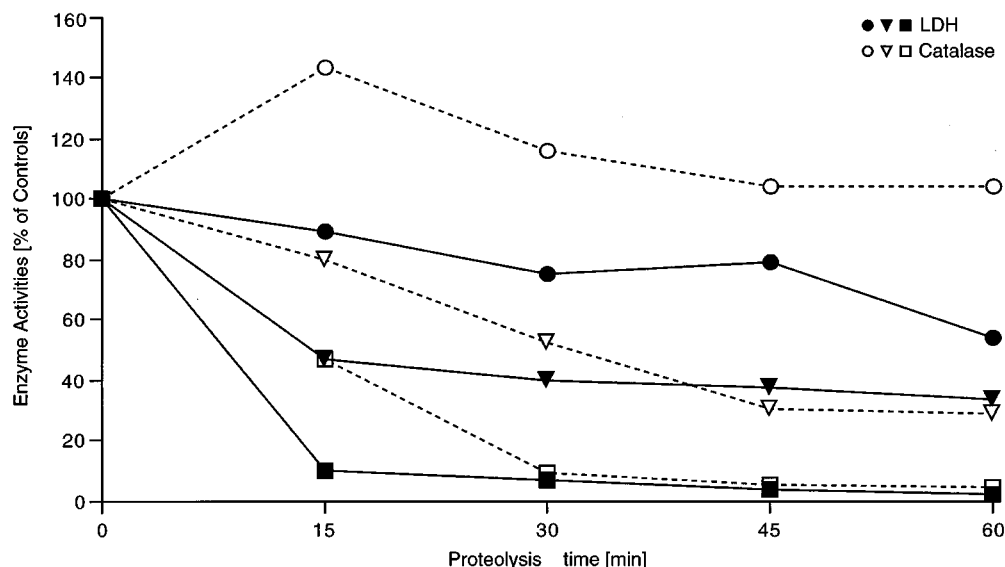
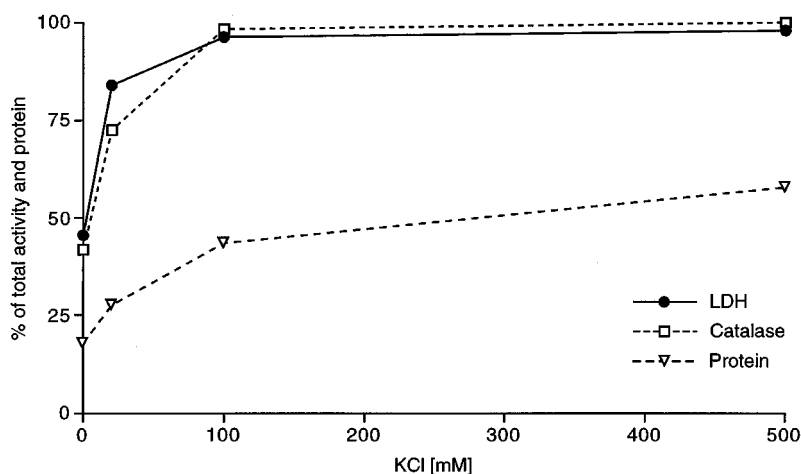


FIG. 6. Influence of different physical pretreatment conditions on the accessibility of LDH and catalase in PO as revealed by proteolysis. ○, ●, intact PO diluted in gradient medium for the assay; ▼, ▽, frozen/thawed (4×) PO in isotonic homogenization buffer; ■, □, frozen/thawed (4×) PO in hypotonic TVBE buffer.

due to a better accessibility of the enzyme to its substrate  $H_2O_2$ . Whereas LDH activity was reduced to 60% after 60 min of digestion, the catalase activity was only moderately affected. This suggests that the effect on LDH could be due in part to the removal of LDH associated with the cytosolic surface of peroxisomes. After complete lysis of PO by freeze-thawing in hypotonic buffer, the activity of both enzymes was completely abolished after 30 min of protease digestion.

Substantial evidence for the intraperoxisomal localization of LDH was provided by the differential kinetics of degradation of the cytosolic and peroxisomal enzyme as revealed by IEF (Fig. 7). Whereas the cytosolic LDH activity was completely abolished after 15 min of protease treatment, the peroxisomal LDH was only slightly affected even after 60 min (Fig. 7A). Only after lysis of PO with 1% Triton X-100 did the particle-bound LDH also disappear with similar kinetics as its cytosolic counterpart (Fig. 7B).

