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Fatal lipid storage myopathy with deficiency of cytochrome-c-oxidase and carnitine

A contribution to the combined cytochemical-finestructural identification of cytochrome-c-oxidase in longterm frozen muscle

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Summary. Two newborn female siblings fell ill with apathy, failure of suckling and a generalized progressive muscular hypotonia. Death occured at the age of 7 weeks, obviously caused by impairment of respiratory musculature. Biochemical studies in one child revealed carnitine deficiency especially in skeletal muscle; hepatic encephalopathy was absent. Both children had a generalized hyperaminoaciduria, an unusual finding in primary carnitine deficiency.

Besides fatty metamorphosis of the liver, bilateral hydroureters and tubular calcifications of both kidneys, morphological studies showed a generalized lipid storage myopathy which predominated in Type-I-fibres and was accentuated in the muscles of the neck. Enzymehistochemical electron microscopy in longterm frozen muscle demonstrated that cytochrome-c-oxidase activity was absent not only in myopathic but also in most of the morphological unchanged muscle fibres. Only some fibres and endothelial cells displayed normal activity of mitochondria. Biochemically no cytochrome aa_3 (cytochrome-c-oxidase) could be found in skeletal muscle; cytochrome b was almost undetectable. – In newborns with fatal lipid storage myopathy and carnitine deficiency it seems necessary to look for additional defects in the respiratory chain. Enzyme histochemical electron microscopy is a sensitive method in identifying cytochrome-c-oxidase even after a 12 months period of storage.

Key words: Lipid storage myopathy – Carnitine deficiency – Mitochondrial myopathy – Respiratory chain – Cytochrome-c-oxidase – Autopsy

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Lipid droplets are present under physiological conditions in striated muscle, particularly in Type I fibres (Prineas and Ng 1967; Engel 1970). A great number of pathological conditions are accompanied by a moderately increased deposition of lipids in the striated muscle fibre, i.e. storage diseases such as glycogenosis Type I, endocrine disturbances, infectious or toxic processes, and enzyme deficiencies affecting the pyruvate metabolism or the transport of fatty acids (Engel 1966; Prineas et al. 1968; Angelini 1976; Scholte et al. 1979; Di Mauro et al. 1980b). Marked lipid storage is often seen in mitochondrial myopathies (Harriman and Reed 1972, Walter 1981). However lipid droplets are the leading morphological abnormality especially in idiopathic lipid storage myopathies (Gulotta et al. 1974; Jerusalem et al. 1975; Slavin et al. 1975; Miranda et al. 1979; Carrier et al. 1980; Sengers et al. 1980; Schröder 1981) and in primary carnitine deficiency.

Carnitine is a quaternary amine which is partly derived from food, but also synthesized in the liver from lysine and methionine. It is required for the transport of long-chain fatty acids across the inner mitochondrial membrane to the site of beta-oxidation in the mitochondrial matrix.

Besides secondary, often clinically silent cases of carnitine deficiency, e.g. in cirrhosis of the liver (Rudman et al. 1977) or chronic haemodialysis (Böhmer et al. 1978) there are two clinical variants of primary carnitine deficiency: One with depletion of carnitine only in striated muscle, and another one with low carnitine levels not only in striated muscle but also in the serum and further organs, particularly the liver (Di Mauro et al. 1980b; Engel 1980). The myogenic variety has a predominantly favourable outcome, the systemic form instead is often fatal. Both variants have in common a progressive, proximally pronounced lipid storage myopathy which, particularly in the case of the systemic manifestation of carnitine deficiency, can involve respiratory muscles and the cardiac muscle too. In addition an encephalopathy of the hepatic type, similar to that in the syndrome of Reye (Chapoy et al. 1980) is frequently seen in the generalized form. It usually becomes manifest intermittently, often in the course of some banal infection.

