Biochem. J. (1992) 287, 91–100 (Printed in Great Britain)

Evidence that β-hydroxyacyl-CoA dehydrase purified from rat liver microsomes is of peroxisomal origin

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The present study provides strong evidence that the previously isolated hepatic microsomal β-hydroxyacyl-CoA dehydrase (EC 4.2.1.17), believed to be a component of the fatty acid chain-elongation system, is derived, not from the endoplasmic reticulum, but rather from the peroxisomes. The isolated dehydrase was purified over 3000-fold and showed optimal enzymic activity toward β-hydroxyacyl-CoAs or *trans*-2-enoyl-CoAs with carbon chain lengths of 8–10. The purified preparation (VDH) displayed a pH optimum at 7.5 with β-hydroxydecanoyl-CoA, and at 6.0 with β-hydroxystearoyl-CoA. Competitive-inhibition studies suggested that VDH contained dehydrase isoforms, and SDS/PAGE showed three major bands at 47, 71 and 78 kDa, all of which reacted to antibody raised to the purified preparation. Immunocytochemical studies with anti-rabbit IgG to VDH unequivocally demonstrated gold particles randomly distributed throughout the peroxisomal matrix of liver sections from both untreated and di-(2-ethylhexyl) phthalate-treated rats. No labelling was associated with endoplasmic reticulum or with the microsomal fraction. Substrate-specificity studies and the use of antibodies to VDH and to the peroxisomal trifunctional protein indicated that VDH and the latter are separate enzymes. On the other hand, the VDH possesses biochemical characteristics similar to those of the D-β-hydroxyacyl-CoA dehydrase recently isolated from rat liver peroxisomes [Li, Smeland & Schulz (1990) J. Biol. Chem. **265**, 13629–13634; Hiltunen, Palosaari & Kunau (1989) J. Biol. Chem. **264**, 13536–13540]. Neither enzyme utilizes crotonoyl-CoA or *cis*-2-enoyl-CoA as substrates, but both enzymes convert *trans*-2-enoyl substrates into the D-isomer only. In addition, the VDH also contained β-oxoacyl-CoA reductase (β-hydroxyacyl-CoA dehydrogenase) activity, which co-purified with the dehydrase.

INTRODUCTION

The mammalian hepatocyte contains several distinct enzymes which catalyse the reaction:

$$R\text{-}CH_2\text{-}CH{=}CH\text{-}CO\text{-}SCoA + H_2O \rightarrow R\text{-}CH_2\text{-}CHOH\text{-}CH_2\text{-}CO\text{-}SCoA$$

Depending on the direction of the catalysed reaction, these enzymes are called β-hydroxyacyl-CoA dehydrase (dehydratase) or enoyl-CoA hydratase.

The first mammalian hydratase to be purified to homogeneity was the bovine liver mitochondrial enzyme, crotonase, involved in the β-oxidation of fatty acids [1–3]. This enzyme was shown to have broad substrate specificity, demonstrated greatest activity with crotonoyl-CoA and progressively decreased in activity with increasing chain length of *trans*-2-enoyl-CoAs [3,4]. A similar substrate specificity was reported for the purified pig heart crotonase [5]. The isolation and partial purification of another hydratase, termed long-chain enoyl-CoA hydratase, from pig heart was reported by Schulz [6]. Unlike the crotonase, this enzyme was virtually inactive with crotonoyl-CoA, and the highest $V_{max.}$ was obtained with *trans*-2-octenoyl-CoA. Furthermore, whereas crotonase activity was significantly inhibited by acetoacetyl-CoA [4], the long-chain enzyme was relatively unaffected by acetoacetyl-CoA [6]. Schulz [6] reported that this enzyme was also associated with the mitochondrial fraction, and, on the basis of sequential extraction experiments, indicated that the enzyme was more tightly associated with the membranes than was crotonase.

Although microbodies (peroxisomes) were identified morphologically in liver in the mid-1950s (for review see [7]), their important role in lipid metabolism was not discovered until 1976, when peroxisomes were reported to possess an acyl-CoA-oxidation system different from that of the mitochondria [8]. One of the enzymes of the peroxisomal system, enoyl-CoA hydratase, was isolated and purified by several laboratories [9–13], and possessed molecular and catalytic properties which differed from those of the mitochondrial hydratase (crotonase). The peroxisomal hydratase had been described as a bifunctional protein [9,10], since it was shown also to have β-hydroxyacyl-CoA dehydrogenase activity. More recently, this same protein was found to contain isomerase activity (Δ^3–Δ^2-enoyl-CoA isomerase), making this polypeptide a trifunctional enzyme [14]. A second peroxisomal hydratase, called D-3-hydroxyacyl-CoA dehydratase, has been purified recently and shown to have a subunit molecular mass of 44 kDa, and is active with *trans*-2-enoyl-CoA substrates of greater than 4 carbon atoms (inactive toward crotonoyl-CoA) and catalyses the conversion of D-3-hydroxyacyl-CoA into *trans*-2-enoyl-CoA [15]; this hydratase may be similar to that described by Hiltunen *et al.* [16].

The soluble portion of the cytoplasm of the cell also contains a D-3-hydroxyacyl dehydratase activity, the component enzyme of the multifunctional fatty acid synthase [17]. Thus a total of five separate enzymes (two mitochondrial, two peroxisomal and one cytosolic) which catalyse reversible hydration reactions involved in fatty acid metabolism have been isolated from the hepatocyte.

A sixth enzyme is associated with the fatty acid chain-elongation system (FACES) present in the endoplasmic reticulum of the hepatocyte. A single report on the solubilization and

Abbreviations used: FACES, fatty acid chain-elongation system; DEHP, di-(2-ethylhexyl) phthalate; VDH, fraction V dehydrase; TFP, trifunctional protein; PBS, phosphate-buffered saline (1.0 mM-sodium phosphate/2.6 mM-KCl/140 mM-NaCl, pH 7.4).

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partial purification of this enzyme, called β-hydroxyacyl-CoA dehydrase, was published in the late 1970s [18], a period during which increasing interest in the isolation of peroxisomes and their constituent enzymes was just developing. For the past 5 years, our laboratory has focused on the purification of the microsomal FACES dehydrase [19]. The purpose of the present study is to provide evidence that the previously partially purified microsomal dehydrase [18] is not a component of the FACES of the endoplasmic reticulum. Rather, it is an enzyme associated with the peroxisomes and possesses catalytic properties similar to the enzyme recently isolated by Schulz's laboratory [15].

MATERIALS AND METHODS

Chemicals

All biochemicals, including Reactive Red Type 3000-CL agarose, were obtained from Sigma (St. Louis, MO, U.S.A.); chemicals used in gel electrophoresis and Bio-Gel HTP (hydroxyapatite) were obtained from Bio-Rad (Rockville Centre, NY, U.S.A.); general chemicals were obtained from Baker (Phillipsburg, NJ, U.S.A.). Sephadex G-200 and chromatofocusing supplies were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ, U.S.A.). D-β-Hydroxyoctanoyl-CoA, L-β-hydroxyoctanoyl-CoA and *cis*-2-octenoyl-CoA were kindly provided by Professor Horst Schulz (City University of New York).

