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Peroxisomal localization of the immunoreactive β-oxidation enzymes in a neonate with a β-oxidation defect

Pathological observations in liver, adrenal cortex and kidney

M. Espeel[1], F. Roels[1], L. Van Maldergem[2], D. De Craemer[1], G. Dacremont[3], R.J.A. Wanders[4], and T. Hashimoto[5]

[1] Department of Human Anatomy and Embryology, Free University of Brussels, Laarbeeklaan 103, B-1090 Brussels, Belgium
[2] Institute of Pathological Morphology, Gerpinnes-Loverval, Belgium
[3] Department of Pediatrics, State University of Ghent, Academic Hospital, Ghent, Belgium
[4] University of Amsterdam, Academisch Medisch Centrum, Amsterdam, The Netherlands
[5] Department of Biochemistry, Shinshu University School of Medicine, Nagano, Japan

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Summary. A boy born to healthy, unrelated parents, presented at birth with hypotonia and seizures. Very long chain fatty acids in the plasma were strongly elevated; bile acid intermediates and plasmalogen biosynthesis were normal. Acyl-CoA oxidase activity was normal. The patient died at the age of 3 months. The cerebellum and medulla oblongata showed neuronal migration defects. The specific biochemical basis for the impaired peroxisomal β-oxidation has not been found. The three immunoreactive peroxisomal β-oxidation enzymes and catalase were localized in the hepatocellular peroxisomes. Aberrant features of the peroxisomes included: a subpopulation of organelles larger than 1 μm, an amorphous nucleoid in many organelles, and invaginations of the peroxisomal membrane into the matrix. Peroxisomes in the proximal renal tubules also contained the three immunoreactive β-oxidation enzymes. Regularly spaced trilamellar inclusions were seen in hepatic macrophages; they were much more abundant in adrenocortical macrophages. The inclusions were birefringent and resistant to acetone extraction. Distinct hepatic fibrosis had developed over a period of 2.5 months. We speculate that the impaired β-oxidation is due to a defect at the level of the peroxisomal carnitine octanoyl or -acetyl transferase, responsible for the export of β-oxidation products.

Key words: Peroxisomal β-oxidation – Immunocytochemistry – Trilamellar inclusions – Neuronal migration – Carnitine octanoyl transferase

Introduction

Peroxisomes are involved in several metabolic functions, one of which is the β-oxidation of very long chain fatty acids (VLCFA). The importance of the peroxisomal β-oxidation system is emphasized by the identification of a growing number of inherited diseases in man in which peroxisomal β-oxidation is impaired. In recent years our knowledge of peroxisomal disorders has increased greatly (Wanders et al. 1990).

Peroxisomal β-oxidation defects may result from:
1. The failure to assemble the peroxisome organelle leading to subsequent degradation of the normally synthesized but mislocalized enzymes: Zellweger syndrome; infantile Refsum disease; neonatal adrenoleukodystrophy (NALD); hyperpipecolic acidaemia; generalized peroxisomal disorders.
2. The absence or deficiency of a single β-oxidation enzyme: acyl-CoA oxidase, 'pseudo-NALD' (Poll-Thé et al. 1988), bifunctional enzyme (Watkins et al. 1989), 3-ketoacyl-CoA thiolase, 'pseudo-Zellweger' (Schram et al. 1987).
3. The absence of all three β-oxidation enzymes, "Zellweger-like snydrome" (Suzuki et al. 1988).
4. The absence or deficiency of the enzyme VLCFA-CoA ligase, X-linked ALD (Hashmi et al. 1986).

These all are devasting diseases with severe neurological involvement; Lazarow and Moser (1989) and Wanders et al. (1990) have described their clinical presentation.

Biochemically, the peroxisomal β-oxidation disorders are characterized by elevated amounts of VLCFA and, except for X-linked adrenoleukodystrophy (X-ALD) and of acyl-CoA oxidase deficiency, of bile acid intermediates in the plasma (Wanders et al. 1990). The presence of the peroxisomal β-oxidation enzymes can be examined by immunoblotting of homogenates or peroxisomal fractions (Tager et al. 1985); immunocytochemistry in addition reveals the subcellular localization of the enzyme proteins (Litwin et al. 1987, 1988; Espeel et al. 1990).

Apart from the above-mentioned defects, Clayton et al. (1988) reported three siblings showing impaired