There are a number of reports of cases with atypical clinical and biochemical findings, that are difficult to classify (Hart et al. 1978; Scarlato et al. 1978a; Scholte et al. 1979a; Carroll et al. 1980; Tripp et al. 1981). They show, that the magnitude of carnitine deficiency does not always correlate with the clinical findings. This fact, as well as the great variability in the response to treatment (Carrol et al. 1980) gives reason to suppose that primary carnitine deficiency is heterogeneous not only from a clinical but also from a pathophysiological point of view. Reports of carnitine deficiency myopathies with glycogen storage, increased pyruvate-lactate levels and well marked alterations in mitochondrial morphology (Di Donato et al. 1978; Di Mauro cit. in Willner et al. 1979) particularly point to wideranging defects, above all in the respiratory chain. This was the reason why we carried out biochemical and enzyme histological investigations to evaluate mitochondrial function in two new-born siblings with fatal lipid storage myopathy and carnitine deficiency.

Table 1. Carnitine content[a] and activity of carnitinepalmityltransferase (CPT)[b]

		Total carnitine	Free carnitine	CPT
Case I	Muscle	8.2	3.2	9.4
	Liver	3.2	2.8	58.2
	Kidney	6.6	3.4	10.8
Control	Muscle (*n* 13)	23±5.9	17.2±4.0	5.5±2.8
	Liver[c]	5.8	5.4	5.4
	Kidney[c]	7.7	6.1	1.3

[a] μmol/g non-collagen containing protein
[b] U/g protein
[c] 1 autopsy sample

Case reports

Case 1

A.S., a girl, the fifth child of Turkish parents, was delivered by vacuum extraction after an uneventful 42 week gestation. She weighed 3,350 g. It was soon noticed that the apathetic infant had marked muscular hypotonia and difficulty in swallowing. The liver was slightly enlarged. Serum transaminases, gammaglutamyl transferase, glucose and ammonia were in the normal range. There were no cardiac symptoms. Further investigation revealed a refractory metabolic acidosis, lactate-pyruvate aciduria in the presence of normal concentrations of serum lactate and pyruvate, and a general hyperaminoaciduria. The infant died of respiratory insufficiency aged seven weeks.

Biochemical investigations. Carnitine estimations done on fresh postmortem tissues showed a marked reduction in the level of both free and total carnitine in striated muscle and a less marked reduction in the liver. In the kidneys it was mainly the level of the free carnitine that was low. The activity of carnitinepalmityltransferase was at twice the normal level in skeletal muscle (Table 1).

Investigations carried out later failed to show any cytochrome aa_3 (cytochrome-c-oxidase) in skeletal muscle; only traces of cytochrome b were present. In the liver there were normal levels of cytochrome (Endres et al. to be published).

Case 2

A sister of A.S., the parents fourth child, also died of respiratory insufficiency aged seven weeks. The clinical picture was similar, hyperaminoaciduria and a metabolic acidosis were present.

Case 3

Another sister of the above cases also died aged about seven weeks. There are no further clinical details, a postmortem examination was not done. – The parents of the deceased children, as well as their adolescent son and daughter, are healthy.

Material and methods

Light microscopy. Postmortem tissues (Case 1 and 2) were fixed in 10% formol saline, embedded in paraffin and stained with haematoxylin-eosin, PAS-alcian blue and van Gieson. Unfixed frozen sections were stained with Sudan black and scarlet red.

Electron microscopy. Fixation in glutaraldehyde, 6.25% in phosphate buffer pH 7.4 for 2 h, followed by washing for more than 24 h in 0.2 M buffered sucrose solution. Postfixation

was carried out with 2% osmic acid. The tissue was embedded in Epon, contrast was enhanced in the ususal way. Thus prepared were: 1. Fresh postmortem skeletal muscle from case 1. 2. Skeletal muscle from case 1 and 2, obtained at autopsy. 3. Autopsy tissue of liver and kidney from case 1.

Enzymehistochemistry (Case 1). Myofibrillary adenosinetriphosphatase pH 9.4; NADH-tetrazolium reductase.