Isolation of hepatic microsomes

Male Sprague–Dawley rats (200–250 g) were given access *ad libitum* to Purina rat chow and tap water. For studies involving treatment with di-(2-ethylhexyl) phthalate (DEHP, peroxisomal proliferator), rats were fed on ground Purina rat chow containing 2% (w/w) DEHP for 8 days. All animals were killed by decapitation between 07:30 and 08:00 h. Livers were removed, perfused with 0.9% NaCl and homogenized in 5 vol. of 0.25 M-sucrose containing 0.01 M-potassium phosphate, pH 7.4. Initial centrifugation was performed at 700 *g* for 10 min, and the pellet was discarded. The supernatant was then centrifuged at 7500 *g* for 10 min, followed by 17500 *g* for another 10 min. Centrifugation of the supernatant fraction at 105000 *g* for 45 min yielded the microsomal pellet, which was rehomogenized in 0.1 M-potassium phosphate, pH 7.4, containing 0.6 M-KCl, recentrifuged at 105000 *g* for 45 min, and resuspended in 0.25 M-sucrose/0.01 M-potassium phosphate, pH 7.4, giving a final protein concentration of 25 mg/ml; this suspension was freeze-dried as reported by Bernert & Sprecher [18]. The homogenization of the microsomal pellet in 0.6 M-KCl removes more than 90% of the contaminating peroxisomal trifunctional *trans*-2-enoyl-CoA hydratase, which was released from peroxisomes during subcellular fractionation and was found to have a high affinity for both free and membrane-bound ribosomes [13,20]. This contaminating peroxisomal enzyme can catalyse the reversible hydration of *trans*-2-enoyl-CoAs with chain lengths of up to 12–14 carbons, and hence can cause misleading results during catalytic analysis of microsomal dehydrase activity.

Peroxisomes used for electron-microscopy immunocytochemistry studies were isolated from the 7500 *g* pellet and layered on a discontinuous sucrose gradient as described by Cook *et al.* [21]. The protein was determined by the method of Bradford [22].

Purification of the β-hydroxyacyl-CoA dehydrase

Freeze-dried microsomes were solubilized with 0.5% sodium deoxycholate and fractionated with $(NH_4)_2SO_4$ by the procedure of Bernert & Sprecher [18]. The 33%-satd-$(NH_4)_2SO_4$ pellet was resuspended in a minimum volume of 0.01 M-potassium phosphate buffer, pH 7.0, containing 2% Triton X-100. This mixture was dialysed for 18 h against 2 × 4 litres of 0.01 M-potassium phosphate, pH 7.0, containing 0.2% Triton X-100 (buffer A). After centrifugation at 105000 *g* for 1 h, the supernatant fraction containing the dehydrase activity was applied to a Whatman CM-cellulose (CM-52) column (1.6 cm × 20 cm), previously equilibrated with buffer A, to which 70–80% of the dehydrase activity adsorbed. The column was washed with buffer A until the baseline analysed with an Altex u.v. monitor (280 nm) returned to 0 (approx. 75 ml) and the adsorbed dehydrase activity was eluted with a linear gradient of 0.01–0.20 M-potassium phosphate in 200 ml of buffer A. The fractions containing dehydrase activity, which was eluted with 0.025 M-phosphate, were then pooled and dialysed overnight against buffer A. {It should be noted that the DEAE-cellulose (DE-52) column employed in the Bernert & Sprecher [18] procedure did not yield reproducible results. There was always a wide range of enzyme activity in the void volume (40–70%). More importantly, this chromatographic step never resulted in a greater than 2–3-fold purification, and therefore was omitted.}

After dialysis, the enzyme fraction was applied and 100% of the dehydrase activity was adsorbed to a 1 ml Reactive Red column which had been equilibrated with buffer A. After washing the column with 20 ml of buffer A, the dehydrase activity was eluted with 0.8 M-KCl in buffer A. After overnight dialysis, the dehydrase preparation (≤ 3 ml) was applied to a calibrated Sephadex G-200 (2.6 cm × 53 cm) column equilibrated in buffer A. The eluted Sephadex G-200 fractions containing dehydrase activity were pooled and 0.025 M-ethanolamine, pH 10.0, containing 0.2% Triton X-100 (buffer B) was added until pH 9.5 was obtained. The pH-adjusted dehydrase fraction was then applied to a Pharmacia PBE 94 column (0.9 cm × 20 cm) equilibrated with buffer B for chromatofocusing. The adsorbed dehydrase activity was eluted when a pH gradient (10.0–6.3) was formed by passing through the column 200 ml of 30% Polybuffer 96, pH 6.0, containing 0.2% Triton X-100. Fractions containing dehydrase activity were pooled, glycerol was added to a final concentration of 5%, and they were then dialysed for 24 h in 50 kDa-cut-off dialysis tubing against 0.01 M-potassium phosphate, pH 7.0, containing 0.2% Triton X-100 and 5% glycerol (buffer C) to remove Polybuffer 96. The pH of all the fractions was measured within 12 h after elution from the column. The enzyme was then applied to a 1 ml hydroxyapatite column previously equilibrated with buffer C, washed with 100 ml of buffer C to remove further Polybuffer 96, and eluted with buffer C containing 0.2 M-potassium phosphate. The dehydrase fractions eluted from the hydroxyapatite column were pooled, dialysed overnight against buffer C and used for the biochemical studies. Fraction V dehydrase (VDH) is the final preparation obtained from the hydroxyapatite column and used to prepare antibody (described below).

All purification steps were performed at 4 °C. The method involving chelation of bicinchoninic acid with copper ions developed by Pierce Chemical Co. (Rockford, IL, U.S.A.) [23] was employed for protein determination of the column fractions, since detergent interfered with the Bradford procedure.

Enzyme assays

The β-hydroxyacyl-CoA dehydrase activity was assayed by recording the initial increase in A_{280} [24] resulting from the dehydration of β-hydroxyacyl-CoA, and the reverse reaction was determined by measuring a decrease in A_{280} after conversion of *trans*-2-enoyl-CoA into the β-hydroxy derivative. The assay mixture in the cuvette contained 0.1 M-potassium phosphate,

pH 7.4, 250 μg of Triton X-100 (during measurement of dehydrase), and 5–25 μl of the enzyme sample in a total volume of 1.0 ml. After equilibration at 37 °C, 50 μM-β-hydroxyacyl-CoA or -*trans*-2-enoyl-CoA, unless otherwise indicated, was added directly to the cuvette, mixed rapidly, and a change in A_{280} was recorded with a Gilford 240 spectrophotometer with a kinetic recorder. The enzyme activity was expressed as nmol or μmol/min per mg of protein, by using an absorption coefficient of 4.4 $mM^{-1} \cdot cm^{-1}$ [4]. Condensation, NADPH:β-oxoacyl-CoA reductase, NADPH:*trans*-2-enoyl-CoA reductase and total elongation activities were measured as described previously [25]. Stearoyl-CoA (Δ^9) desaturase activity was determined as described by Montgomery & Cinti [26]. The method of Yasukochi & Masters [27] was used to measure NADPH:cytochrome *c* (*P*-450) reductase activity, and the procedure of Strittmatter [28] was employed to determine NADH:cytochrome b_5 reductase activity. Both cytochrome b_5 and *P*-450 contents were measured as described by Omura & Sato [29].