**Immunoelectron Microscopic Localization of LDH in Peroxisomes: Evidence for a Heterogeneous Distribution in the Peroxisomal Population**—Postembedding protein A-gold immunocytochemistry of liver sections revealed a predominantly cytosolic localization of LDH. In addition, a heterogeneous labeling of PO is observed, with most of the PO exhibiting only a few gold particles in their matrix. A selected area of a liver cell is shown in Fig. 8a, where the PO are intensively labeled with gold

particles representing LDH antigen. The membranes and cores are not stained in these organelles, whereas the peroxisomal matrix is strongly labeled. Other cell organelles such as mitochondria, lysosomes, and ER are negative (Fig. 8b). As controls for the specificity of the immunocytochemical detection of LDH, liver sections were incubated in parallel with an anti-catalase antibody, which revealed exclusive peroxisomal labeling (Fig. 8c) or with an appropriate LDH-preimmune serum, which showed no labeling (Fig. 8d).

The presence of LDH protein in isolated peroxisomal fractions is demonstrated in Fig. 9. The heterogeneity of LDH labeling in the peroxisomal fraction is clearly visible in Fig. 9, a and b. Cores and contaminating mitochondria are not labeled (Fig. 9b). As revealed by higher magnification, only few gold particles are attached to the cytosolic surface of the peroxisomal membrane (Fig. 9c), suggesting that the bulk of LDH in the isolated fractions is intraperoxisomal. In quantitative counts of gold particles, approximately 20% of all gold particles were associated with either side of the peroxisomal membrane, thus confirming that at least 80% of labeling was truly intraperoxisomal. The appropriate controls with anti-catalase and anti-LDH preimmune serum are shown in Fig. 9, d and e.

**Peroxisomal LDH Is Increased by Bezafibrate Treatment**—In Table II the activities of palmitoyl-CoA oxidase and LDH in total homogenates and highly purified PO obtained from livers

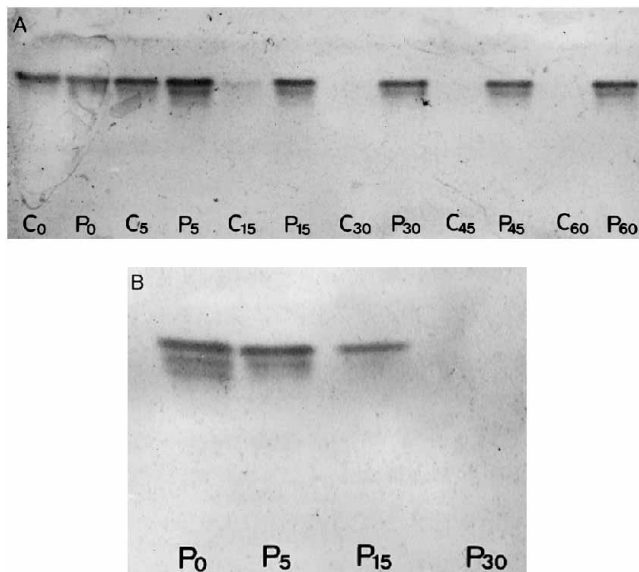


FIG. 7. *A*, kinetics of proteolysis on cytosolic and peroxisomal LDH as revealed by IEF. Freshly isolated undiluted PO and cytosolic fractions exhibiting the same activities (10 milliunits) were treated for different time intervals with 0.33 mg/ml proteinase K, followed by IEF and activity staining. Protein amounts corresponding to 5 milliunits of LDH activity were loaded per lane: cytosol (C), 1.5  $\mu$ g; highly purified peroxisomes (P), 12  $\mu$ g. The incubation times are indicated by the numbers given in subscript. *B*, kinetics of proteolysis of peroxisomal LDH (P) after lysis of the cell organelles by 1% Triton X-100. The incubation times are given by the numbers in subscript.

of rats treated for 7 and 14 days with bezafibrate are compared with those of control animals. Whereas the palmitoyl-CoA oxidase activity in total homogenates was increased up to 9-fold by the treatment, that of LDH was only slightly elevated. In highly purified peroxisomal fractions on the other hand, the elevation of LDH activity was much more pronounced than in homogenates, reflecting a selective induction of the peroxisomal LDH. Similarly, in Western blots the amount of LDH protein was significantly increased in peroxisomal fractions of bezafibrate-treated animals (Fig. 10).