Cytochemical-ultrastructural demonstration of cytochrome-c-oxidase: Unfixed thin sections were preincubated in H_2O_2-free incubation medium for 2 h followed by incubation in complete medium (3 mg DAB/ml, 0.3% H_2O_2 in 10 ml tris stock-solution 0.1 M and destilled water to 50 ml), pH 8.5 (Herzog and Fahimi 1974). This was followed by fixation with 3% glutaraldehyde, 0.05% $CaCl_2$ in 0.1 M sodium cacodylate buffer pH 7.4, for 2 h at 4° C. Sections were rinsed in 0.15 M tris-HCl pH 7.4, post-fixed in unbuffered 1% osmium tetroxide for 1 h at 4° C, dehydrated in ethanol, followed by propylene, propylene/Epon (50/50) and Epon. Sections were examined directly or after treatment with lead citrate.

The following material was examined: Fresh postmortem skeletal muscle from case 1, stored frozen for one year (kept at about −80° C), control tissue (muscle biopsy kept under the same conditions, taken from an infant of the same age, with spinal muscular atrophy of Werdnig-Hoffmann) and fresh rat muscle.

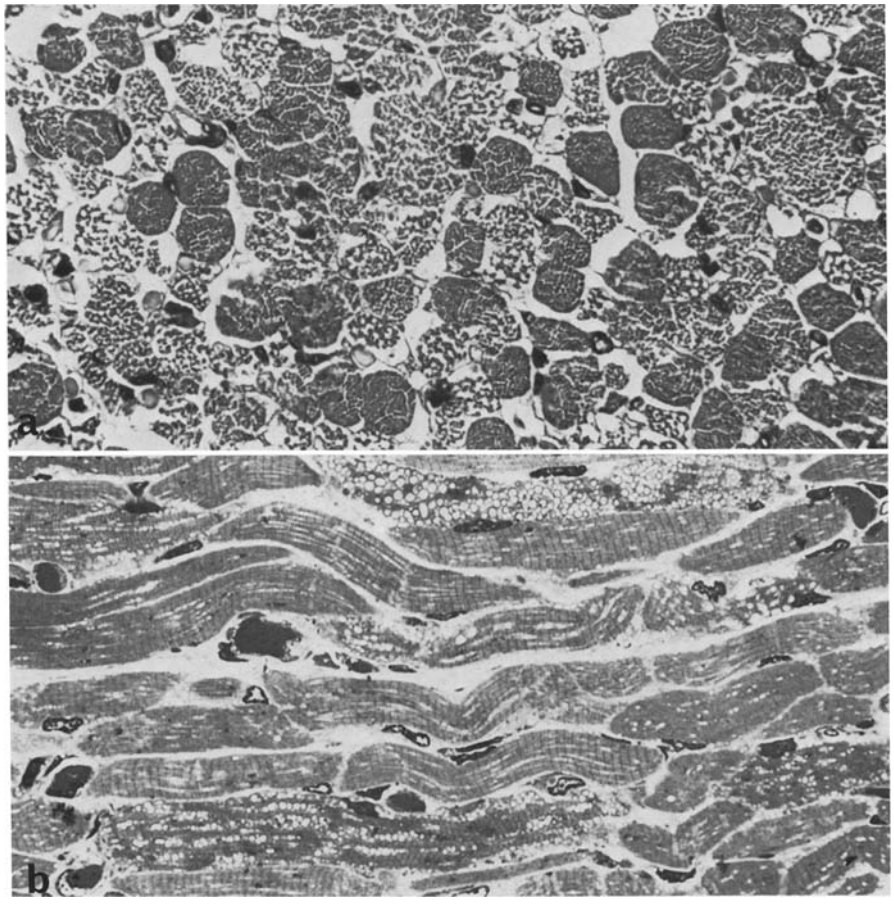

Fig. 1 a, b. a Loose arrangement of fibrils in many muscle fibres (HE). **b** Vacuolar degeneration of some fibres (Semithin, Azurmethylenblue). **a, b** × 640