Offprint requests to: M. Espeel

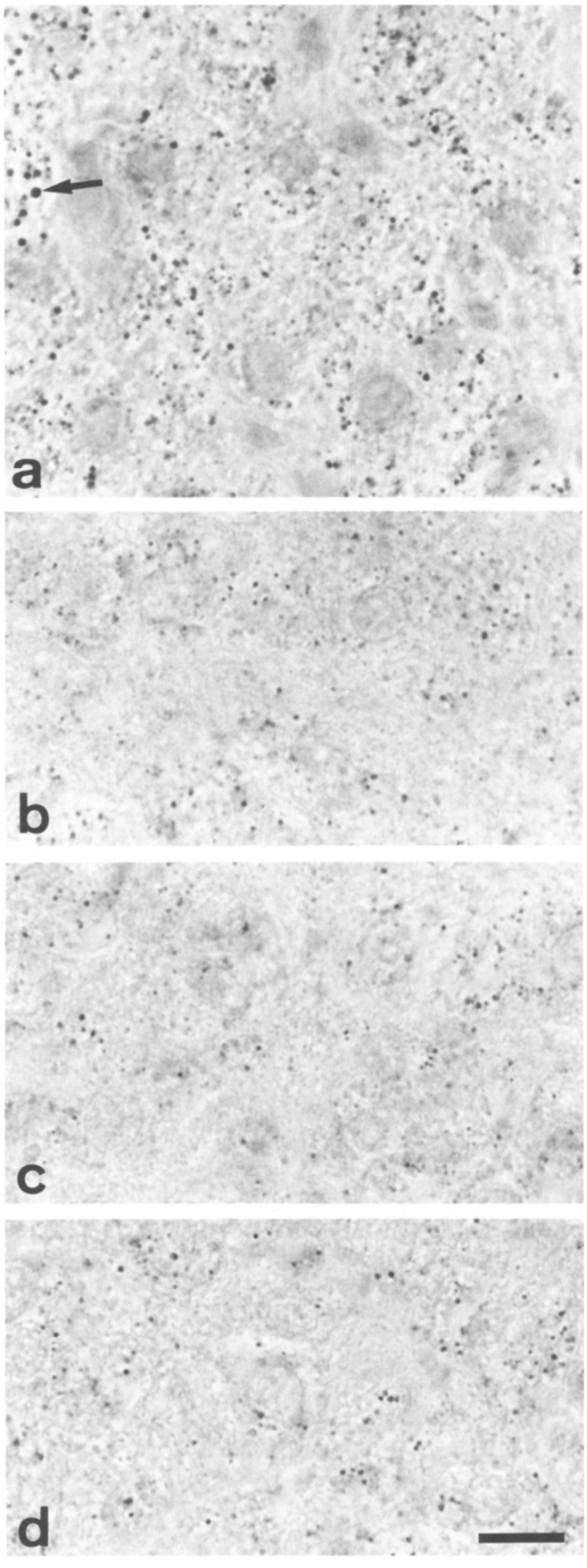

Fig. 1. a Hepatic peroxisomes, visualized by their catalase activity, in the liver biopsy; the distribution of the organelles is normal. The *arrow* indicates an enlarged organelle (phase contrast); **b–d** Immunostaining against acyl-CoA oxidase, bifunctional enzyme and 3-keto-acyl-CoA thiolase protein respectively. Peroxisomes are distinctly visualized as dark granules and show a distribution pattern which is similar to that with diaminobenzidine staining (bright field illumination). *Bar* = 10 μm. × 1250

