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# Immunocytochemical localization of 2,4-dienoyl-CoA reductase in the liver of normal and di-(2-ethylhexyl)phthalate-fed rats

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## Summary

Localization of 2,4-dienoyl-CoA reductase (DCR) in rat liver was studied using immunoenzyme and immunogold techniques. The animals were fed on a laboratory diet with or without 2% di-(2-ethylhexyl)phthalate (DEHP), a peroxisome proliferator, for two weeks. For light microscopy (LM), semithin Epon sections were stained by immunoenzyme technique after removal of the epoxy resin. For electron microscopy (EM), ultrathin Lowicryl K4M sections were stained by the protein A-gold technique. By LM, in untreated rats reaction deposits showing the antigenic sites for DCR were present in the cytoplasmic granules. Hepatocytes, epithelial cells of interlobular bile duct, and sinus-lining cells contained these granules. After administration of DEHP, the cytoplasmic granules stained similarly. The staining intensity of the hepatocytes increased markedly, but that of the other cells decreased. The sinus-lining cells became mostly negative. By EM, gold particles indicating the antigenic sites for DCR were present in both the mitochondria and peroxisomes of hepatocytes of untreated rats. In the other cells, the gold label was confined to the mitochondria. After administration of DEHP, labelling intensity of the hepatocyte mitochondria increased markedly, but that of the peroxisomes conversely decreased. Quantitative analysis of labelling density showed that the mitochondrial DCR increased to about three times that in the untreated rat, but the peroxisomal DCR decreased to 1/6. The results show that in the rat liver, DCR exists in both mitochondria and peroxisomes. DEHP can induce mitochondrial DCR, but not peroxisomal DCR.

## Introduction

2,4-Dienoyl-CoA reductase [(DCR) EC 1.3.1.34] is important in the  $\beta$ -oxidation system of fatty acids. The system is necessary for the degradation of unsaturated fatty acids with *cis* double bonds extending from even numbered carbon atoms (Kunau & Dommers, 1978). DCR is active in both mitochondria and peroxisomes, as are other  $\beta$ -oxidation enzymes (Dommers *et al.*, 1981; Mizugaki *et al.*, 1982). Moreover, DCR activities in rat liver increase by feeding of clofibrate, an inducer of peroxisomal  $\beta$ -oxidation enzymes in rat liver (Lazarow & de Duve, 1976).

Each enzyme in the mitochondrial  $\beta$ -oxidation system of saturated fatty acids is immunologically distinct from the corresponding enzyme in the peroxisomal system (Furuta *et al.*, 1980; Osumi & Hashimoto, 1980; Miyazawa *et al.*, 1980). We recently purified hepatic DCR from rats fed on a diet containing 0.3% (w/w) clofibrate (Kimura, *et al.*, 1984), and obtained polyclonal antibodies from rabbit against this enzyme. We have considered that DCR was present in peroxi-

somes at that time. However, we have found by immunoblot analysis that most of the enzyme activity is in the mitochondrial fraction. We will report and discuss in detail these results elsewhere. In the present study, we show the localization of DCR in rat liver using immunocytochemical techniques.

## Materials and methods

### Antibodies used

DCR was purified from rat liver to homogeneity (Kimura *et al.*, 1984). The purified enzyme (1 mg ml<sup>-1</sup>) was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected at multiple subcutaneous sites on the back of New Zealand white rabbits (3.0 kg) every two weeks, four times. Two weeks after the booster injection, blood was drawn from the carotid artery. The antibodies were partially purified by fractionation with ammonium sulphate and were dialysed against 10 mM potassium phosphate, pH 7.5, containing 0.15 M NaCl. We tested the specificity of the antibodies by agar gel-diffusion and immunoblot analysis.

### *Immunoblot analysis of rat liver homogenates*

Liver (5 g) from rats which were fed on a diet with or without 2% DEHP was homogenized in 5 ml of the homogenizing solution, consisting of 0.05 M potassium phosphate buffer (pH 7.4), 0.1% Triton X-100, 10 mM diisopropylfluorophosphate (Sigma, USA), 62.5 µg pepstatin (Sigma, USA), and 125 µg leupeptin (Peptide Institute, Osaka, Japan). Homogenate was centrifuged at 100 000 g for 1 h and the supernatant was used for electrophoresis. Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis in gradient slab gel (10–20%) was carried out as described by Laemmli (1970). For molecular weight markers, we used bovine serum albumin (66 kDa), ovalbumin (45 kDa), porcine pepsin (34.7 kDa), bovine milk lactoglobulin (18.4 kDa) and egg white lysozyme (14.3 kDa).