**NADH Provided by Peroxisomal  $\beta$ -Oxidation Is Reoxidized by LDH in Peroxisomes**—The cyanide-insensitive  $\beta$ -oxidation system (5 mM palmitoyl-CoA, 100 mM KCN, 37  $^{\circ}$ C) of highly purified PO of bezafibrate-treated animals (75 mg/kg/day) produced 179.7 nmol of NADH/min/mg of protein. Under similar assay conditions (2 mM pyruvate, 100 mM KCN, 37  $^{\circ}$ C), the peroxisomal LDH oxidized 930 nmol of NADH/min/mg of protein, suggesting that it is capable to reoxidize completely the NADH produced by the peroxisomal  $\beta$ -oxidation system.

In Tables III–V the influence of PO-associated LDH on the reoxidation of NADH produced by peroxisomal  $\beta$ -oxidation is summarized. Table III shows that the degree of reoxidation of NADH was clearly dependent on the concentration of pyruvate added to the mixture used for assaying the  $\beta$ -oxidation activity. Reoxidation of NADH is maximal at 2 mM pyruvate, a concentration that results in optimal LDH rates and is diminished at higher pyruvate concentrations known to inhibit LDH activity. Thus, it seems that NADH produced in PO is reoxidized indeed by intraperoxisomal LDH. This notion is further supported by

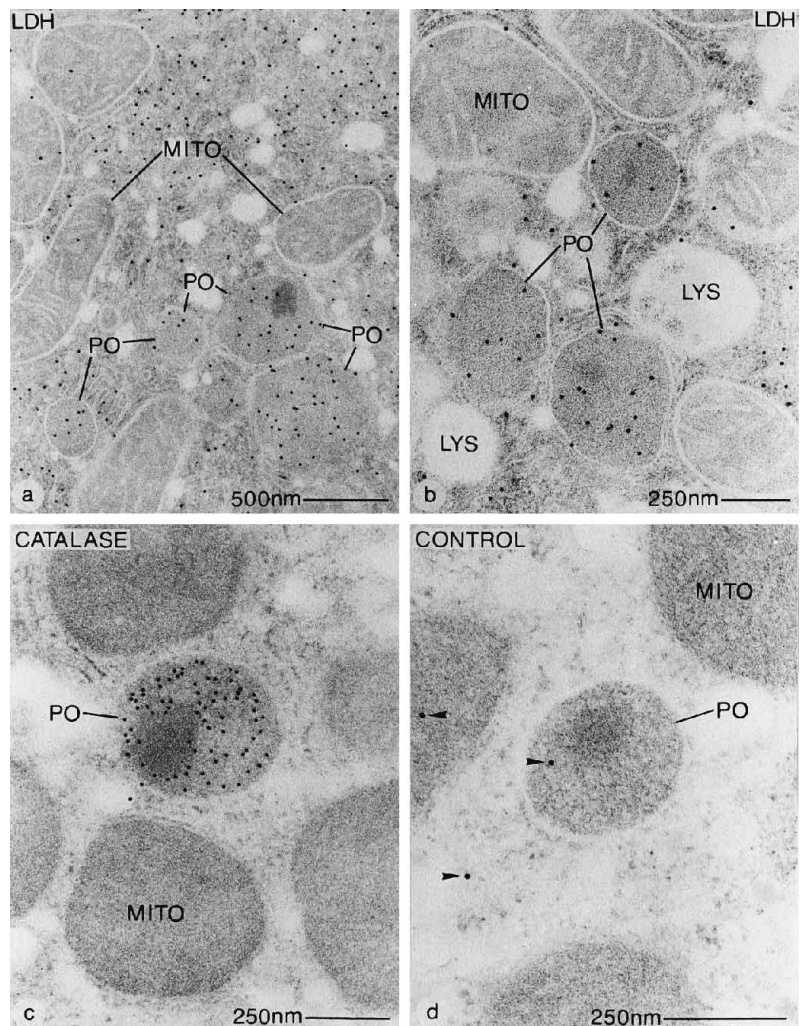


FIG. 8. *a* and *b*, electron micrographs of rat liver sections incubated with the anti-LDH antibody followed by protein A-gold. Note the localization of gold particles in the peroxisomal matrix (PO) and the cytoplasm. Mitochondria (MITO) and lysosomes (LYS) are not labeled. *c*, control section labeled with the antibody to catalase revealing exclusive peroxisomal localization of catalase. *d*, control section incubated with the appropriate LDH-pre-immune serum.