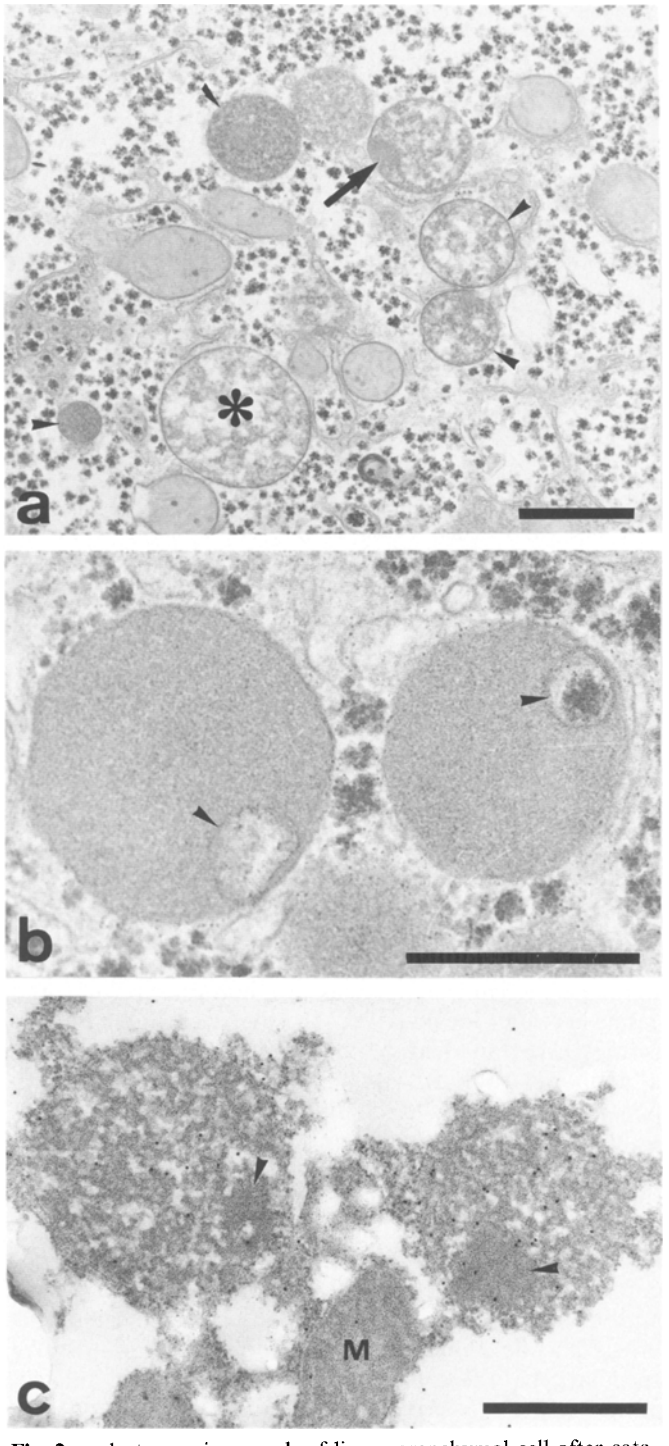

Fig. 2. a electron micrograph of liver parenchymal cell after catalase staining; the peroxisomes (*arrowheads*) show a heterogeneous reaction for catalase activity. An enlarged organelle (> 1 μm) with very weak catalase activity is present (*asterisk*). The *arrow* indicates an amorphous nucleoid inside a peroxisome. *Bar* = 1 μm. × 15000 **b** Two peroxisomes with membrane invaginations (*arrowhead*), one of which contains a glycogen particle. *Bar* = 0.5 μm. × 61600. **c** Immunogold localization of 3-keto-acyl-CoA thiolase on etched Epon section, using protein A-10 nm gold. Labelling over two peroxisomal profiles is obvious; a nucleoid is present in both organelles (*arrowhead*). *M*, Mitochondrion. *Bar* = 0.5 μm. × 43200

peroxisomal β-oxidation (VLCFA and bile acid intermediates elevated) in the presence of the immunoreactive β-oxidation enzyme proteins on blots of liver homogenates. Similar findings in other patients were described by Barth et al. (1990) and Naidu et al. (1988); in the latter case, however, the levels of bile acid intermediates were normal.

Several ultrastructural and cytochemical alterations occur in patients suffering from a congenital peroxisomal β-oxidation defect. The size, shape and number of the hepatic peroxisomes – if present as catalase-containing organelles – is altered (Roels et al. 1988; Hughes et al. 1990); there are polarizing inclusions in liver macrophages, the adrenal gland and the brain (Johnson et al. 1976; Ghatak et al. 1981; Kerckaert et al. 1988) and iron accumulation and development of hepatic fibrosis/cirrhosis occurs (Roels et al. 1991).

In this paper we describe alterations in the hepatocellular peroxisomes and histopathological observations in a newborn with a greatly impaired peroxisomal β-oxidation, although the normal subcellular localization of catalase and the three β-oxidation enzyme proteins in liver and kidney was demonstrated by immunocytochemistry.

Materials and methods

The clinical and biochemical findings in this patient will be described by Van Maldergem et al. (to be published). Briefly, a boy – born to healthy, unrelated parents – presented at birth with severe hypotonia and seizures; the facies was normal. Archaic reflexes could not be evoked. Somaesthetic evoked potentials showed central conductive latency. Auditive and brain-stem evoked potentials were abnormal in the late component. Visual potentials were normal. Cerebral sagittal NMR images revealed diffuse hyperdensities in the frontal and temporoparietal region. The hypotonia persisted; at the age of 2 months a flat nasal bridge and a prominent forehead were noted. Skeletal calcifications were not detected. VLCFA in

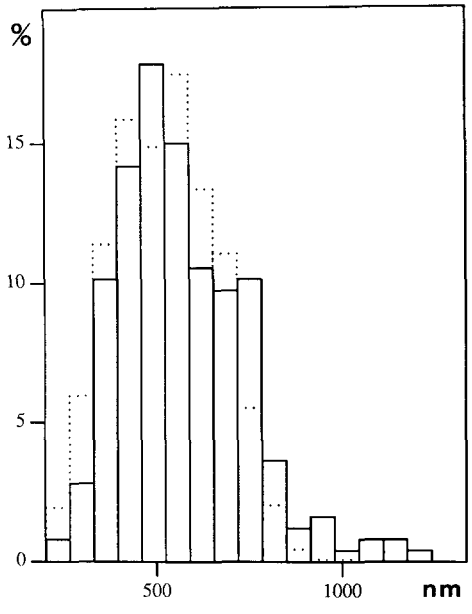

Fig. 3. Histogram of the size distribution (d-circle) of the hepatic peroxisomes. *Dotted lines* represent eight control livers. Note the subgroup with enlarged organelles (>1 μm)