After electrophoresis, proteins were transferred electrophoretically to a Durapore-membrane (Millipore Co., USA) according to the method of Towbin *et al.* (1979). The membrane was first incubated in phosphate buffered saline (PBS) containing 1% gelatin for 30 min, followed by incubation for 18 h with anti-DCR ( $10 \mu\text{g ml}^{-1}$ ) in 1% gelatin-PBS. After being washed in 0.3% Tween 20-PBS, the membrane was soaked in peroxidase-conjugated Fab' of goat anti-rabbit IgG for 1 h. Peroxidase activity was stained by DAB medium consisting of  $0.25 \text{ mg ml}^{-1}$  3,3'-diaminobenzidine tetrahydrochloride, 0.1 M Tris-HCl buffer, pH 7.2, and 0.01%  $\text{H}_2\text{O}_2$  (Graham & Karnovsky, 1966).

### *Preparation of peroxidase-labelled Fab' of goat anti-rabbit IgG*

A goat was intracutaneously injected with purified rabbit IgG (2 mg) emulsified with Freund's complete adjuvant every two weeks. After the fourth injection, the animal was boosted with 4 mg of rabbit IgG. Two weeks after the booster injection, blood was drawn from the carotid vein every week. Specific anti-rabbit IgG was prepared by affinity chromatography using rabbit IgG-coupled Sepharose 4B. We confirmed specificity of the antibody by an Ouchterlony double diffusion test. The affinity-purified goat anti-rabbit IgG was digested with pepsin at pH 4.0. The resulting  $\text{F(ab')}_2$  fragments were isolated by gel-filtration on Ultrogel AcA 40 and then reduced with 2-mercaptoethylamine. The resulting Fab' fragments were conjugated with horseradish peroxidase (HRP, Sigma Type IV) by the method of Hashida *et al.* (1984).

### *Preparation of protein A-gold probe*

Colloidal gold was prepared by reduction of tetrachloroauric acid with sodium ascorbate (Stathis & Fabrikanos, 1958). Conjugation of the colloidal gold with protein A (Pharmacia) was carried out at pH 5.9. The protein A-gold probe with a diameter of about 12 nm was isolated by density gradient centrifugation (Slot & Geuze, 1981) and stored in 40% glycerol at  $-20^\circ\text{C}$ .

### *Tissue preparation*

Male Wistar rats (initial body weight of 180 g) were used. The animals were fed *ad libitum* on a standard diet with or without 2% di-(2-ethylhexyl)phthalate (DEHP) for 2 weeks (Shindo *et al.*, 1978). Liver was fixed by perfusion through the portal vein with the fixative at room temperature for 10

min. The fixative consisted of 4% paraformaldehyde, 0.1% glutaraldehyde, 0.01%  $\text{CaCl}_2$ , and 0.15 M cacodylate-HCl buffer, pH 7.4. The fixed livers were cut into tissue slices (200 µm thick) with a vibratome. The slices were dehydrated in graded dimethylformamide at  $-20^\circ\text{C}$  and embedded in Lowicryl K4M at  $-20^\circ\text{C}$  (Roth *et al.*, 1981). Some tissue slices were fixed again with 2% glutaraldehyde for 1 h, dehydrated in graded ethanol and embedded in Epon. Post-osmication was omitted.

### *Light microscopic immunocytochemistry*

We have described the basic procedures previously (Litwin *et al.*, 1984). Briefly, Epon-embedded materials were cut into 1 µm-thick sections, which were mounted on a clean glass slide. After removal of the epoxy resin with 10% NaOH ethanol solution, the sections were digested with trypsin ( $1 \text{ mg ml}^{-1}$ , Difco, USA) for 3 min. After treatment with 0.5% bovine serum albumin (BSA), the sections were incubated with anti-DCR ( $50 \mu\text{g ml}^{-1}$ ) for 1 h. After being washed, the sections were treated with the HRP-Fab' of goat anti-rabbit IgG for 30 min. This was followed by 10-min incubation with diaminobenzidine- $\text{H}_2\text{O}_2$  medium for HRP. The sections were dried, mounted in Entellan (Merck, Germany) and examined with a Nikon light microscope.

### *Immunoelectron microscopy*

The procedures were based on the method described by Roth (1982). Briefly, thin sections of Lowicryl K4M-embedded materials were treated with 0.5% BSA for 5 min and incubated with anti-DCR ( $50 \mu\text{g ml}^{-1}$ ) overnight at  $4^\circ\text{C}$ . After being washed, the sections were incubated with the protein A-gold probe for 30 min and treated with 2% glutaraldehyde. After being air-dried, the sections were double-stained with 2% uranyl acetate for 3 min and with lead citrate for 20 s. The sections were examined in a Hitachi H600 electron microscope at an accelerating voltage of 100 kV.

### *Immunocytochemical control experiments*

Semithin or ultrathin sections were first treated with the IgG fraction from a non-immunized rabbit and then incubated with the HRP-Fab' conjugate or with the protein A-gold probe. Some sections were directly incubated with the HRP-Fab' conjugate or with the protein A-gold probe.

### *Quantification of labelling density*

To quantify the labelling density of mitochondria and peroxisomes, we selected at random two tissue blocks from each animal. A single section from each block provided 10 electron micrographs, which were enlarged to magnification of  $\times 36000$ . The surface areas of mitochondria and peroxisomes and the area other than of these organelles were estimated by a semi-computing system (Yokota *et al.*, 1986). The number of gold particles in these areas was counted. Labelling density was expressed as the number of gold particles per square micrometre (Bendayan *et al.*, 1980).