FIG. 9. *a* and *b*, highly purified PO incubated with the anti-LDH antibody followed by protein A-gold and silver intensification. Note the heterogeneous labeling of isolated peroxisomes (PO). Two mitochondria (asterisk), and an isolated core (arrowheads) are unlabeled. *c*, higher magnification view of isolated peroxisomes labeled for LDH with 6-nm gold particles. Note the presence of a few gold particles attached to the cytoplasmic surface of the peroxisomal membrane (arrowheads) in addition to the labeling of the matrix. *d* and *e*, purified PO incubated with an antibody to catalase and LDH-preimmune serum.

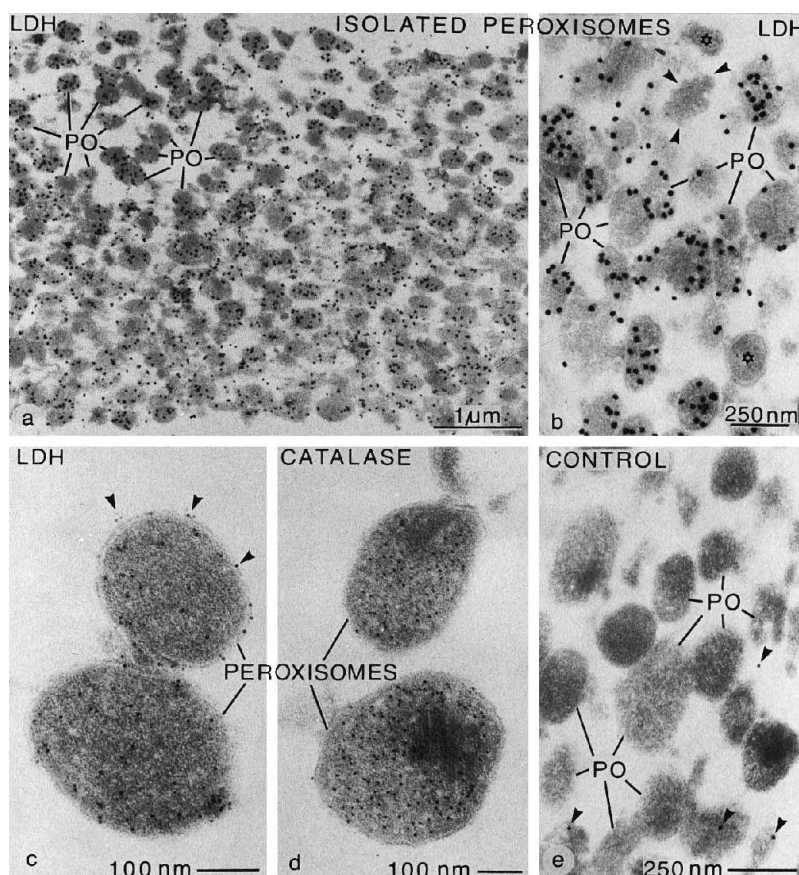


TABLE II  
Specific and relative specific activities of palmitoyl-CoA oxidase and LDH

Enzyme activities were measured in total homogenates and highly purified peroxisomes obtained from livers of control rats and rats treated for 7 (Beza 1) and 14 (Beza 2) days with bezafibrate (75 mg/kg/day). Values are expressed as means for two experiments. RSA, (units/mg peroxisomes)  $\times$  (units/mg homogenate)<sup>-1</sup>.

Source	Palmitoyl-CoA oxidase				LDH			
	Homogenate		Peroxisomes		Homogenate		Peroxisomes	
	mU/gL	mU/mg	mU/mg	RSA	U/gL	U/mg	U/mg	RSA
Control	244.0	1.27	23.66	18.63	464.45	2.31	0.507	0,220
Beza 1	2152.0	8.26	63.39	7.67	487.65	1.84	0.763	0,415
Beza 2	2264.5	6.87	52.23	7.60	527.10	2.18	0.840	0,385