Table 1. Morphometry of peroxisomes

	Patient	Controls[d]
Number of peroxisomes	247	1124
d-Circle (μm)		
mean measured	0.570	0.517±0.018[b] (0.445–0.620)[c]
mean corrected[a]	0.712	0.635±0.021 (0.555–0.753)
max. measured	1.230	1.027
Volume density (%)	0.80	1.03±0.10 (0.71–1.44)
Numerical density (μm^{-3})	0.070	0.102±0.009 (0.053–0.132)
Surface density (μm^{-1})	0.087	0.108±0.01 (0.083–0.152)

[a] Corrected for sectioning effect, according to formula 9 from Abe et al. (1983) with $R = d$ circle/2
[b] Mean±SEM
[c] Range
[d] Eight samples (De Craemer et al. 1991 b)

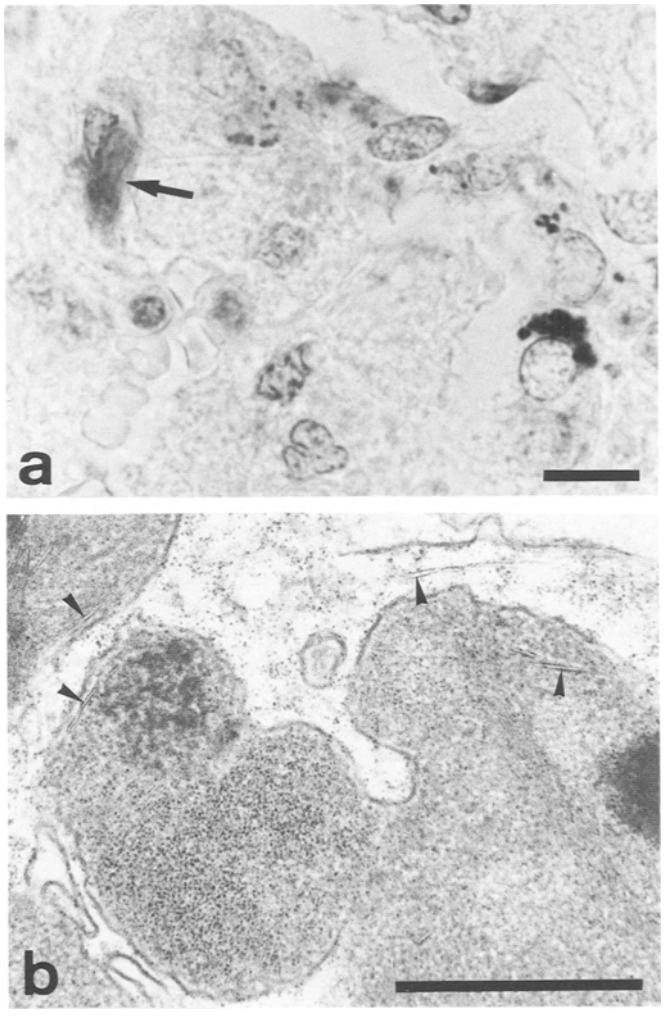

Fig. 4. a Iron (revealed by Perl's stain) in the liver is present in granules, but also in diffuse form in macrophages (*arrow*). *Bar* = 10 μm. ×1250 **b** Detail of a liver macrophage with short trilamellar inclusions (*arrowheads*) in lysosomes and in the cytoplasm. Iron particles are present inside the lysosomes and in the cytoplasm. *Bar* = 0.5 μm. ×64800