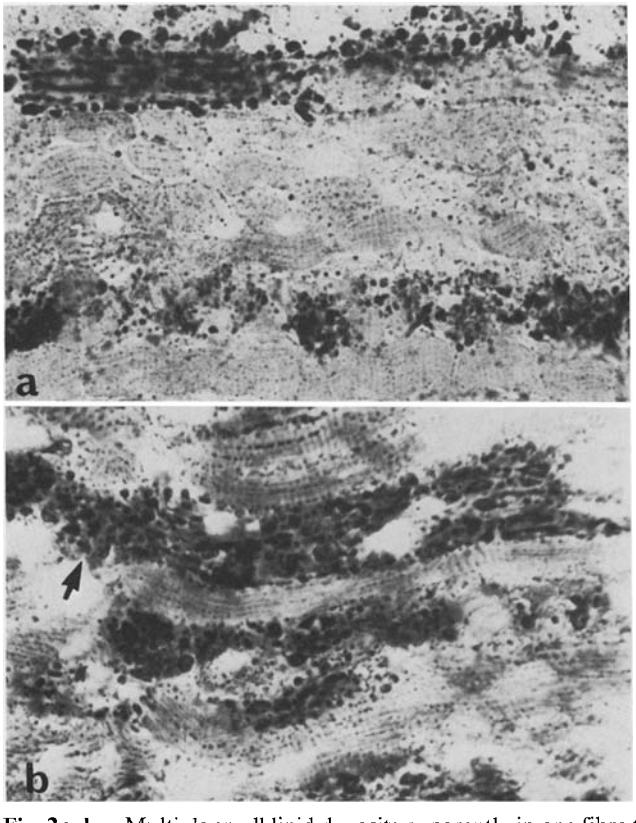

Fig. 2a, b. a Multiple small lipid deposits apparently in one fibre type (Sudan black). **b** NADH-Reductase Reaction. Increased enzyme activity with a rough reaction product in type I fibres (→). **a, b** × 640

Biochemical investigation. Demonstration of free and total carnitine and carnitine palmityltransferase as described previously (Pongratz et al. 1979; Deufel 1981).

Results

A. Muscle pathology

Macroscopic Findings. Marked hypotrophy was noted at autopsy. The two infants had a length of 54 cm, the weight of case 1 was 2,490 g, case 2 3,400 g. There was a generalized decrease in muscle mass, most noticeable in the neck.

Light microscopy. Many muscle fibers had a coarsened appearance with loose arrangement of their fibrils (Fig. 1a). Some of the nuclei were in a central position, frequently there were enlarged nucleoli. The semithin sections showed a vacuolated myopathy (Fig. 1b). There was an increase in fine lipid droplets, distributed in a random manner, but only within one fibre-type, corresponding to a lipid storage myopathy (Fig. 2a). The