## **Results**

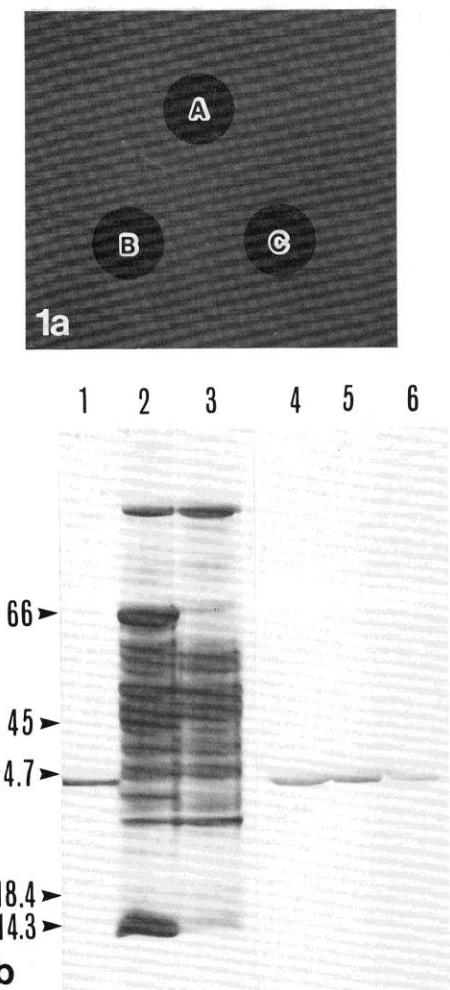
### *Antibody specificity*

Immunodiffusion tests showed that there is a single precipitation line between anti-DCR antiserum and

the purified DCR (Fig. 1a). Between the antiserum and rat liver extract there was also a single line. Both lines fused with each other showing no spur (Fig. 1a). Western blotting showed a single signal in the liver extracts from rats treated with or without DEHP. The molecular weight of the signal was 33 kDa which corresponded to that of the purified DCR (Fig. 1b). Furthermore, marked induction of DCR was noted in the liver extract of the DEHP-treated rat.

*Light microscopic localization of DCR in rat liver*

*Normal rat:* A discrete granular staining, which showed the antigenic sites for DCR, was present in



**Fig. 1.** (a) Immunodiffusion analysis of antibody. Well A, antibody (200 µg); well B, purified DCR (2.7 µg); well C, liver extract (850 µg) from DEHP-treated rat. (b) Immunoblot analysis. Lanes 1-3, staining with Coomassie Brilliant Blue; lanes 4-6, immunoblot with rabbit anti-DCR and HRP-conjugated Fab' of goat anti-rabbit IgG. Lane 1, the purified rat liver DCR (0.5 µg); lanes 2 and 5, liver homogenate of rat treated with DEHP (50 µg), lanes 3 and 6, liver homogenate of normal rat (50 µg); lane 4, purified rat liver DCR (0.25 µg).