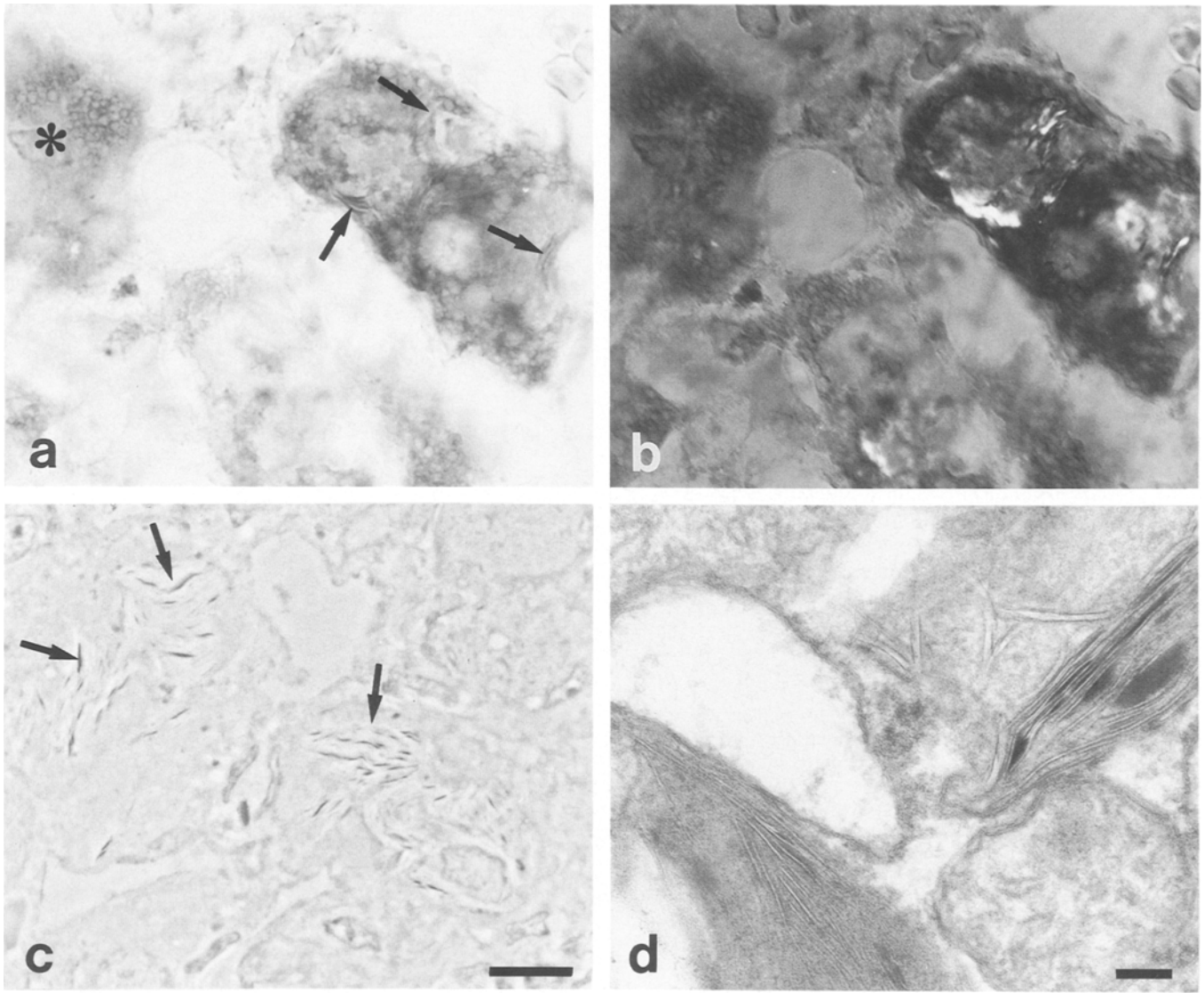

Fig. 5. a Cryostat section of adrenal gland (inner side of the cortex), stained with Sudan black B (SBB) after acetone treatment; bright field illumination. The cytoplasm of one cell still shows a positive reaction, while fat is dissolved in the neighbouring cell (spongious cytoplasm; *asterisk*); the SBB-positive cell also contains striated birefringent material (*arrows*), as revealed in polarized light (**b**). **c** Epon section (2 μm) of adrenal gland in the same zone as **a**; ballooned cells contain numerous inclusions with striated appearance (*arrows*) (phase contrast). **a–c** ×1250. *Bar* = 10 μm. **d** Regularly spaced trilamellar inclusions, constituting the striated birefringent material. *Bar* = 0.1 μm. ×81000

the plasma were strongly elevated ($C_{26:0}$: 3.5 μg/ml and $C_{26:0}/C_{22:0}$: 0.38; controls 0.3–0.8 μg/ml and 0.012–0.035 respectively) without organic or pipecolic aciduria. Total β-oxidation activity of peroxisomes from cultured fibroblasts was impaired: 0.7 pmol/min per mg protein (controls: 3.0–7.5 pmol/min per mg protein; with $C_{26:0}$ as substrate and measured according to Wanders et al. 1987). Bile acid intermediates and plasmalogen biosynthesis were normal. Death occurred at the age of 3 months during a prolonged apnoeic spell. Autopsy was performed immediately. Neuropathological findings included pachygyria of the bulbar olivae and cerebellar neuronal heterotopias, as seen typically in Zellweger disease (Caviness and Lyon 1978).

At the age of 15 days a liver biopsy was taken. It was fixed in 4% formaldehyde in 0.1 M sodium cacodylate containing 1% calcium chloride. Peroxisomes were visualized by staining for catalase activity with diaminobenzidine (Roels et al. 1988). Autopsy material (liver, kidney, brain, adrenal gland, myocardium and skeletal muscle) was fixed in glutaraldehyde. Histological stains, performed on cryostat sections, included periodic acid-Schiff (PAS; after borohydride treatment), acid phosphatase (Lojda et al. 1979), trichrome, Perl's, Holcinger, Sudan black B and oil red O (ORO).

Immunostaining for the peroxisomal β-oxidation enzymes [acyl-CoA oxidase; bifunctional enzyme (enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase); 3-keto-acyl-CoA thiolase] was performed on paraffin (5 μm) sections from the liver biopsy and kidney autopsy specimen using protein A-gold (5 nm) and silver enhancement (Intense M, Janssen Life Sciences, Beerse, Belgium) according to Litwin et al. (1988). For ultrastructural localization, etched Epon sections on nickel grids were stained with anti-thiolase antibodies according to Litwin et al. (1987), using protein A-gold (10 nm) to reveal the antigen-antibody complex. Protein A-gold probes were prepared according to Slot and Geuze (1985). Antibodies were raised in rabbits; purification of the enzymes, preparation and specificity testing of the antibodies were described by Osumi et al. (1980), Furuta et al. (1980) and Miyazawa et al. (1980). Anti-thiolase serum was affinity purified. As controls, sec-

tions were incubated with non-immune rabbit serum (Dakopatts, Copenhagen, Denmark).