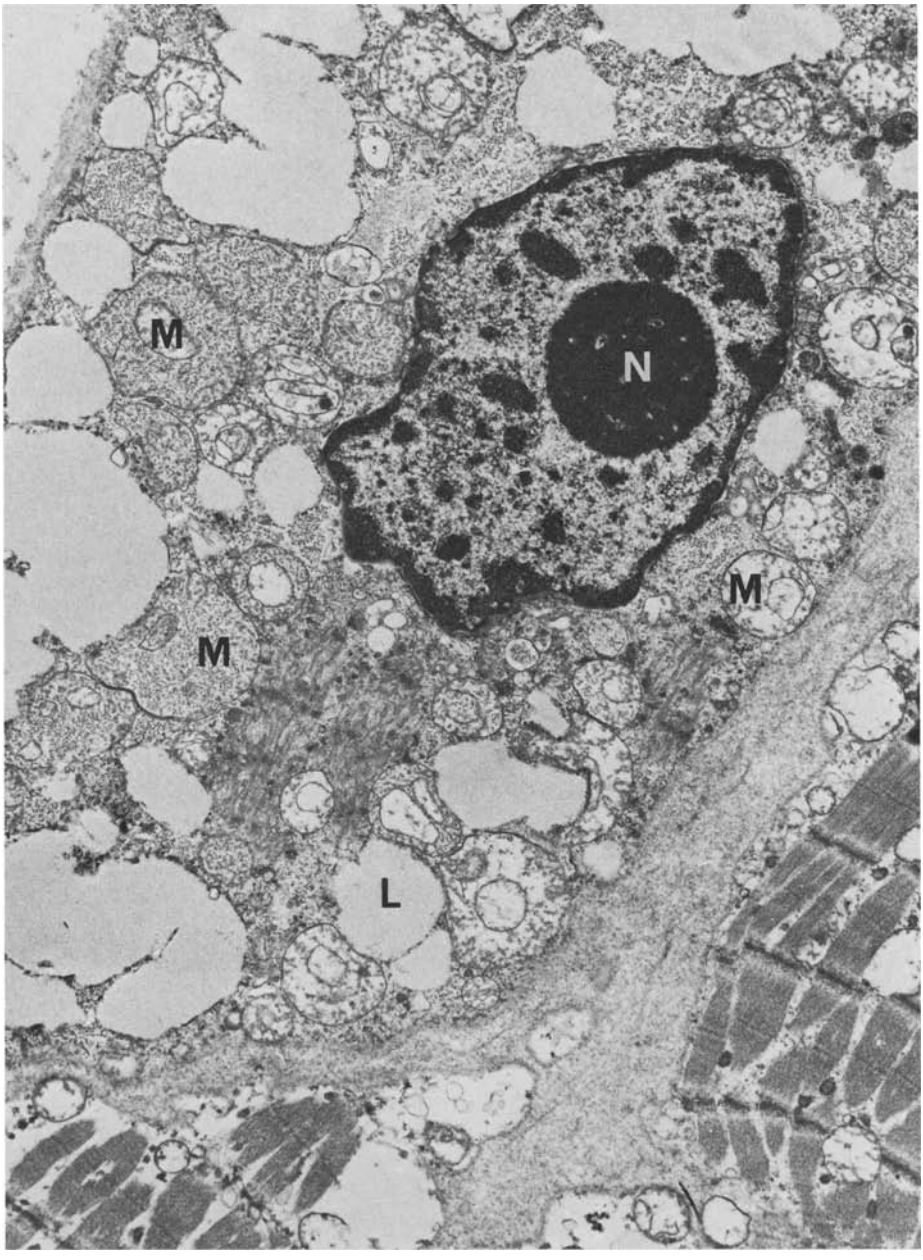

Fig. 3. Muscle fibre containing only a few fibrils, with many enlarged mitochondria (*M*) which show slight morphological anomalies and store glycogen. In the cytoplasma many lipid vacuoles (*L*) and glycogen particles; nucleus with enlarged nucleolus (*N*). × 10,000