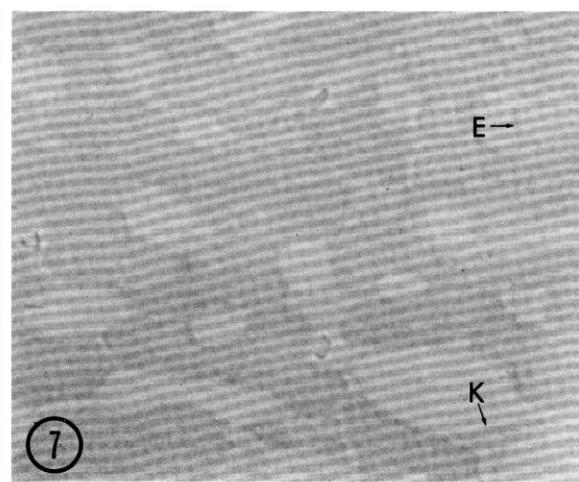
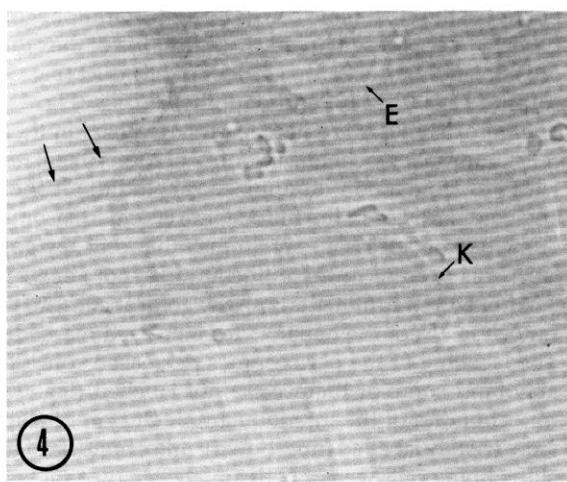
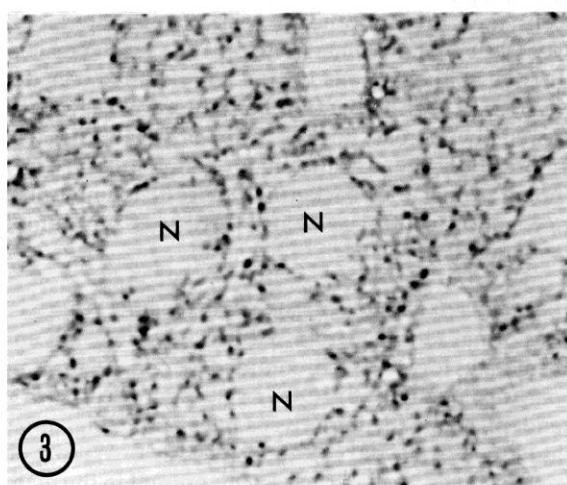
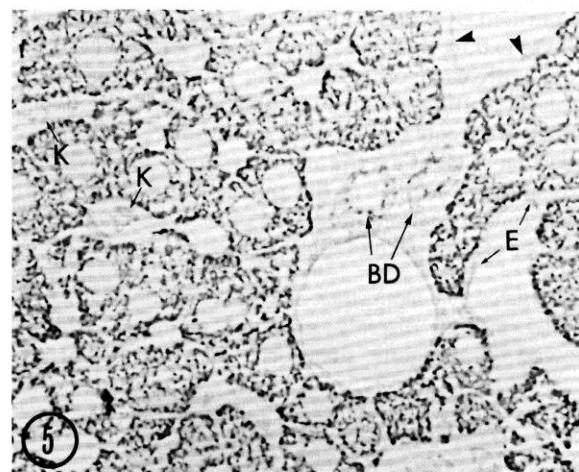
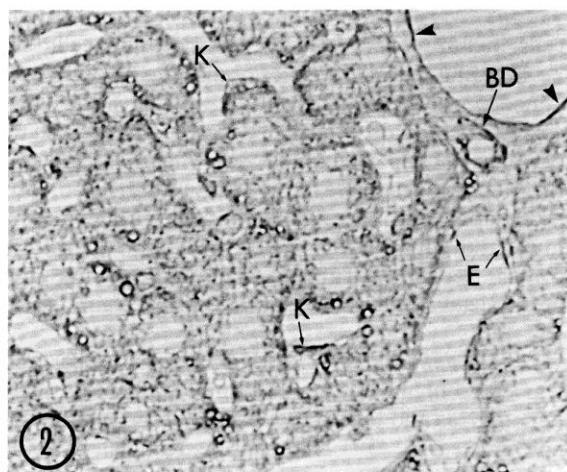
various liver-forming cells. They were parenchymal cells, including endothelial cells and Kupffer cells (Fig. 2). Endothelial cells of interlobular vessels also contained positive granules (Fig. 3, arrowheads). There was no staining difference between the centre and the periphery of hepatic lobules. At higher magnification, the hepatocytes were crowded with the round or rod-shaped granules stained for DCR (Fig. 3). Cell nuclei did not stain for DCR. In immunocytochemical control sections incubated with IgG from a non-immunized rabbit, there was no positive staining (Fig. 4).

*DEHP-administered rat:* A reaction product for DCR was present in the cytoplasmic granules of the hepatocytes and the epithelial cells of the interlobular bile duct (Fig. 5). The staining intensity of the hepatocytes increased markedly. The sinus-lining cells and the endothelial cells of the interlobular vessels were not stained (Fig. 5). The staining reaction in the epithelial cells of the interlobular bile duct decreased slightly (Fig. 5). In high power view of the parenchymal cells, most of the positive granules were short-fibrils among which many round granules were noted as unstained background (Fig. 6). In immunocytochemical control sections, there was no granular staining (Fig. 7).

*Immunoelectron microscopic localization of DCR*

In normal rat liver, gold particles showing the antigenic sites for DCR were confined to mitochondria and peroxisomes (Fig. 8). Other cell organelles, such as endoplasmic reticulum, lysosomes, and Golgi apparatus, were devoid of gold particles. Within the peroxisomes, gold particles were present in the matrix but not in the crystalloid core (Fig. 8). In addition, gold particles were present in the mitochondria of the other cells, including the epithelial cells of bile duct (Fig. 9), Kupffer cells (Fig. 10) and endothelial cells (Fig. 11). The peroxisomes were not clearly visualized in these cells. Immunocytochemical control sections incubated with IgG from a non-immunized rabbit did not show any gold particles in the mitochondria or peroxisomes of the hepatocytes (Fig. 12). In the other cells, no gold label was present in the mitochondria (not shown).

After DEHP administration, the gold labelling in the mitochondria of the hepatocytes increased markedly. The labelling of the peroxisomes decreased considerably, and some of them were not labelled (Fig. 13). Mitochondria of the interlobular bile duct epithelium were weakly labelled (not shown); mitochondria of the sinus-lining cells were mostly unlabelled (not shown). Within the mitochondria, most of the gold particles were associated with the matrical surface of the cristae (Fig. 14). In peroxisomes, a few gold particles were present in the matrix and had no association with crystalloid core or membrane (Fig. 13). Other organelles were not labelled with gold particles. In



immunocytochemical control sections, no gold label was present in either of the organelles (Fig. 15).