Morphometry of the hepatic peroxisomes from the biopsy sample was performed according to De Craemer et al. (1991a). As controls, liver biopsies from eight normal adults were used.

Results

Peroxisomes in the liver showed a weak staining for catalase activity. Their distribution was normal. The population contained rare, obviously enlarged organelles (Fig. 1a). At the ultrastructural level, an amorphous nucleoid was present in the matrix of many peroxisomes; it was situated in the outer margin of the organelle (Fig. 2a). A tubular invagination of the peroxisomal membrane was seen in 2.9% of the organelles (Fig. 2b). In the autopsy liver 5 nm particles were found inside several peroxisomes.

By immunocytochemistry the three β-oxidation enzymes were localized in organelles compatible with peroxisomes according to their size, shape and distribution (Fig. 1b–d). In comparison with normal human liver, the staining reaction was weak for all three enzymes: the granules were not black but brown to dark brown. Control incubations were negative. The peroxisomal localization of the thiolase enzyme protein was confirmed by electron microscopy (Fig. 2c).

Morphometry showed that the size distribution of the peroxisomes was bimodal: the population contained a small group of organelles larger than 1 μm, which were absent in controls (Fig. 3). Numerical density, volume density and surface density were low, but within the range of controls (Table 1).

Weakly polarizing granules were found in macrophages; these granules were orange after PAS and did not react with lipid stains. By electron microscopy, rigid, trilamellar inclusions were found inside the lysosomes and cytoplasm of these cells (Fig. 4b). The inclusions are composed of an electron-lucid inner sheet against which electron-dense amorphous to fine granular material is apposed. The thickness of the lamellae (including the three layers) was 11.422 ± 0.378 nm SEM ($n=25$).

Trichrome staining revealed a moderate but distinct fibrosis at autopsy, which was not detected in the biopsy specimen. Micro- and macroglobular fat droplets with birefringent periphery were present in a minority of parenchymal cells in the central area; fat was dissolved by acetone. Iron (Perl's staining) was demonstrated in granules in parenchymal cells and macrophages as well as in diffuse form in the latter (Fig. 4a); reaction product was also present in sinusoidal cells. At the ultrastructural level iron was present as 5 nm particles in the cytoplasm of parenchymal cells; it was proment in the siderosomes and cytoplasm of macrophages (Fig. 4b).

In the adrenal gland the glomerulosa contained abundant fat droplets; these were completely dissolved after acetone treatment, causing the appearance of foam cells. Fat was almost absent in the fasciculata. Large solitary, PAS-positive cells at the inner side of the cortex contained birefringent inclusions. Some of these cells showed a diffuse reaction for fat not extracted by acetone but soluble in *n*-hexane and xylene. The inclusions had a striated appearance in phase contrast microscopy; they were resistant to acetone and some of them reacted with fat stains (Fig. 5a–c).

By electron microscopy, these cells were identified as macrophages containing peculiar "angulate" lysosomes, crowded with stacks of regularly spaced trilamellar structures (Fig. 5d), similar to those found in the liver macrophages. Their thickness is not significantly different (10.444 ± 0.277 nm SEM; $n=66$).

In the kidney focal proliferative glomerulonephritis was noticed. All tubules showed a diffuse ORO reaction; in some tubules microglobular fat was present. Peroxisomes were normally distributed; as in the liver, many organelles contained an amorphous nucleoid (Fig. 6a). The presence of the three β-oxidation enzymes and their peroxisomal localization was demonstrated by immunocytochemistry (Fig. 6b).