β-Hydroxyacyl-CoA dehydrogenase activity was determined by addition of 20–50 μM-β-hydroxyacyl-CoA and 5–25 μl of enzyme preparation to a cuvette containing 0.1 M-potassium phosphate, pH 7.4, in a total volume of 1.0 ml. The reaction was initiated by addition of 5 mM-NAD^+ and the increase in A_{340} was recorded. For the measurement of NADH:β-oxoacyl-CoA reductase activity, 50 μM-β-oxoacyl-CoA was added to a cuvette containing 5–25 μl of enzyme preparation in 0.1 M-potassium phosphate, pH 7.4, in a final volume of 1.0 ml. A decrease in A_{340} was recorded after addition of 100 μM-NADH. Both enzymic assays were performed at 37 °C and the activities expressed as μmol/min per mg of protein by using an absorption coefficient of 6.2 $mM^{-1} \cdot cm^{-1}$. Δ^3–Δ^2-enoyl CoA isomerase activity was measured by the method of Palosaari & Hiltunen [14].

Substrate synthesis

The procedure described by Stoffel & Pruss [30] was used to synthesize the β-hydroxyacyl acids, which were then converted into CoA esters by the mixed-anhydride procedure [31]. Chemical synthesis of the *trans*-2-enoyl-CoAs was as described previously [32], and the β-oxoacyl-CoAs were synthesized by the procedure of Al-Arif & Blecher [33]. Δ^3-*cis*-$C_{10:1}$ acid was also chemically synthesized as described by Stoffel & Ecker [34] and then converted into the CoA esters [31]. The concentration of the synthesized CoAs was measured by the method of Ellman [35], after cleavage of the thioester bond with hydroxylamine.

Isolation of enoyl-CoA hydratase (trifunctional protein, TFP) and preparation of antibody

The peroxisomal TFP was purified and anti-TFP IgG was prepared as described previously [13]. The quantities of enzyme and IgG used in several experiments are indicated in the appropriate Figure legends.

Gel electrophoresis

SDS/PAGE (9% gel) was performed in a discontinuous buffer system by the method of Laemmli [36] and then gels were stained with Coomassie Brilliant Blue R.

Immunization of rabbits with VDH and isolation of IgG fraction

Before vaccination, rabbits were bled to obtain pre-immune sera. The purified VDH (100 μg/0.5 ml) isolated from rat liver microsomes was mixed with Freund's complete adjuvant (1:1, v/v) and injected subcutaneously into 20 different shaved areas on the back of an adult male New Zealand White rabbit, with boosters given 7, 14 and 21 days after the initial vaccination. The rabbit was bled 36 days after the initial injection and the serum produced immunoprecipitate bands on Ouchterlony plates.

Separation and concentration of the IgG fraction were performed with a 1 ml Protein A–Sepharose column as described previously [13].

Immunodiffusion and electrophoretic blotting

For immunodiffusion, 1 g of agarose was dissolved in 100 ml of water with constant stirring at 90 °C and then prepared as described by Bailey [37]. For electrophoretic blotting, the proteins were first subjected to PAGE (9% gel) as described by Laemmli [36]. The proteins were then electroblotted on to nitrocellulose and developed as described previously [13].

Electron-microscopic immunocytochemistry

Livers from control or DEHP-treated rats were fixed by perfusion of room-temperature fixative solution containing 1% glutaraldehyde or 3% paraformaldehyde/0.15% glutaraldehyde in 0.1 M-sodium cacodylate buffer, pH 7.4. After perfusion, part of the major anterior lobe was cut into small pieces and immersed in fresh fixative for a total of 60–80 min. The fixed liver samples were rinsed and stored at 4 °C in 0.1 M-cacodylate buffer, pH 7.4, containing 7% (w/v) sucrose. Some samples were treated with aq. 0.5% uranyl acetate for 1 h. Tissue blocks were either (*a*) dehydrated in ethanol solutions and embedded in LR White resin, with polymerization at 60 °C overnight, or (*b*) dehydrated through 90% methanol, embedded in Lowicryl K4M at −20 °C, and polymerized at −20 °C for 5–7 days under u.v. light (wavelength 360 nm). Peroxisomal and microsomal fractions, prepared as described above, were fixed for 1 h in 1% glutaraldehyde/0.1 M-cacodylate, pH 7.4, then dehydrated in ethanol and embedded in LR White resin. Thin sections were cut with a diamond knife and collected on uncoated (LR White) or Formvar-coated (Lowicryl) nickel grids.

Immuno-gold labelling was performed essentially as described by Roth *et al.* [38] and Bendayan *et al.* [39]. The thin sections were treated with 1% BSA or 1% BSA/1% skim milk in phosphate-buffered saline (PBS) for 30 min, then incubated at 4 °C for 16–22 h with antiserum to VDH, diluted 1:100 in PBS containing 5% (v/v) normal goat serum. After rinsing in PBS and treatment with 1% BSA for 10 min, the sections were incubated for 1 h at room temperature with colloidal gold coated with goat anti-rabbit IgG (10 nm diameter; Amersham), then diluted in PBS. After thorough rinsing with PBS and distilled water, the sections were stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope.

RESULTS AND DISCUSSION

Enzyme isolation and purification

The early stages of purification through the CM-cellulose step followed the procedure developed by Bernert & Sprecher [18], which included (*a*) freeze-drying of the microsomal fraction, (*b*) solubilization by 0.5% sodium deoxycholate, (*c*) 20%- and 33%-$(NH_4)_2SO_4$ fractionation and (*d*) CM-cellulose CM-52 chromatography. Bernert & Sprecher [18] reported a 95-fold purification of the β-hydroxyacyl-CoA dehydrase, yielding a specific activity of 4.2 μmol of *trans*-2-octadecenoate formed/min per mg of protein after CM-cellulose chromatography. As shown in Table 1, our results were similar after CM-cellulose chromatography, generating 120-fold purification and a specific activity of 2.8 μmol/min per mg of protein. The dehydrase activity was eluted as a single peak (Fig. 1*a*) between fractions 20 and 31 with

Table 1. Purification of β-hydroxyacyl-CoA dehydrase

The data represent means of six different preparations. The dehydrase activity was measured with 50 μM-β-hydroxyacyl CoA as described in the Materials and methods section, except that pH 6.5 was employed to mimic the conditions used by Bernert & Sprecher [18] when β-hydroxystearoyl-CoA ($C_{18:0}$) dehydration was measured. The β-hydroxydecanoyl-CoA (C_{10}) dehydration was measured at pH 7.4.

Substrate...	β-Hydroxydecanoyl-CoA			β-Hydroxystearoyl-CoA		
Purification step	Specific activity (μmol/min per mg)	Total activity (μmol/min)	Purification (fold)	Specific activity (μmol/min per mg)	Total activity (μmol/min)	Purification (fold)
Freeze-dried microsomes	0.116	315.5	1.0	0.022	59.84	1.0
33%-$(NH_4)_2SO_4$ pellet	0.234	275.3	2.0	0.028	32.93	1.3
CM-cellulose (fraction I)	26.80	243.9	231	2.79	25.39	127
Reactive Red Agarose (fraction II)	35.55	203.7	306	4.19	24.01	190
Sephadex G-200 (fraction III)	71.10	150.0	613	8.80	18.57	400
Chromatofocusing (fraction IV) Hydroxyapatite (fraction V)	402	60.3	3466	70	10.50	3182

a phosphate concentration of 25–50 mM, results similar to those reported by Bernert & Sprecher [18]. This single peak contained both β-hydroxydecanoyl-CoA (C_{10}) and β-hydroxystearoyl-CoA (C_{18}) dehydrase activities. No dehydrase activity could be detected between fractions 40 and 120.