*Quantitative analysis of labelling density in mitochondria and peroxisomes of normal and DEHP-administered rats*

Table 1 shows the results. In the liver of untreated (normal) rat, the labelling density of peroxisomes was about twice as much as that of mitochondria. The labelling density of these organelles was significantly higher than that of the background. After administration of DEHP the labelling density of mitochondria increased to about three times that of the normal rat, but that of peroxisomes inversely decreased to about 1/6. The labelling density of both organelles was significantly higher than that of the background.

## Discussion

This immunoenzyme study shows that DCR is present in most liver-forming cells, that is hepatocytes, epithelial cells of interlobular bile duct, endothelial cells of the sinus and capillaries and Kupffer cells. The results suggest that most of the liver-forming cells metabolize unsaturated fatty acid by DCR. Since the hepatocytes were stained most strongly, these cells would seem to be a main site for the metabolism of fatty acids in the rat liver.

It was difficult to identify peroxisomes from mitochondria at the light microscope level when both

**Table 1.** Labelling density in mitochondria and peroxisomes of hepatocytes from normal and DEHP-administered rat (gold particles  $\mu\text{m}^{-2} \pm \text{s.d.}$ )

Rat	Mitochondria	Peroxisomes	Background
Normal	23.36 $\pm$ 8.59	44.18 $\pm$ 3.82	1.55 $\pm$ 0.13
DEHP	65.85 $\pm$ 16.37	7.58 $\pm$ 2.25	0.81 $\pm$ 0.19

**Fig. 2.** Section from normal rat liver which was stained for DCR by immunoenzyme technique after removal of epoxy resin. Reaction deposits are seen in cytoplasmic granules of parenchymal cells, Kupffer cells (K), endothelial cells (E). In the epithelial cells of interlobular bile duct (BD) and in the endothelial cells (arrowheads) of interlobular vein, the positive reaction is present.  $\times 700$ .

**Fig. 3.** High power of normal rat liver after immunoenzyme staining. The cytoplasmic granules of hepatocytes are stained, but the cytoplasmic matrix and nuclei (N) are not.  $\times 1600$ .

**Fig. 4.** Immunocytochemical control section. Note that no reaction deposits are present in hepatocytes, Kupffer cell (K), and endothelial cell (E). In the epithelial cell (arrows) of interlobular bile duct, no positive reaction is noted.  $\times 700$ .

**Fig. 5.** Section from liver of DEHP-administered rat, stained for DCR. Heavy reaction deposits are present in the cytoplasmic granules of hepatocytes and epithelial cells of the interlobular bile duct (BD). Kupffer cells (K), endothelial cells (E) and vessel endothelial cells (arrowheads) are mostly negative.  $\times 700$ .