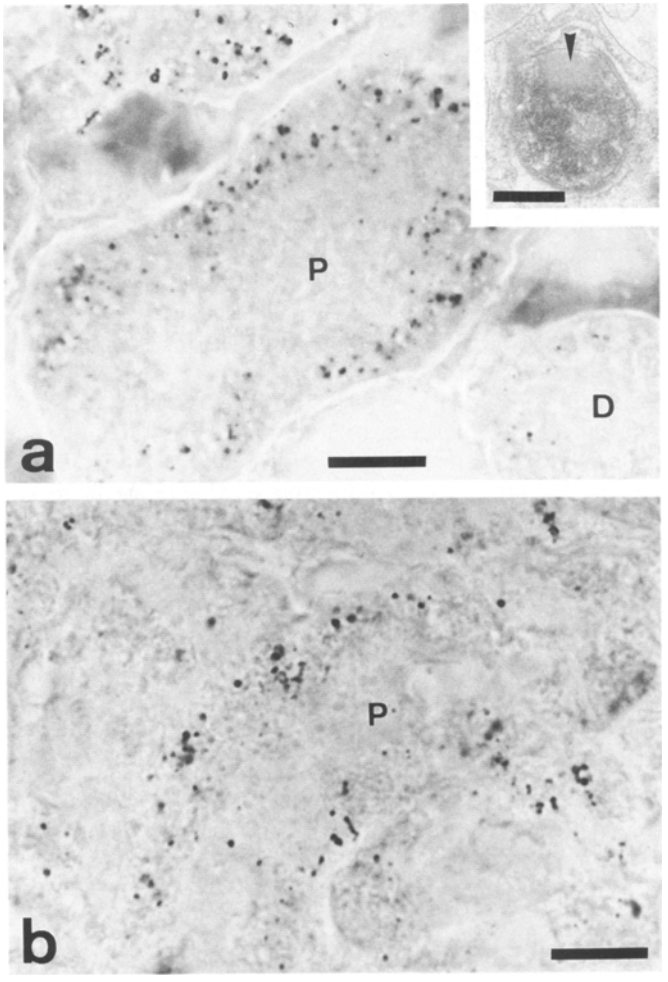

Fig. 6. a Staining for catalase activity in the kidney (autopsy). Distribution of the peroxisomes, visualized as dark granules, is normal; they are large and numerous in the proximal tubules (*P*) and scarce and smaller in the distal tubules (*D*). *Bar* = 10 μm. × 1250. *Inset:* peroxisome from the proximal tubules with a nucleoid (*arrowhead*) and surrounded by endoplasmic reticulum cisternae. *Bar* = 0.5 μm. × 18500. **b** Peroxisomes in the proximal tubules visualized as dark granules after immunostaining against bifunctional enzyme protein. *Bar* = 10 μm. × 1250

Microglobular fat was present, resistant to acetone extraction, in both the myocardium and skeletal muscle. The droplets showed birefringency, which remained present after acetone treatment.

Discussion

The intriguing characteristic of this patient is the occurrence of a peroxisomal β-oxidation defect together with the normal subcellular localization of the peroxisomal β-oxidation enzymes, as shown by immunocytochemistry. This is the first time such a situation has been reported. In contrast to most peroxisomal disorders, which are considered as defects of peroxisome biogenesis (Lazarow and Moser 1989), enzyme import – at least of the β-oxidation enzymes – is not defective in this patient. In Zellweger syndrome, Zellweger-like syndrome, infantile Refsum disease, NALD, β-oxidation enzymes and the sterol carrier protein 2 (nsLTP) are synthesized but not imported into the peroxisomes (Schram et al. 1986; Suzuki et al. 1990). A plausible explanation for the β-oxidation defect in this case could be the inactivity of at least one β-oxidation enzyme. However, the activity of acyl-CoA oxidase in vitro was found to be normal (Van Maldergem et al., to be published). In addition, the enzymatic deficiency of bifunctional enzyme and/or thiolase has – according to current knowledge (Wanders et al. 1990) – to be excluded because of the normal level of bile acid intermediates. Therefore, an enzyme defect at other levels should be considered.

The deficiency of VLCFA acyl-CoA ligase, which catalyses the VLCFA-CoA ester formation, is unlikely in view of the different clinical course and presentation of X-ALD (Moser et al. 1984). In search of the cause of the defect, the absence of a co-factor or the presence of an inhibitor inside the peroxisomes should also be taken into account. Goldfischer et al. (1986) have reported a case in which several peroxisomal oxidases were inactive; the deficient enzymes all shared flavin adenine dinucleotide as a co-factor. The lack of a co-factor inside the peroxisomes of this case is unlikely; deficiencies of several peroxisomal enzymes depending on the same co-factor did not occur (Van Maldergem et al., to be published).

Finally, a defect at the level of metabolite export should not be overlooked. The accumulation inside the perosixome of octanoyl-CoA or acetyl-CoA, which are the products of β-oxidation, might inhibit the β-oxidation sequence by negative feedback. This hypothesis is suggested by the fact that peroxisomal β-oxidation by intact fibroblasts is impaired (0.7 pmol/min per mg protein (controls: 3.0–7.5; substrate $C_{26:0}$) while in homogenates a normal acyl-CoA oxidase activity was found. Farrell et al. (1984) have suggested that carnitine octanoyl and acetyltransferase, which are present in peroxisomes, are involved in the evacuation of chain-shortened fatty acids out of the peroxisomes. After induction of peroxisomal β-oxidation by drugs and high fat diets in rats, peroxisomal carnitine acyltransferase showed a higher activity, increased levels of mRNA and immunoreactive protein and enhanced transcription rates (Brady et al. 1989). Buechler and Löwenstein (1990) have recently given evidence that peroxisomal β-oxidation requires carnitine intermediates. The peroxisomal carnitine transferases have not been examined in peroxisomal disorders.