the data presented in Table IV. A dose-dependent inhibition of NADH-reoxidation was noted with increasing concentrations of oxamic acid, an inhibitor of LDH. In Table V, the production rates of NADH by the peroxisomal  $\beta$ -oxidation system in the presence of pyruvate and other  $\alpha$ -ketoacids are compared. Even though glyoxylate can be converted by LDH to oxalate, 2 mM glyoxylate in the  $\beta$ -oxidation assay mixture exerted no effects on NADH production rates. Only at higher concentrations (5 mM), 36.4% of the NADH produced was reoxidized. These data are consistent with 470 times higher  $K_m$  values of peroxisomal LDH for glyoxylate compared with pyruvate ( $K_m$ -glyoxylate:  $5.83 \times 10^{-2}$  mol/liter;  $K_m$ -pyruvate:  $1.24 \times 10^{-4}$  mol/liter). On the other hand, oxaloacetate proved to be almost as effective as pyruvate, suggesting the presence of an additional dehydrogenase (possibly malate dehydrogenase) in peroxisomes.

#### DISCUSSION

In the present study, the intracellular distribution of L-lactate dehydrogenase in rat hepatocytes was studied by three different approaches: (a) analytical subcellular fractionation with determination of enzyme activity, (b) immunodetection of LDH in isolated subcellular fractions using a monospecific an-

tibody, (c) immunoelectron microscopy applied to liver sections and to isolated peroxisomal fractions.

The results clearly demonstrate that LDH is present in the matrix of rat liver peroxisomes in addition to the cytosol. Moreover, the data presented in this study suggest strongly that the peroxisomal LDH is directly coupled to the reoxidation of the NADH generated by the palmitoyl-CoA  $\beta$ -oxidation system present in this cell organelle.

**LDH Is Associated with Different Cell Organelles**—An association of LDH with different cell organelles such as the nucleus, mitochondria or microsomes was proposed already in the sixties by Agostini *et al.* (40). Since then the debate on the subcellular distribution of LDH has continued mainly because the LDH-isoforms found in the different cell compartments were similar and the percentage of total activity associated with the different organelles was very low (*e.g.*  $\sim 1\%$  for nuclear and 1–1.6% for mitochondrial LDH) (6, 9, 10). In the meantime, only the nuclear localization of LDH has been confirmed by immunoelectron microscopy (41). In addition, this nuclear enzyme was shown to be posttranslationally modified by the phosphorylation of the tyrosine residue 238 and to behave like

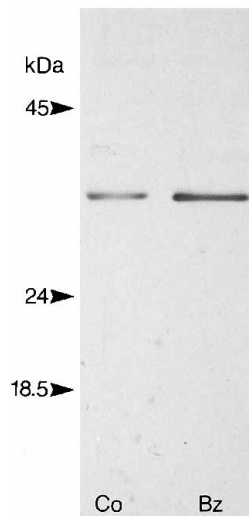


FIG. 10. Western blot of highly purified peroxisomal fractions (2  $\mu$ g of protein) from control rats (Co) and rats treated for 14 days with 75 mg/kg/d bezafibrate (Bz) incubated with the anti-LDH antibody. Molecular mass standards in kDa are as follows: 45, ovalbumin; 24, trypsinogen; 18.5,  $\beta$ -lactoglobulin.

TABLE III

Reoxidation of NADH produced by peroxisomal  $\beta$  oxidation in the presence of pyruvate

Pyruvate	NADH production	NADH reoxidized by LDH
mM	nmol/min/mg	%
0	179.75	0
0.1	92.59	48.49
0.5	39.22	78.18
1.0	32.68	81.82
2.0	0	100
5.0	39.22	78.18
10.0	49.02	72, 73
20.0	74.08	58, 79

TABLE IV

Effect of the LDH-inhibitor oxamate on NADH reoxidation in isolated peroxisomes

Pyruvate	Oxamate	NADH production	NADH reoxidized by LDH
mM	mM	nmol/min/mg	%
2.0		0	100
2.0	0.05	21.79	87.9
2.0	0.5	23.02	84.8
2.0	1	21.79	87.9
2.0	2	34.86	80.6
2.0	5	59.75	65.5
2.0	10	228.77	0
	10	232.04	0
		179.75	0

TABLE V

Effect of different  $\alpha$ -ketoacids on peroxisomal NADH-production rates

2-Oxoacid	NADH production	NADH reoxidized by LDH
	nmol/min/mg	%
	179.75	0
2 mM pyruvate	0	100
2 mM glyoxylate	180.65	0
5 mM glyoxylate	114.38	36.4
2 mM oxaloacetate	28.83	84.0

a single-stranded DNA-binding protein (6).