Fraction I (CM-cellulose) was concentrated on a Reactive Red–agarose column and then applied to a Sephadex G-200 column. The elution profile (Fig. 1*b*) shows a single activity peak, and, since purity rather than yield was of prime interest, only fraction samples between 152 and 174 were pooled. This purification step generated a 2-fold increase in specific activity, to 71 and 9 μmol/min per mg of protein with the C_{10} and C_{18} substrates respectively. The selection of the Sephadex G-200 column was based on the fact that, under our experimental conditions, the use of Sephadex G-100 resulted in the elution of the dehydrase in the void volume, suggesting that the enzyme was a large polypeptide, protein aggregate or an aggregate of Triton X-100 and protein. In the next purification step, chromatofocusing (Fig. 1*c*) yielded a fraction containing the peak dehydrase activity which was eluted at pH 8.7–8.8. This step (Table 1) increased the specific activity of the dehydrase with the C_{18} substrate to 70 μmol/min per mg of protein and to 400 with the C_{10}, achieving a purification of greater than 3000-fold, or 30 times greater than the previously published purification [18]. The low yield of 15–20% was, in large part, due to a decrease in the number of pooled fraction samples to achieve greater purity.

All ensuing studies were performed with VDH (Table 1). Neither TFP activity, a peroxisomal enzyme, nor crotonase activity, a mitochondrial enzyme, both of which possess dehydrase activity, was present in the purified fraction. In addition, there was no detectable (1) Δ^9 desaturase activity, (2) microsomal electron-transport components and (3) elongation components (condensing enzyme, NADPH:β-oxoacyl-CoA and *trans*-2-enoyl-CoA reductases).

Biochemical properties of β-hydroxyacyl-CoA dehydrase fraction V (VDH)

The dehydrase activity was linear with protein concentration ranging from 25 ng to at least 450 ng (highest concentration tested; results not shown). Non-linear concave curves, as reported by Bernert & Sprecher [18] with their partially purified enzyme, were not observed with our purified enzyme preparation. Those authors were able to achieve linearity, however, when optimal amounts of Triton X-100 were included in the dehydrase assay. Furthermore, they observed that addition of 0.5–0.6 mg of Triton X-100 to the dehydrase assay mixture containing 1.5 μg of protein resulted in a stimulation of activity (2.5-fold with respect to β-hydroxystearoyl-CoA). The addition of increasing amounts of Triton X-100 up to 500 μg to our purified enzyme did not produce a further stimulation of dehydrase activity. The reason for the lack of Triton X-100-induced stimulation with our enzyme preparation may reside in the fact that the enzyme (50 ng in the assay) is already associated with 20 μg of Triton X-100, representing a 400:1 ratio of detergent to protein. The enzyme sample used by Bernert & Sprecher [18] contained a detergent/protein ratio of approx. 100:1. Interestingly, calculations made from their results [18] indicate that maximal stimulation of dehydrase activity was obtained when the ratio reached approx. 400:1.

When a series of β-hydroxyacyl-CoAs of carbon chain lengths (Fig. 2) ranging from C_4 to C_{18} was tested, the purified dehydrase was unable to dehydrate β-hydroxybutyryl-CoA (C_4). However, an additional two-carbon increase in chain length (C_6) did result in significant dehydrase activity. The enzyme was most active towards a carbon chain length of 10, progressively decreasing in activity as the chain length increased to 18 carbons. In their study, Bernert & Sprecher [18] evaluated carbon chain lengths of 12–20 and found that C_{14} generated the highest activity, followed in decreasing order by 12, 16, 18 and 20. None of the short-chain derivatives was tested by those authors.

As also shown in Fig. 2, the purified dehydrase was very active in catalysing the reverse reaction, i.e. the conversion of *trans*-2-enoyl-CoA into the β-hydroxyacyl-CoA. Interestingly, the reverse reaction was faster with C_6, C_8, C_{10} and C_{12}; hence the ratio of activities of the reverse to the forward reaction was greater than 1.0 for each substrate from C_6 to and including C_{14}, but with a declining ratio. These results raise two perplexing questions: (1) why is the dehydrase more active toward short- to intermediate-chain substrates? and (2) why is the reverse reaction faster for substrate of chain length C_6–C_{14}? Some insight into these questions is provided below.

Kinetic parameters were determined for two substrates, namely β-hydroxydecanoyl-CoA, the most active substrate, and β-hydroxystearoyl-CoA, the intermediate in the elongation of palmitoyl-CoA (Table 2). As expected the $V_{max.}$ was greatest for the shorter-chain CoA derivative, whereas the K_m values were similar. In general, the reverse reaction provided similar results.

The effect of pH on the rate of reaction catalysed by the dehydrase was also analysed with the two substrates, β-hydroxydecanoyl-CoA and β-hydroxystearoyl-CoA. Using the

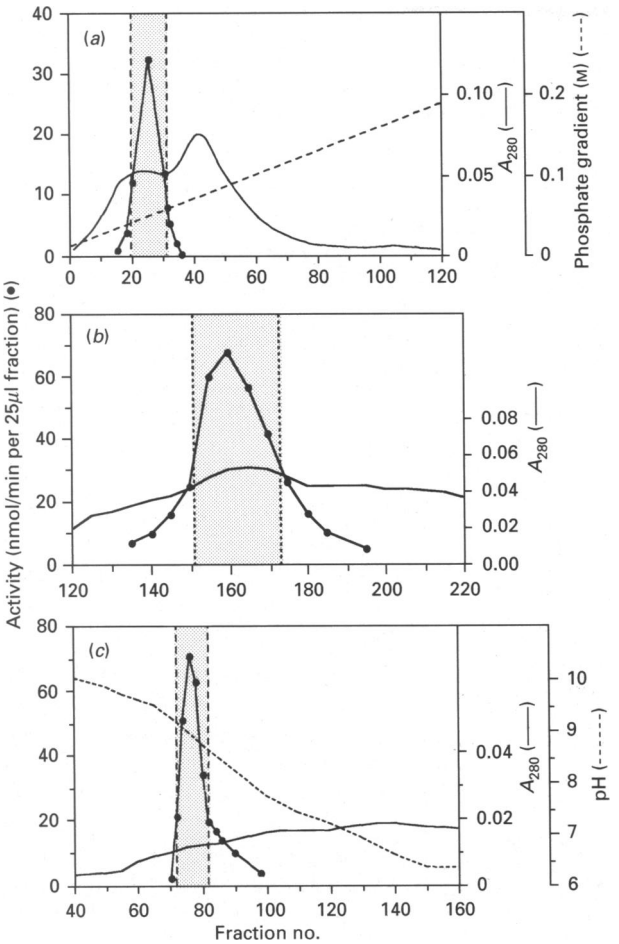

Fig. 1. Column-chromatography elution profiles of β-hydroxyacyl-CoA dehydrase activity

(*a*) The 33%-$(NH_4)_2SO_4$ pellet fraction was applied to a CM-cellulose (CM-52) column (1.6 cm × 20 cm) and the adsorbed dehydrase activity (●) was eluted with a linear gradient of 0.01–0.2 M-potassium phosphate (----) in 200 ml of buffer A as described in the Materials and methods section. Approx. 2 ml fractions were collected and tubes 20–31 were pooled (shaded area). (*b*) Dialysed Reactive Red pooled fraction was applied to a calibrated Sephadex G-200 column (2.6 cm × 52 cm) equilibrated with buffer A. Approx. 1 ml fractions were collected and tubes 152–174 were pooled (shaded area). (*c*) Pooled Sephadex G-200 fraction was applied to a chromatofocusing column (0.9 cm × 20 cm) developed with a pH gradient 9.0–6.3 (----). Approx. 1.5 ml fractions were collected and tubes 72–82 were pooled (shaded area) and dialysed as described in the Materials and methods section. The dehydrase activity for all three columns was measured with β-hydroxydodecanoyl-CoA and β-hydroxystearoyl-CoA as substrates. Protein was monitored continuously at 280 nm (——) with an Altex u.v. monitor for all three chromatographic steps. The data represent the means of 5–12 separate columns run under identical conditions.