In our patient the early presence of abundant trilamellar storage material is an indication, in comparison with other peroxisomal disorders, of a general impairment of fatty acid metabolism (see below; trilamellar inclusions). The neuronal heterotopias indicate that the pathogenic factor was active during the fetal period. This makes our case phenotypically most similar to Zellweger syndrome, which is characterized by neuronal migration disorders, or to the pseudo-Zellweger patient (Goldfischer et al. 1986). In contrast to the latter syndromes, our patient did not have abnormal bile acid intermediates, which therefore can be ruled out as the common cause of the migration defects.

Although the primary defect of the present peroxisomal β-oxidation disorder could not be elucidated, involvement of the peroxisomes is demonstrated by morphological alterations of the organelles. Three abnormalities are found in hepatic peroxisomes of this patient. Firstly, the size distribution of the organelles is aberrant: the population contains a group of very large organelles, which are absent in control livers. Enlarged peroxisomes – in co-existence with normally sized ones – occur in several cases of peroxisomal deficiencies; the possible significance of the phenomenon is discussed by Roels and Cornelis (1989) and Roels et al. (1988, 1991). Secondly, there is the presence of a nucleoid in the matrix of many peroxisomes. Interestingly, it was present in the same form in both the liver and kidney peroxisomes. A similar amorphous type of nucleoid was observed in a case, clinically diagnosed as NALD (patient 1 of Roels et al. 1988) and in a case of Wilson's disease and of chronic active hepatitis (Sternlieb and Quintana 1977). The amorphous nucleoid is absent in normal human liver peroxisomes and differs markedly from the structured core (crystalline urate oxidase) in rodent liver peroxisomes. Finally, there are the cylindrical invaginations of the peroxisomal membrane. Morphologically, they are the reverse of the tail-like protrusions and membrane loops, which are found in rapidly dividing peroxisome populations (fetal liver peroxisomes, Kerckaert 1990) or after peroxisome proliferation induced by drugs (Hruban et al. 1974; Baumgart et al. 1989; De Craemer et al. 1991a, b). The protrusions represent newly synthesized or reservoir membrane, ready for importation of the matrix enzymes (Baumgart et al. 1989). Functionally the invaginations also represent an excess of membrane material; however, they are less accessible for import from the cytoplasmic pool and they are only present in 2.9% of the population. They give evidence that the functional deficiency is not caused by a shortage of peroxisomal membrane material. They were also found in other peroxisomal disorders: in NALD-like (Roels et al. 1988), in thiolase deficiency (3.4%), in one case of infantile refsum disease with peroxisomal ghosts (3.4%) and in two cases of acyl-CoA oxidase deficiency (0.5% and 1.2%) (Roels et al. 1991).

Hepatic fibrosis progressing into cirrhosis is typical in Zellweger syndrome and hyperpipecolic acidaemia but not in other congenital peroxisomal disorders (Roels et al. 1991). In the present case fibrosis developed rapidly over a time span of 2.5 months.

Iron accumulation in liver macrophages was observed in thiolase deficiency (Van Hoof and Roels 1989) and in all Zellweger syndromes. In the latter it is maximal between the 5th and 20th week and drops to normal levels afterwards; it is not associated with necrosis (Danks et al. 1975; Gilchrist et al. 1976). Storage is present in the normal fetus. Post-natally and into adulthood it is found in macrophages and parenchyma of primary hyperoxaluria type I liver (De Craemer et al. 1989).

The significance of the fat in the renal tubules is unclear. According to Kaufmann (1957) the presence of fat in renal epithelium is common. However, Waugh (1966) stated that fat is normally absent. In three control kidney samples of neonates we could not demonstrate any fat after ORO staining. Interestingly, like in our patient, Colevas et al. (1988) observed lipid accumulation in the kidney, the myocardium and skeletal muscle in two neonates affected by glutaric acidemia type II. The authors related this finding to the metabolic block, but the resistance of the fat to acetone extraction was not examined. In our patient, we believe that this insolubility could be related to the accumulation of VLCFA (Johnson et al. 1976).

The occurrence of trilamellar inclusions in the liver varies amongst the peroxisomal disorders (Kerckaert et al. 1988; for review, see Roels et al. 1991). They are most abundant in Zellweger syndrome and infantile Refsum disease and in many cases of NALD; their amount increases with age. Hepatic inclusions are absent in many patients with X-ALD and in acyl-CoA oxidase deficiency. In the adrenal gland, they are found in X-ALD, NALD and Zellweger syndrome. Their chemical nature is still unclear; Johnson et al. (1976) concluded from extraction experiments in adrenal gland from X-ALD cases that they result from the storage of VLCFA. Their early and abundant presence in this patient reflects a general disturbance of the peroxisomal metabolism.

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Note added in proof

Bile acid intermediates have been determined in frozen liver tissue by R.B.H. Schutgens and R.J.A. Wanders, Amsterdam, who found strongly elevated levels of trihydroxycholestanoic acid (THCA), dihydroxycholestanoic (DHCA) and a C_{29} acid. The inactivity of bifunctional enzyme or thiolase protein therefore can no longer be excluded, and our speculation that bile acid intermediates cannot be responsible for defective neuronal migration is not valid. The new results are still compatible with an export impairment.