An association of LDH with PO was first reported by the group of Tolbert and co-workers (10). They stated, however, that the possibility of LDH being associated to the surface of PO could not be ruled out by the methods used in their studies. They found only 0.6% of the total LDH activity in intact PO,

which after correction for particle breakage during subcellular fractionation could make up as much as 1.5% of the total activity. Since the peroxisomal isoform (LDH-A<sub>4</sub>) described by McGroarty *et al.* (10) was identical to that of the cytosolic fraction and their kinetic properties were similar, it has since been generally concluded that the LDH in peroxisomal fractions is a cytosolic contaminant (11).

In our earlier studies (18), we consistently found 1.5–2% of the total LDH activity in the crude peroxisomal fraction. The latter is separated into two peaks after density gradient centrifugation in an exponential metrizamide gradient (23). The first peak of LDH colocalized with the major peroxisomal peak (fractions 2 and 3;  $\rho = 1.23$ – $1.24$  g/cm<sup>3</sup>) at the bottom of the gradient, whereas the second peak was associated with the microsomal fractions (fractions 15 and 16;  $\rho = 1.13$ – $1.14$  g/cm<sup>3</sup>) at the top of the gradient immediately below the soluble components containing the so called 178 light peroxisomes 178 (Fig. 2). Recently, Schrader *et al.* (24) have demonstrated, that this second peak with low density contains a large number of small PO that exhibit a relatively high ratio of  $\beta$ -oxidation enzymes to catalase. Moreover, Wilcke *et al.* (39) demonstrated by postembedding immunocytochemistry of the low density fractions obtained from di(ethylhexyl)phthalate-treated animals, that indeed the small peroxisomal vesicles present in these fractions contain significant amounts of  $\beta$ -oxidation enzymes.

*LDH Is a Bona Fide Peroxisomal Matrix Enzyme and Is Distributed Heterogeneously in the Peroxisomal Population*—Since it was reported that all LDH-isoforms present in different cell organelles in hepatocytes resembled the cytosolic ones (40), we decided to use the isolated cytosolic LDH for generation of a rabbit anti-rat liver-LDH antibody. After confirmation of the monospecificity of our antibody for LDH, it was used for the immunocytochemical detection of the LDH protein in rat liver sections. The results clearly indicate the presence of LDH in the peroxisomal matrix, in addition to the staining of the cytoplasm and the nucleus, whereas the remainder of the cell organelles such as mitochondria, lysosomes, or ER appeared negative (see Fig. 9, a and b). In addition, the morphological data provide strong evidence for a heterogeneous distribution of the LDH in PO. These results are in full agreement with those obtained after subfractionation of PO, where both LDH activity and LDH protein detected by Western blotting were found in the matrix fraction of PO. Although the isoenzyme composition of the cytosolic and peroxisomal LDHs are very similar, as shown by IEF and gel electrophoresis, highly purified PO contained relatively more LDH-A<sub>3</sub>B than the cytosolic fraction (Fig. 4B). This isoenzyme pattern was also confirmed by blotting of the IEF-gels and immunodecoration of the blots for LDH (data not shown). The LDH-A<sub>3</sub>B band in PO became even more prominent after mild “proteinase K-stripping” of intact PO (Fig. 7A, compare P<sub>0</sub> with P<sub>5</sub>-P<sub>60</sub>).

*At Least 80% of the LDH Activity in Isolated Peroxisomal Fractions Is Truly Intraperoxisomal*—As shown by selective salt extraction, limited proteolysis of intact and partially extracted PO and separately by immunoelectron microscopy, approximately 10–20% of the peroxisomal LDH is bound to the cytosolic surface of the peroxisomal membrane. Even though only 60% of the LDH activity is retained after 60 min of protease treatment of freshly isolated intact PO (Fig. 6), a higher intraperoxisomal LDH-percentage can be assumed, since after this time period, the catalase activity was reduced by about 25% also. Additional support for the presence of more than 80% of peroxisomal LDH activity being in the matrix is provided by the kinetics of proteolytic degradation of LDH as shown by IEF (Fig. 7A). Whereas the cytosolic LDH was completely degraded