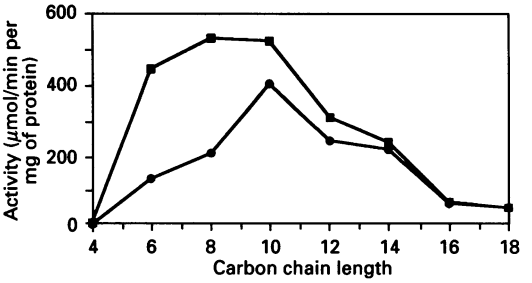

Fig. 2. Substrate specificity of VDH

The substrate specificity was determined with β-hydroxyacyl-CoAs and *trans*-2-enoyl-CoAs ranging in chain length from C_4 to C_{18}. The β-hydroxyacyl-CoA dehydrase activity was determined as described in the Materials and methods section, with 50 μM substrate for C_{12}–C_{18} and 100 μM for C_4–C_{10} (●). The reverse reaction, *trans*-2-enoyl-CoA hydratase activity (■), was measured with 50 μM substrate for C_{12}–C_{18}, 100 μM for C_6–C_{10} and 200 μM for C_4 as described in the Materials and methods section.

Table 2. Kinetic parameters of the purified dehydrase

β-Hydroxyacyl-CoA dehydrase and *trans*-2-enoyl-CoA hydratase (backward reaction) activities were determined as described in the Materials and methods section. The substrate concentration used ranged from 2.5 μM to 100 μM.

Substrate	K_m (μM)	$V_{max.}$ (μmol/min per mg)
β-Hydroxydecanoyl-CoA	40.0	450.0
β-Hydroxystearoyl-CoA	37.0	45.3
trans-2-Decenoyl-CoA	15.9	537.6
trans-2-Octadecenoyl-CoA	33.3	46.8

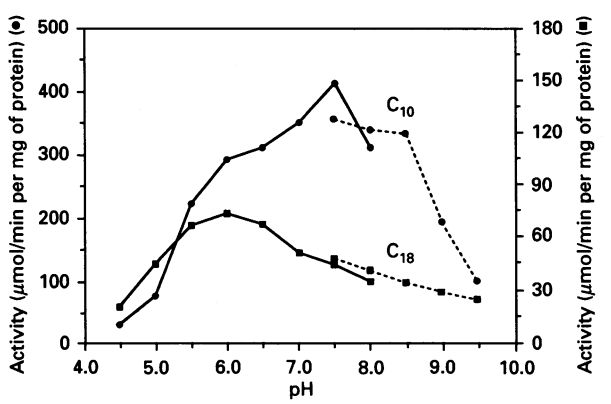

Fig. 3. Effect of pH on the β-hydroxyacyl-CoA dehydrase activity of VDH

By using 50 ng of fraction V enzyme, the β-hydroxydecanoyl-CoA dehydrase activity was measured with 0.1 M-potassium phosphate (●——●), pH 4.5–8.0, or 0.1 M-Tris/HCl (●---●), pH 7.5–10.0. The β-hydroxyoctadecanoyl-CoA dehydrase activity was measured with 0.1 M-potassium phosphate (■——■) or 0.1 M-Tris/HCl (■---■), by using 350 ng of fraction V enzyme. Activities were measured as described in the Materials and methods section, with 100 μM-hydroxydecanoyl-CoA or 50 μM-hydroxyoctadecanoyl-CoA as substrate.

latter substrate, Bernert & Sprecher [18] found three pH optima, at 6.5, 8.5 and 10, for their dehydrase, although the specific activities at each pH optimum were very similar. A pH/activity analysis using VDH showed that the pH optimum for β-hydroxystearoyl-CoA was approx. 6.0, whereas the enzymic activity with β-hydroxydecanoyl-CoA showed a somewhat broader pH optimum between 7.0 and 8.5 (Fig. 3). These results prompted us to perform competitive-inhibition studies using 20 μM-[1-^{14}C]-*trans*-2-octadecenoyl-CoA (*trans*-2-18:1) as the substrate and the more reactive *trans*-2-decenoyl-CoA (*trans*-2-10:1) as the competitive inhibitor. Since *trans*-2-10:1 had a $V_{max.}$ value 10 times that for *trans*-2-18:1, and a K_m value that was similar (Table 2), it was expected that *trans*-2-10:1 would be a potent inhibitor of the hydration of *trans*-2-18:1. On the contrary, it was observed that in the presence of radiolabelled 20 μM-*trans*-

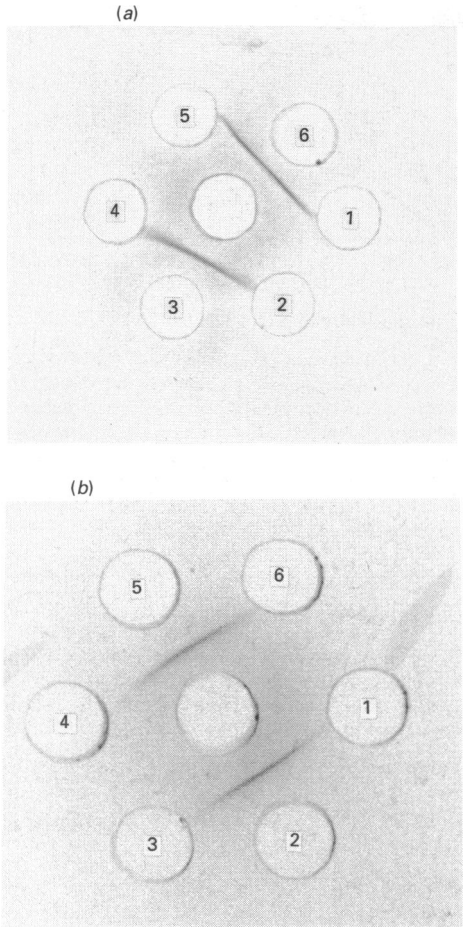

Fig. 4. Ouchterlony double-diffusion immunoprecipitation analysis using antiserum towards purified peroxisomal TFP on mitochondrial (crotonase), TFP and VDH

Wells 1 and 4 contained 4 and 8 μg respectively of crotonase from bovine liver (Sigma); wells 2 and 5 contained 4 and 8 μg respectively of VDH; and wells 3 and 6 contained 4 and 8 μg respectively of purified TFP. The centre well contained 25 μl of antiserum (*a*) to peroxisomal TFP or (*b*) to VDH. Ouchterlony conditions were as described in the Materials and methods section.