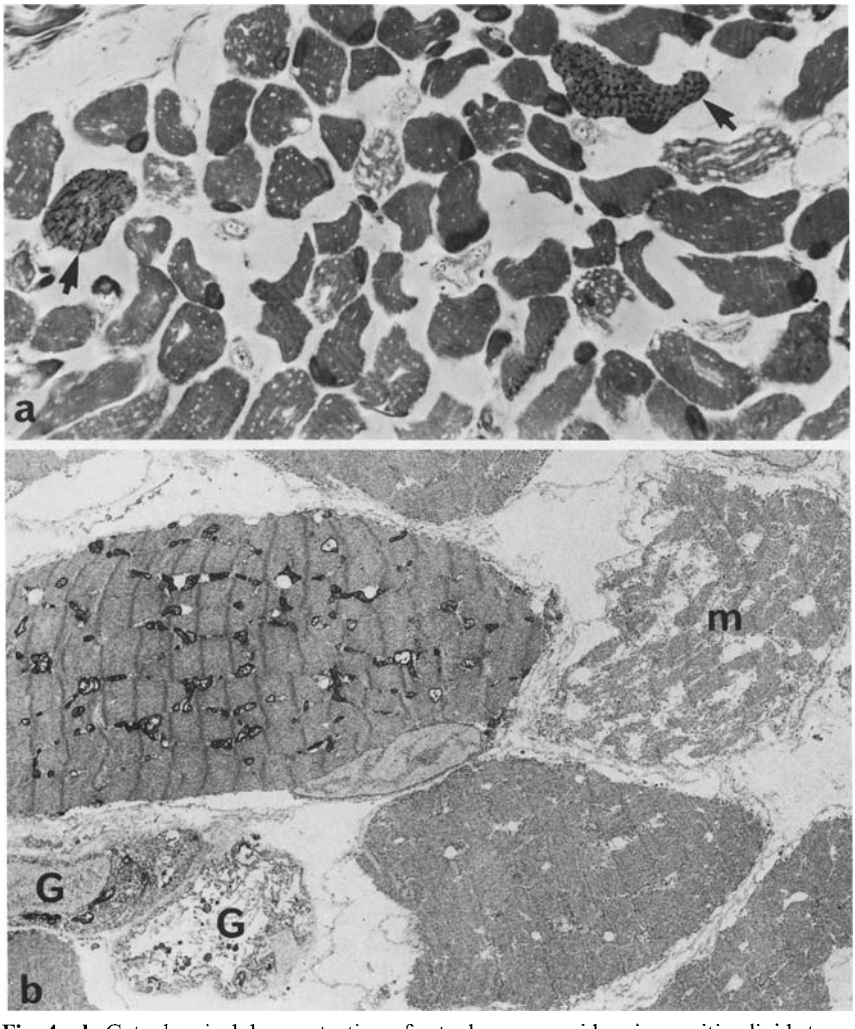

Fig. 4a, b. Cytochemical demonstration of cytochrome-c-oxidase in carnitine lipid storage myopathy after a 12 months period of storage. **A** Reaction product only in some fibres (→, Semithin, Azurmethylenblue). **b** No enzyme reaction in myopathic (*m*) and in many of the morphologically unchanged fibres. One fibre as well as endothelial cells of blood vessels (*G*) with tightly packed reaction product in the mitochondria. **a** × 640, **b** × 2,600

above changes were seen in skeletal muscle, muscles of respiration including accessory muscles, muscles of tongue and pharynx and striated muscle fibres in the oesophagus.

Electron microscopy. In the muscle tissue examined the main findings were an increase in both number and size of the mitochondria; other findings were loss of fibrils, numerous lipid vacuoles, abundant glycogen (Fig. 3) and occasional cytoplasmic bodies. Only minor structural abnormalities were seen in mitochondria, but glycogen was often present in their matrix.

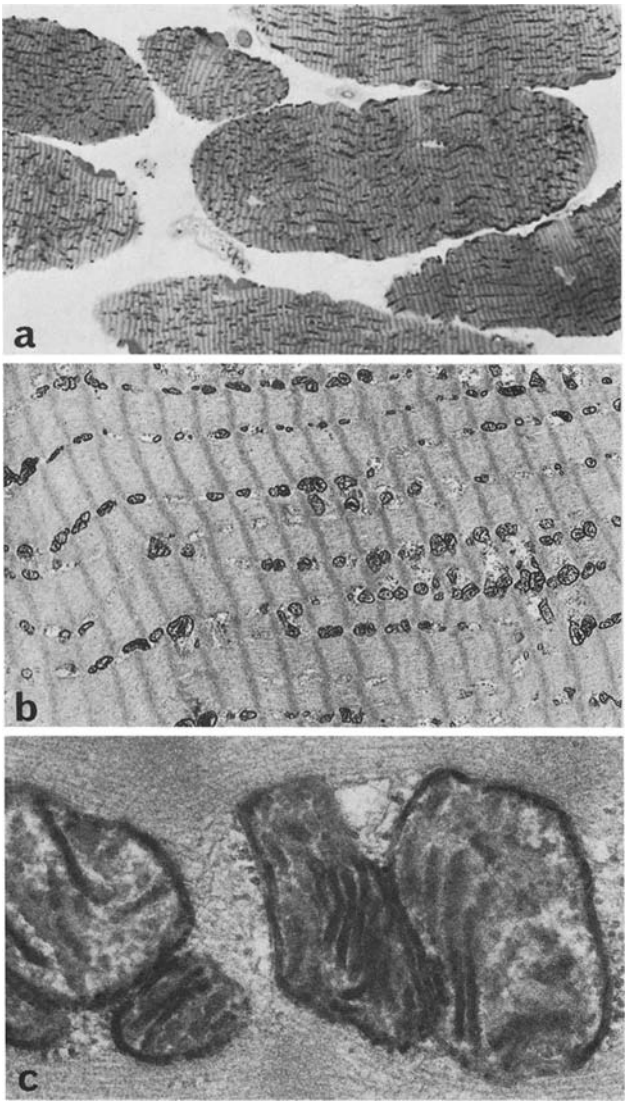

Fig. 5a–c. Cytochemical demonstration of cytochrome-c-oxidase in control muscle after a 12 months period of storage. **a** Uniform distribution of the reaction product without diffusion artefacts. **b, c** strong density of mitochondria by the deposition of the enzyme product. **a** ×640, **b** ×4,000, **c** ×52,000