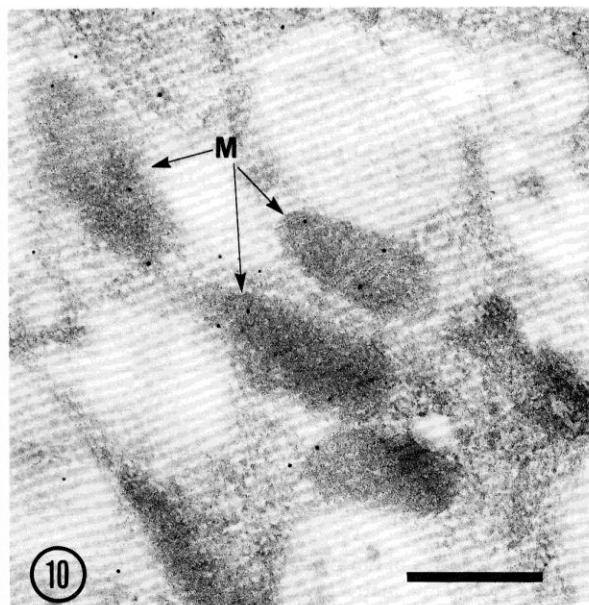
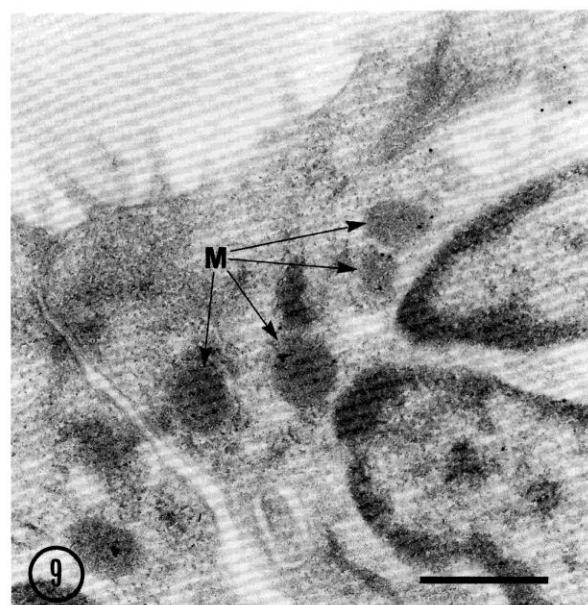
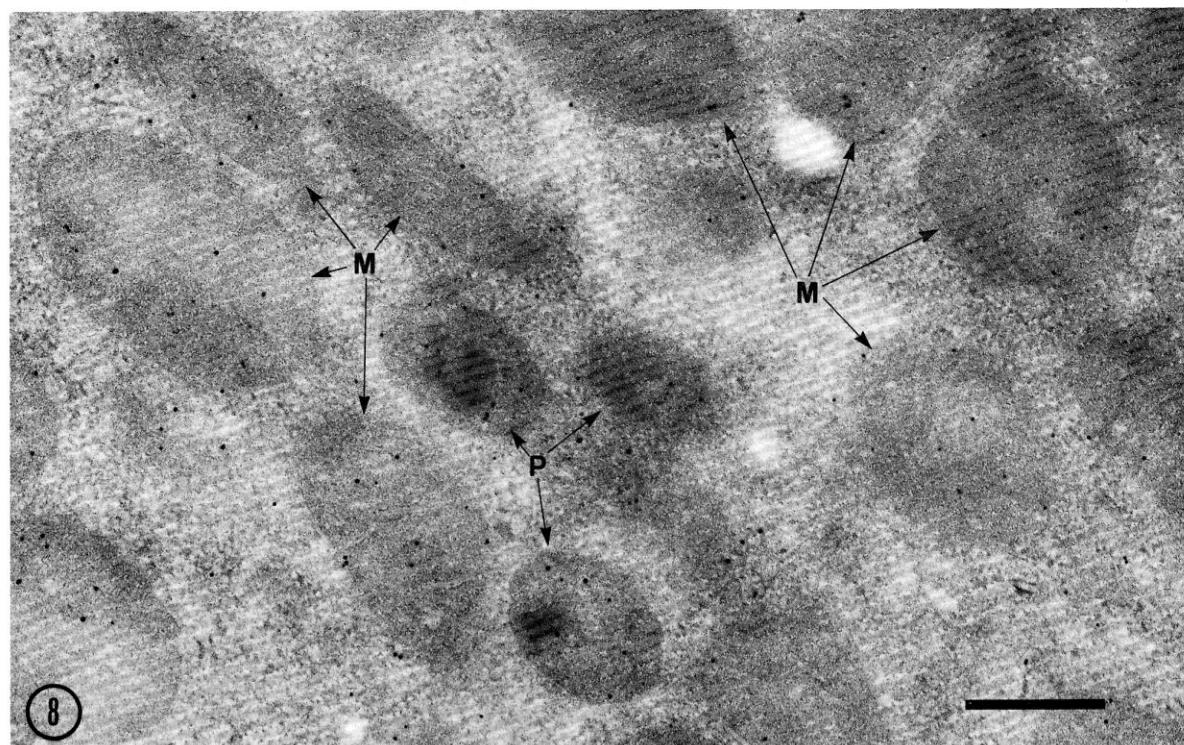
**Fig. 6.** High power view of liver from DEHP-treated rat. Reaction deposits are seen in short fibrillar granules of hepatocytes. Nuclei (N) and cytoplasmic matrix are negative for staining. In the cytoplasm of hepatocytes, unstained granules are noted (arrows).  $\times 1600$ .