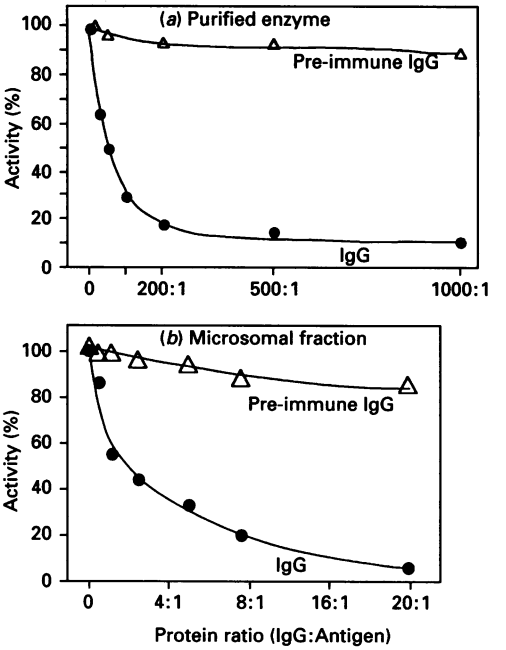

Fig. 5. Effect of anti-VDH IgG and pre-immune IgG on purified VDH or microsomal β-hydroxydodecanoyl-CoA hydratase activity

(*a*) Increasing amounts of pre-immune IgG (△) or immune IgG (●) were preincubated for 10 min at room temperature with 50 ng of purified VDH. (*b*) Increasing amounts of pre-immune IgG or anti-IgG were preincubated for 10 min at room temperature with 50 μg of microsomes from liver of untreated rats; 500 μg of Triton X-100 was included in the assay. The β-hydroxydodecanoyl-CoA dehydrase activity was measured as described in the Materials and methods section.

2-18:1 and either 20 μM or 80 μM unlabelled *trans*-2-10:1, there was zero and at most 9% inhibition of the formation of β-hydroxystearoyl-CoA respectively, suggesting that more than one dehydrase form existed in fraction V.

Although VDH was unable to catalyse NADPH:β-oxoacyl-CoA reduction, there was NADH-dependent β-oxoacyl-CoA reductase activity (also called NAD^+:β-hydroxyacyl-CoA dehydrogenase) associated with this fraction. With β-oxododecanoyl-CoA (the shortest carbon-chain length chemically synthesized in our laboratory), the specific activity of the β-ketoacyl-CoA reductase in fraction V was 50 μmol/min per mg of protein; this high activity was approx. 3000-fold greater than the reductase activity in the solubilized microsomes, which was 15 nmol/min per mg of protein. The reductase activity co-purified with the dehydrase at a dehydrase/reductase activity ratio of 4–9:1 at each purification step; the final step (fraction V) had an activity ratio of 6:1. These results suggest at least two interpretations: (1) that a second and separate enzyme has co-purified with the dehydrase, or (2) that the dehydrase and the reductase (dehydrogenase) represent two separate activities associated with a single enzyme, similar to the peroxisomal TFP. Although the latter protein was most active toward crotonoyl-CoA hydration and NADH-dependent acetoacetyl-CoA reduction, VDH exhibited virtually no activity toward these two substrates. Recently, Palosaari & Hiltunen [14] reported the presence of Δ^3–Δ^2-enoyl-CoA isomerase activity associated with the purified peroxisomal dehydrase. Using their assay procedure [14] and *cis*-3-decenoyl-CoA as substrate, we did not find any Δ^3–Δ^2-enoyl-CoA isomerase activity in VDH. Other differences between our purified preparation and TFP are presented below.

Studies with antibodies to TFP and to fraction V

On the basis of differences in substrate specificities and the absence of isomerase activity in fraction V, TFP and VDH appear to be separate enzymes. As reported previously [13], antibody raised to TFP inhibited both the hydration of crotonoyl-CoA and the reduction of acetoacetyl-CoA, two major reactions catalysed by TFP. Pre-immune IgG had no effect on either enzyme activity. When the antibody to the TFP was tested against VDH, neither the forward reaction, employing β-hydroxydecanoyl-CoA, nor the reverse reaction, employing *trans*-2-hexenoyl-CoA, was affected by the anti-TFP IgG. *trans*-2-Hexenoyl-CoA was selected because it is an excellent substrate for both TFP [10,13] and VDH (Fig. 2). Ouchterlony immunodiffusion analysis with TFP and immune serum depicted distinct precipitin lines between the antibody in the centre well and the purified TFP (wells 3 and 6), whereas none was observed against either VDH (wells 2 and 5) or purified mitochondrial crotonase (wells 1 and 4) (Fig. 4*a*); pre-immune serum did not show any precipitin bands with the TFP.

Antibody raised to the VDH formed precipitin lines with the VDH enzyme preparation (Fig. 4*b*, wells 2 and 5). However, none formed against either the purified TFP (wells 3 and 6) or the

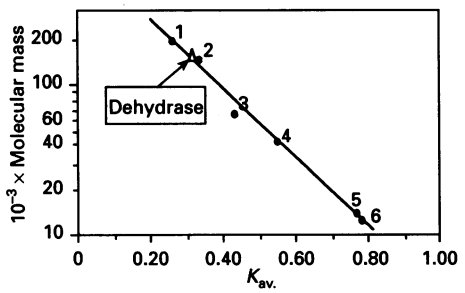

Fig. 6. Calibration curve for Sephadex G-200: $K_{av.}$ versus log of the molecular mass of standard proteins applied to a Sephadex G-200 column

Standard proteins in order of decreasing molecular mass were (1) β-amylase (200 kDa), (2) alcohol dehydrogenase (150 kDa), (3) BSA (66 kDa), (4) ovalbumin (45 kDa), (5) RNAase A (13.7 kDa) and (6) cytochrome *c* (12.4 kDa). The molecular mass of the purified VDH (△) is the average of ten separate preparations chromatographed on Sephadex G-200.

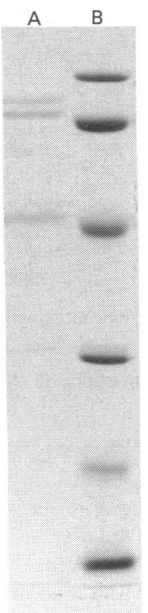

Fig. 7. SDS/PAGE of VDH

Lane A contains 9 μg of purified VDH. The standard proteins (7 μg each) in lane B are (from top to bottom) phosphorylase *b* (97 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

mitochondrial crotonase (wells 1 and 4), establishing that VDH is distinct from either the peroxisomal TFP or the mitochondrial crotonase. Anti-VDH IgG was also effective in blocking β-hydroxyacyl-CoA dehydrase activity. As shown in Fig. 5(*a*), an IgG:antigen ratio of 200:1 resulted in over 80% inhibition of β-hydroxydodecanoyl-CoA conversion into *trans*-2-dodecenoyl-CoA. When tested against the dehydrase activity in the microsomal fraction, 80% inhibition was observed with a ratio of 8:1 (Fig. 5*b*). Pre-immune IgG showed less than 20% inhibition with either preparation. In addition, anti-VDH IgG had no affect on TFP dehydrase activity (results not shown). Hence, on the basis of antibody studies as well as the substrate-specificity analysis, the data strongly support the conclusion that TFP and VDH are separate enzymes.