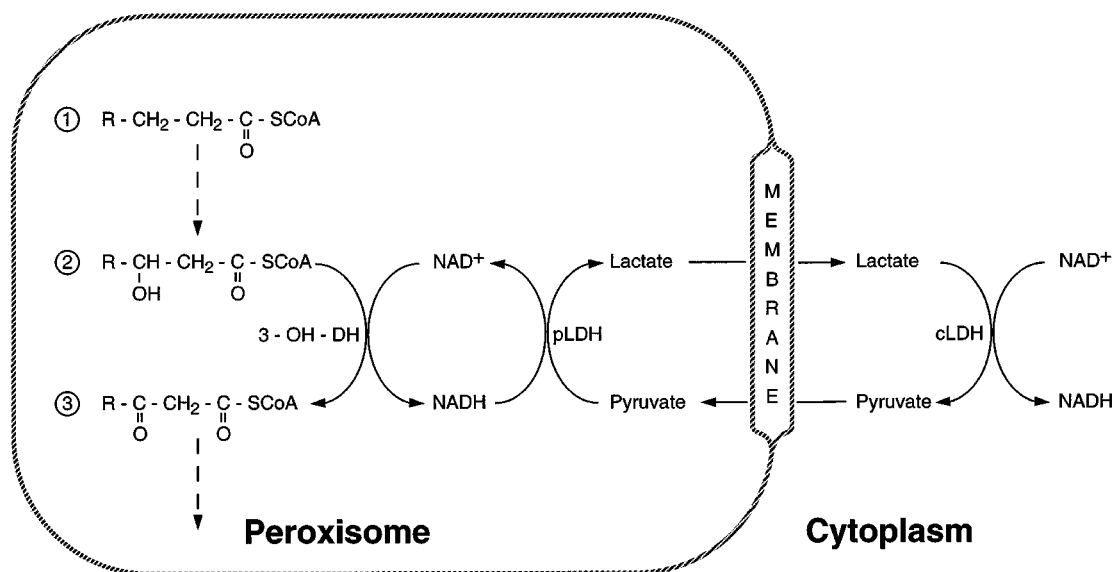


FIG. 11. **Proposed shuttle mechanism for the transfer of reducing equivalents between PO and cytoplasm.** 1–3, intermediates of the  $\beta$ -oxidation spiral; 1, activated long chain fatty acid; 2, 3-hydroxyacyl-CoA; 3, 3-ketoacyl-CoA; 3-OH-DH, 3-hydroxyacyl-CoA dehydrogenase; pLDH, peroxisomal LDH; cLDH, cytoplasmic LDH.

after 15 min of protease treatment, that of intact PO persisted more than 30 min and indeed made up to 80% of the total activity. Most convincingly, quantitative analysis of immunoelectron microscopic preparations of isolated peroxisomal fractions showed that about 20% of the gold particles are attached to either side of the peroxisomal membrane with 80% being localized in the matrix (Fig. 9c).

**Peroxisomal LDH Is Coupled to the  $\beta$ -Oxidation System and Reoxidation of NADH in Peroxisomes**—Whereas the presence of several  $\text{NAD}^+$ -linked dehydrogenases, such as  $\alpha$ -glycerol phosphate dehydrogenase (13), alcohol dehydrogenase (14), and different 3-hydroxyacyl-CoA dehydrogenases (12) has been well established in mammalian PO, no NADH-reoxidizing system has been described in this cell organelle. Since the peroxisomal membrane seems to be permeable *in vitro* for small solutes and coenzymes (15), the reoxidation of NADH in the cytosol after passage across the peroxisomal membrane has been considered and discussed (11). In view of the clear compartmentation and strict regulation of cytosolic and mitochondrial  $\text{NAD}^+/\text{NADH}$ -pools by the malate-aspartate,  $\alpha$ -glycerol-phosphate, and malate-pyruvate shuttle systems (42), it seemed very unlikely to us that a membrane-bounded organelle such as the PO should be permeable under *in vivo* conditions to these cofactors. In plants a malate-oxaloacetate-aspartate shuttle between glyoxysomes, chloroplasts, and mitochondria during the photosynthetic respiration has been envisaged with glyoxysomal malate dehydrogenase being the enzyme reoxidizing the reduced NADH in this cell organelle (43, 44). Finally, at the time of preparation of this manuscript van Roermund *et al.* (16) reported that PO in *S. cerevisiae* are not permeable to  $\text{NAD}^+/\text{NADH}$  *in vivo* and that a malate dehydrogenase-linked shuttle system is present in them. Support for the coupling of LDH to the  $\beta$ -oxidation system and the reoxidation of NADH in mammalian PO is provided by the following lines of evidence: (a) peroxisomal fractions of animals treated with bezafibrate exhibited higher levels of peroxisomal LDH activity (Table II) and contained higher amounts of LDH protein (Fig. 10), (b) in highly purified peroxisomal fractions from obese mice containing enhanced levels of  $\beta$ -oxidation enzymes also elevated LDH-levels were reported (45), and (c) pyruvate stimulated the  $\beta$ -oxidation of palmitoyl-CoA and erucoyl-CoA in peroxisomal fractions, whereas the addition of exogenous LDH did not lead