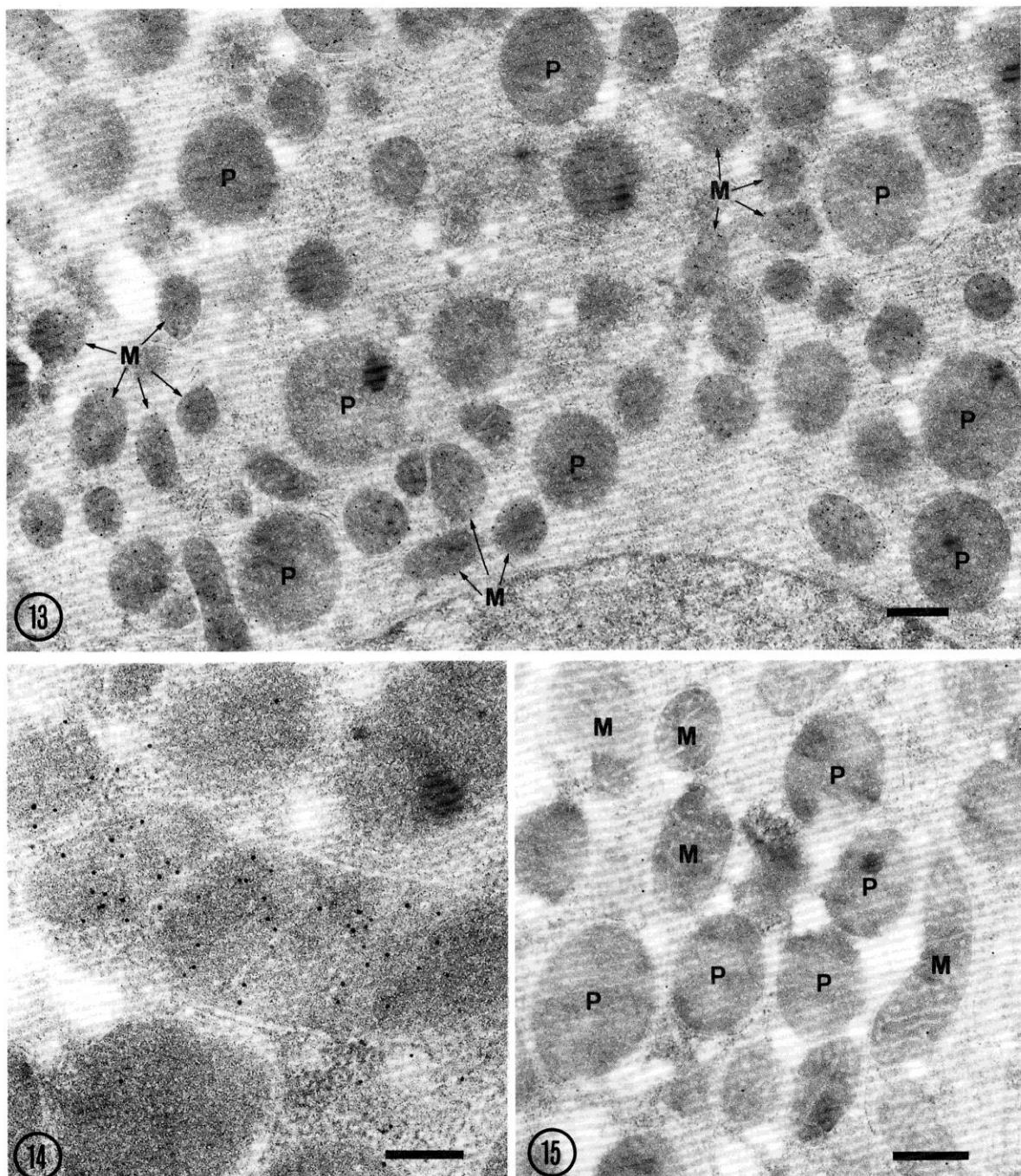
**Fig. 7.** Immunocytochemical control section of liver from DEHP-treated rat. No positive staining is seen in hepatocytes, Kupffer cell (K), and endothelial cell (E).  $\times 700$ .

organelles are positively stained; we therefore used the immunoelectron microscopical technique. Using this technique, we have demonstrated that, in the parenchymal cells of normal rat liver, mitochondria and peroxisomes are the cytoplasmic granules positive for DCR. Quantitative analysis of the gold labelling showed that the labelling density of both the organelles is significantly higher than that of the background noise. The data show that DCR is present in both mitochondria and peroxisomes, a result consistent with biochemical data (Dommers *et al.*, 1981; Mizugaki *et al.*, 1982).

Our quantitative analysis also showed that, in normal rat liver, labelling density of the peroxisomes was about twice as high as that of the mitochondria. After administration of DEHP the labelling density of the mitochondria increased about 3-fold, but inversely, that of the peroxisomes decreased to about 1/6. According to a stereological analysis of rat liver (Blouin *et al.*, 1977), the volume % of the mitochondria is about 12 times as that of the peroxisomes. Therefore, in normal rat liver, the mitochondrial DCR is much more than the peroxisomal DCR.

This study shows also that DEHP reduced peroxisomal DCR somewhat, although it markedly induced mitochondrial DCR. DEHP is one of the peroxisome proliferators and can markedly induce the enzymes concerned with peroxisomal  $\beta$ -oxidation of fatty acids in rat liver (Hashimoto, 1982). We have shown previously that volume and numerical densities of peroxisomes increased about 3-fold, but those of mitochondria increased only slightly (Yokota, 1986). Therefore, it is likely that the dilution of the enzyme by the proliferated peroxisomes results in the decrease in the peroxisomal DCR. Both unstained granules (Fig. 6), and some unlabelled peroxisomes (Fig. 13) were observed. In this case, DEHP does not stimulate the synthesis of the peroxisomal DCR. The increase in the mitochondrial DCR seems to be caused