Analysis of VDH by SDS/PAGE and Western blotting

When either Fraction II or VDH was applied separately to a Sephadex G-200 column standardized with proteins ranging in molecular mass from 12.4 kDa (cytochrome *c*) to 200 kDa (β-amylase), the enzyme activity was eluted in both cases shortly after the void volume in the same fraction in which the standard, alcohol dehydrogenase, was eluted. A plot of $K_{av.}$ versus the log of the molecular mass (Fig. 6) yielded a value of 150 kDa for VDH. When VDH was subjected to SDS/PAGE, three major bands appeared, corresponding to molecular masses of 47, 71 and 78 kDa (Fig. 7). In addition, two other faint bands at 30 kDa and 50 kDa were apparent. However, Western immunoblotting revealed only the three bands corresponding to 47, 71 and 78 kDa. In an attempt to correlate the size of the native enzyme (approx. 150 kDa) with bands on the gel, VDH was subjected to two-dimensional gel electrophoresis. However, the protein was not able to penetrate the non-denatured gel; application of the Ames & Nikaido [40] modification of the O'Farrell [41] procedure did not correct the problem. It was not simply a technical difficulty, since two-dimensional gels were obtained (1) with the purified TFP, whose size is 76 kDa and has a pI of 8.6 [13], and (2) with crotonase, a hexamer of approx. 160 kDa having a 27 kDa subunit. Lowering the acrylamide concentration to 5% did not affect the penetration. At present we are unable to establish unequivocally whether the dehydrase is a single polypeptide or a protein of two or more subunits.

Immunocytochemical localization of VDH

Labelling of thin sections of livers from DEHP-treated rats embedded in LR White resin with antibody to VDH, followed by colloidal gold coated with goat anti-rabbit IgG, revealed gold particles concentrated over peroxisomes (Fig. 8). Within the peroxisomes, the label appeared to be randomly distributed throughout the matrix. Few particles were observed over mitochondria, smooth and rough endoplasmic reticulum, or nuclei. Treatment of fixed liver slices with 0.1% Triton X-100 in 0.01 M-phosphate buffer for 1 h at 4 °C before dehydration and embedding did not appreciably alter the immunoreactivity or distribution of the gold label. A similar distribution of immunoreactivity was observed with livers from control rats. Identical results were obtained with sections of Lowicryl-embedded liver, and with ultrathin frozen sections labelled by a similar procedure (results not shown).

Immunocytochemical control incubations were performed with preimmune serum instead of the primary antibody, or by omitting the primary antibody from the labelling sequence. Under these conditions, only a few randomly distributed gold particles were seen over the sections (results not shown).

Examination of peroxisomal pellets prepared from livers of control and DEHP-treated rats revealed that most of the peroxisomes were apparently disrupted or leaky, as they contained only scattered clumps of dense matrix material. Nonetheless, immunolabelling of these sections with antibody to VDH revealed gold particles over the residual peroxisomal content, and over apparent matrix material present outside the peroxisomes. In contrast, sections of microsomal pellets labelled with the antibody to VDH demonstrated virtually no immunoreactivity, even in the presence of Triton X-100 (results not shown). These immunochemical studies with liver slices and the antibody to VDH demonstrate unequivocally that the β-hydroxyacyl-CoA dehydrase isolated and purified from the microsomal fraction is not a component of FACES, but instead is peroxisomal.

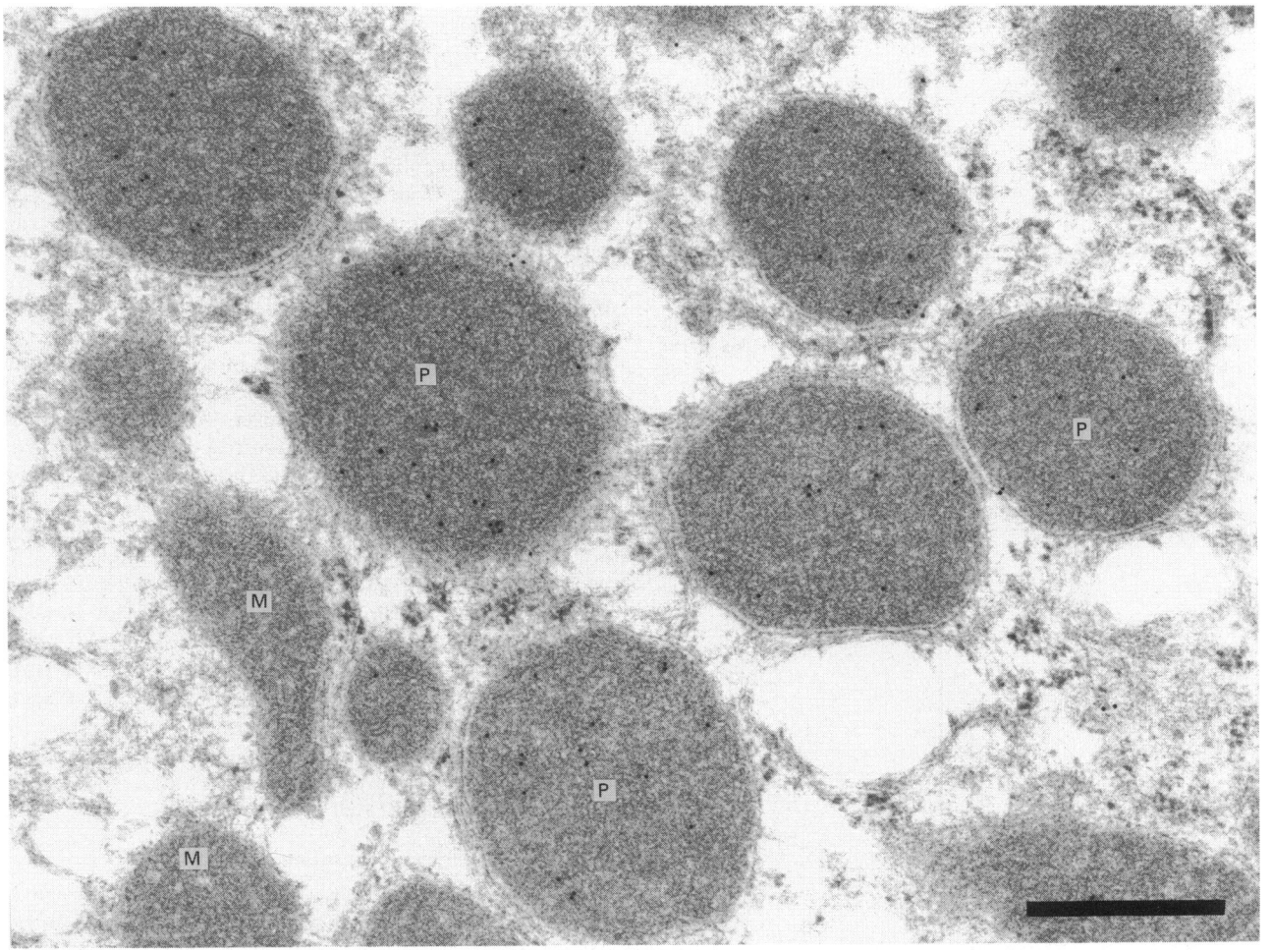

Fig. 8. Thin section of DEHP-treated rat liver fixed in 1% glutaraldehyde and embedded in LR White resin, incubated with antibody to VDH and 10 nm goat anti-(rabbit IgG)–gold complex

Gold particles are concentrated over peroxisomes (P); few particles are present over other structures. Mitochondria (M). Magnification × 53000; scale bar = 0.5 μm.