to further stimulation (17).

In a series of separate experiments, a direct involvement of peroxisomal LDH in the reoxidation of NADH produced by the  $\beta$ -oxidation of palmitoyl-CoA was demonstrated (Tables III–V). Thus, NADH-production rates measured in the  $\beta$ -oxidation assay were inversely proportional to the LDH activity in PO (Table III), with NADH reoxidation rates being maximal at maximal LDH activity (2 mM pyruvate). Inhibition of peroxisomal LDH by high pyruvate levels (up to 20 mM) or by the addition of oxamic acid restored NADH production rates (Table IV). A direct inhibition of palmitoyl-CoA oxidase or 3-enoyl-CoA hydratase by pyruvate leading to changes in NADH production could be excluded in our study by measuring the enzymes separately in the presence of increasing amounts of pyruvate (data not shown). Thus, the data demonstrate that peroxisomal LDH is capable of reoxidizing NADH generated in the PO and suggest that LDH may play a role in regulating the peroxisomal  $\text{NAD}^+/\text{NADH}$  ratio and in maintaining the flux of fatty acids through the peroxisomal  $\beta$ -oxidation system.

If indeed the peroxisomal LDH plays a role in regulating the peroxisomal  $\text{NAD}^+/\text{NADH}$  levels, one has to assume that the peroxisomal membrane *in vivo* displays a restricted permeability to these cofactors and that LDH constitutes a component of a shuttle system transferring reducing equivalents across the peroxisomal membrane. Fig. 11 depicts such a putative shuttle mechanism. Lactate generated inside the PO by the action of peroxisomal LDH crosses the peroxisomal membrane and is reoxidized in the cytosol to pyruvate, which reenters the PO, thus resulting in the transfer of the reducing equivalents from the PO to the cytosol.

Several LDH gene-related sequences, which may have arisen by gene duplication of the original functional LDH gene have been reported for the LDH-A and LDH-B genes (2, 46). Until now, however, only a part of these LDH gene-related sequences have been cloned, sequenced, and characterized as nonfunctional, processed pseudogenes (3, 47). Therefore, in spite of similarities in respect to kinetics, electrophoretic mobility and antigenicity between the peroxisomal and cytosolic LDH's, the possibility for the existence of functionally active peroxisomal LDH genes should not be overlooked. For members of the closely related malate dehydrogenase family, a separate gene has been found for each isozyme localized in a different cell

compartment (44).

Two distinct targeting signals for peroxisomal matrix proteins have been identified so far: a C-terminal tripeptide (SKL-variant; PTS1) and an N-terminal PTS2 (48). Furthermore, the peroxisomal proteins do not seem to require unfolding prior to import and can even be translocated over the peroxisomal membrane as oligomers (49–52). In addition, epitope-tagged truncated subunits of peroxisomal thiolase, lacking the PTS2 targeting signal, could be imported into yeast peroxisomes in association with normal subunits containing the N-terminal targeting signal (piggyback import) (51). Similar results were described for trimeric chloramphenicol acetyltransferase-PTS1 ( $\pm$ ) chimeras (52). Thus, one could speculate that a targeting signal in LDH isoform A would be sufficient to direct both A<sub>4</sub> and A<sub>3</sub>B oligomers into peroxisomes.

The cloning and complete sequencing of the cDNA for peroxisomal LDH-A (and -B) may resolve this question and may clarify which type of targeting signal (PTS1 or PTS2) is conducting the specific LDH-isoforms to the peroxisomes.

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