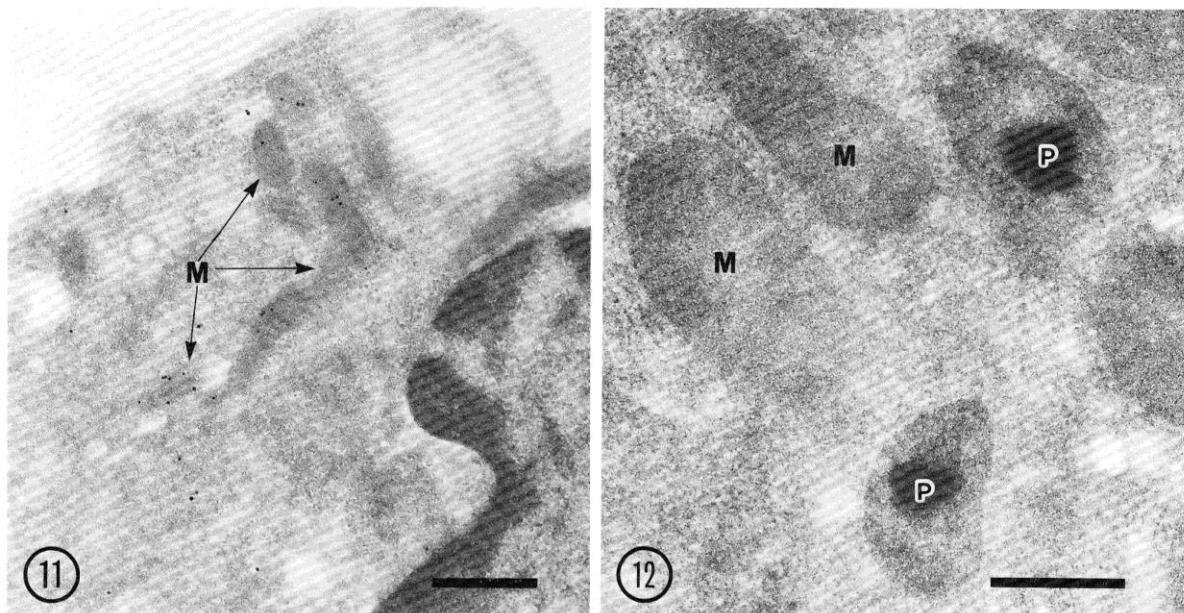




In conclusion, 2,4-dienoyl-CoA reductase (DCR) is present in both mitochondria and peroxisomes in rat liver. The data suggest that both these organelles are concerned with the degradation of unsaturated fatty acids. A peroxisome proliferator, DEHP, can induce the mitochondrial DCR, but it reduces the peroxisomal DCR.

#### Acknowledgement

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by the stimulation of DCR synthesis, since the mitochondria are not proliferated and the labelling density of the individual mitochondria is increased by DEHP.

On the other hand, it is not known whether the mitochondrial DCR and the peroxisomal DCR are the same. The present result showed that our antibody recognizes the mitochondrial and peroxisomal enzyme(s). Serine-pyruvate aminotransferase has shown to be present in both the mitochondria and peroxisomes of rat liver (Oda *et al.*, 1982). The enzymes show no differences in their physicochemical and immunological properties. However, the induction behaviour of the two differs considerably. The peroxisomal enzyme is induced by DEHP, but the

mitochondrial enzyme is induced by glucagon (Yokota, 1986). It is likely that DCR is another such type of enzyme. This requires further study.

The present study showed that weak staining for DCR is present in the Kupffer cells and endothelial cells of normal rat liver. This staining was mostly undetectable in DEHP-treated rats. Although it is unclear why DEHP treatment reduces the DCR in these cells, DEHP seems to inhibit the synthesis of the mitochondrial DCR of these cells. The hepatocytes and the sinus-lining cells derive from different origins: the former differentiates from endoderm and the latter from mesoderm. The different reaction of the cells to chemicals seems in part to be due to such differences in their developmental origins.

**Fig. 8.** Thin section of hepatocyte from normal rat stained by the protein A-gold technique for DCR. Gold particles are present on mitochondria (M) and peroxisomes (P). Scale bar, 0.5 µm; × 41 000.

**Fig. 9.** Epithelial cells of interlobular bile duct. Gold particles for DCR are present on mitochondria (M). Scale bar, 0.5 µm; × 37 000.

**Fig. 10.** Kupffer cell of normal rat liver. Gold particles indicating DCR are seen on mitochondria (M). Scale bar, 0.5 µm; × 41 000.

**Fig. 11.** A sinus-lining endothelial cell stained for DCR. Gold particles are observed on mitochondria (M). Scale bar, 0.5 µm; × 31 000.

**Fig. 12.** Immunocytochemical control section of normal rat hepatocyte. No gold particles are present on mitochondria (M) and peroxisomes (P). Scale bar, 0.5 µm; × 41 000.

**Fig. 13.** Thin section of hepatocyte from DEHP-administered rat stained for DCR by the protein A-gold technique. Mitochondria (M) are heavily labelled by gold particles, but proliferated peroxisomes (P) are slightly or not labelled. Scale bar, 0.5 µm; × 21 000.

**Fig. 14.** High power view of hepatocyte from a DEHP-treated rat. Most of gold particles are closely associated with the inner membrane. Scale bar, 0.25 µm; × 54 000.

**Fig. 15.** Immunocytochemical control section of DEHP-treated rat. No gold particles are seen on mitochondria (M) and peroxisomes (P). Scale bar, 0.5 µm; × 26 000.

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