Gel electrophoresis of peroxisomes (50 μg) followed by immunoblotting with anti-VDH antibody, demonstrated the same three major bands that were seen in Fig. 7. No visible bands were observed when the peroxisomes were replaced by 50 μg of microsomes. This can be explained by the minute amounts of the dehydrase in the microsomes. For example, based on our 3000-fold purification, 1 mg of microsomal protein contains 0.33 μg of VDH; hence 50 μg of microsomes would contain only 0.017 μg of enzyme, or 0.03% of the total microsomal protein, which is consistent with contamination. We propose that during subcellular fractionation by differential ultracentrifugation peroxisomes leak some of their contents, which become sequestered or internalized during the re-formation of the microsomal membrane vesicles. Support for this proposal has been provided in a study [20] in which the peroxisomal TFP was shown to have a high affinity for the RNA of the rough endoplasmic reticulum. Although 0.6 M-KCl can remove the TFP adsorbed to the microsomal fraction, Triton X-100 is needed to release the dehydrase from the microsomes.

Substrate isomers for VDH

Recently, Schulz's laboratory [15] isolated and purified from rat liver peroxisomes a novel enzyme which catalysed the conversion of D-β-hydroxyacyl-CoA into *trans*-2-enoyl-CoA. The enzyme appears to have properties similar to that described by Hiltunen *et al.* [16]. Fig. 9 (a and b) demonstrates the rapid loss of absorbance at 280 nm after addition of *trans*-2-octenoyl-CoA to a cuvette containing VDH. Upon addition of NAD^+ after a change in wavelength to 340 nm (Fig. 9, c and d), an increase in absorbance was observed, indicating that VDH catalysed the conversion of *trans*-2-octenoyl-CoA into β-hydroxyoctanoyl-CoA, and the latter was then converted into β-oxo-octanoyl CoA. In another experiment, when NAD^+ was added to a cuvette containing VDH and D(+)-β-hydroxy-octanoyl-CoA, an increase in absorbance was noted (Fig. 9, e and f). However, replacement of the D-isomer with L(−)-β-hydroxyoctanoyl-CoA resulted in no increase in absorbance upon addition of NAD^+. In a final experiment, *cis*-2-octenoyl-CoA was found not to be a substrate for VDH. These results indicate that VDH, unlike TFP and crotonase [15], is specific for *trans*-2-acyl-CoAs, converting them only into the D-isomer which undergoes further oxidation to the β-oxoacyl-CoA by the dehydrogenase, and that VDH may be related to the peroxisomal dehydrase reported by Li *et al.* [15] and Hiltunen *et al.* [16]. Similarities between the two dehydrase activities include: (1) inability to utilize crotonoyl-CoA as a substrate; (2) ability to convert *trans*-2-enoyl substrates into the D-isomer (not the L-form); (3) lack of activity toward *cis*-2-enoyl substrates; (4) somewhat similar pH profiles, although VDH shows slightly broader activity; and (5) carbon chain lengths above 10 show

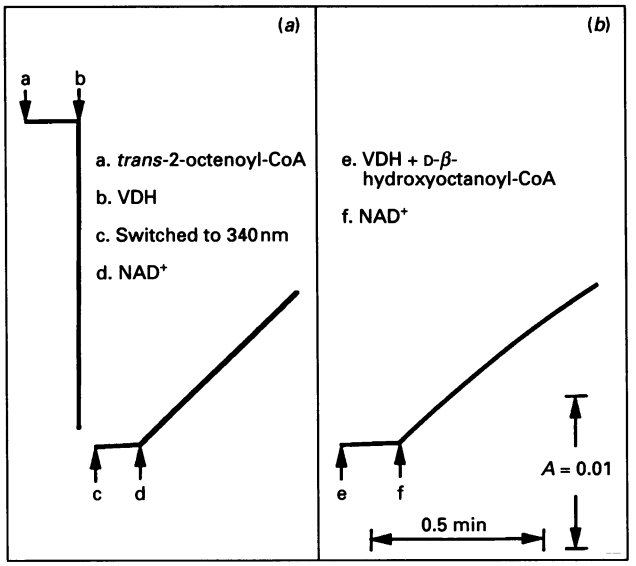

Fig. 9. Isomeric substrates for VDH

(*a*) In a 1.0 ml cuvette containing 0.1 M-potassium phosphate, pH 9.0, a baseline was obtained at 280 nm with 20 μM-*trans*-2-octenoyl-CoA (a); 300 ng of VDH was added at (b) and an absorbance decrease was recorded. After switching to 340 nm (c), 5 mM-NAD^+ was added at (d) and an increase in absorbance recorded. (*b*) After obtaining a baseline with a cuvette containing 0.1 M-potassium phosphate, pH 9.0, 300 ng of VDH and 20 μM-β-hydroxyoctanoyl-CoA (e), the reaction was initiated by addition of 5 mM-NAD^+ (f) and an increase in absorbance at 340 nm was recorded.

declining activity. However, at this stage it should be pointed out that some differences also appear to exist. Firstly, the enzyme reported by Li *et al.* [15] has a subunit molecular mass of 44 kDa and consists of two identical subunits; VDH shows a band at 47 kDa and two additional bands, all of which react with the anti-VDH IgG. Secondly, VDH also contains β-oxoreductase (β-hydroxyacyl-CoA dehydrogenase) activity. Whether this activity resides on the same polypeptide, as occurs with the trifunctional enzyme, is at present uncertain. However, it can be stated that throughout the entire purification the ratio of dehydrase to β-oxoreductase varied only between 4:1 and 9:1 at each purification step, suggesting co-purification. With respect to the purified peroxisomal D-dehydrase [15], no indication was made about the presence or absence of dehydrogenase activity. Thirdly, it is somewhat perplexing at present that VDH contains approx. 5-fold greater activity than the D-dehydrase previously reported [15]. For example, the ostensibly homogeneous preparation of Li *et al.* [15] exhibited a specific activity of 40 μmol/min per mg of protein with D-β-hydroxyoctanoyl-CoA, whereas the VDH specific activity was 200 μmol/min per mg with the same substrate and 500 μmol/min per mg with the *trans*-2-enoyl substrate. A possible explanation may be related to the presence of isoenzymes in our preparation. We cannot exclude this possibility on the basis of two observations: (1) the difference in the pH profile for the C_{18} substrate versus the C_{10} substrate (Fig. 3) and (2) the inability of the C_{10} substrate markedly to inhibit the hydration of the C_{18} substrate.

Finally, it should be pointed out that, although these studies strongly suggest that the previously reported partially purified microsomal β-hydroxyacyl-CoA dehydrase [18] is not the purported component of the microsomal fatty acid chain-elongation system, the latter system does indeed reside in the endoplasmic reticulum (for recent review, see [42]). In 1986, a report appeared [43] describing the purification of a rat liver microsomal *trans*-2-enoyl-CoA hydratase. The molecular and biochemical properties of the enzyme described by that author [43] include a molecular mass of 84 kDa on SDS/PAGE and maximal activity with crotonoyl-CoA, followed by *trans*-2-hexenoyl-CoA. These properties can be equated with those of the peroxisomal TFP rather than with those of the microsomal FACES.

We express our gratitude to Professor Horst Schulz for providing *cis*-2-octenoyl-CoA and the D- and L-isomers of β-hydroxyoctanoyl-CoA and valuable discussions. The assistance of Ms. Christine B. Pearson and Mr. David R. Serwanski in preparing the samples for electron microscopy is gratefully acknowledged. This study was supported by U.S. Public Health Service grant DK21633.

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Received 16 January 1992/6 April 1992; accepted 13 April 1992