Within some muscle fibres electron dense round paracrystalline inclusions (17 nm ∅, case 1) could be found, their significance is not clear.

Enzyme histochemistry revealed a coarsened reaction pattern of NADH-reductase in Type I fibres (Fig. 2b). Staining for myofibrillary ATPase confirmed that the vacuolation noted was present mainly in Type I fibres. In light microscopy a marked defect of cytochrome-c-oxidase was demon-

strated already. Only some individual fibres had normal enzyme activity (Fig. 4a). In the control enzyme activity was evenly distributed throughout the whole muscle (5a). Electron microscopy showed that cytochrome-c-oxidase was undetectable not only in abnormal but also in morphologically normal appearing fibres (4b). On the other hand the mitochondria of endothelial cells had intense deposits of the enzyme reaction product (Fig. 4b just as the mitochondria from the control muscle (Fig. 5b, c).

B. Other tissues

In the liver fatty metamorphosis and occasional cytoplasmic vacuoles of the hypoxic type were found. In the kidneys there was extensive tubular necrosis with calcification and some foreign-body reaction. The bladder and ureters were dilated. On electron microscopy no significant abnormality was noted in the mitochondria of kidney, liver, or in smooth muscle. There was no evidence of fatty change in the heart or the kidneys.

Discussion

Little is known about the pathological processes at the molecular level in primary carnitine deficiency. It has not yet been possible to ascertain whether it is due to faulty synthesis or transport mechanisms (Karpati et al 1975; Willner et al. 1979; Scarlato et al. 1978b; Rebouche and Engel 1980). In addition our two cases with hyperaminoaciduria demonstrate that a proximal tubular defect might be a contributary cause of carnitine deficiency.

Disturbances of carnitine metabolism can occur under a variety of circumstances, e.g. during parenteral feeding of premature infants, (Penn et al. 1980 and 1981), during pregnancy (Scholte et al. 1978), in the course of the vomiting sickness in Jamaica (Karnovsky 1979), in glutaraciduria Type II (Duscheiko et al. 1979) and in connection with methylmalonaciduria (Carrier et al. 1980). Our own observations demonstrate a defect in the cytochrome-c-oxidase in striated muscle, associated with carnitine deficiency.

From the clinical point of view it is remarkable, that in our cases with carnitine deficiency in the skeletal muscle, the liver and kidney, the symptoms do not correspond to those of typical systemic carnitine deficiency. There was no evidence of hepato- or encephalopathy. The rapid progression of our cases is unusual too in typical cases of fatal primary carnitine deficiency (Boudin et al. 1976; Cornelio et al. 1977; Engel et al. 1977; Hart et al. 1978; Scarlato et al. 1978a; Ware et al. 1978; Scholte et al. 1979a). The same is true for the generalized hyperaminoaciduria. Our own clinical and biochemical findings are comparable to those in the literature for two cases with a defect in the cytochrome aa_3 (cytochrome-c-oxidase) and b in skeletal muscle and kidneys (van Biervliet et al. 1977; Di Mauro et al. 1980a). One of the above cases (Di Mauro et al. 1980a) is interesting because there was a lowering of the carnitine level (only on one occasion) in striated muscle. Other cases of carnitine deficiency without typical symptoms and with a rapidly fatal course (Pongratz et al. 1979; Esiri et al. 1979) probably

belong also to this apparently well circumscribed entity which differs from other myopathies with deficiency of cytochrome-c-oxidase (Monnens et al. 1975) by, among other features, the bad prognosis.

To the methods used it should be noted, that enzyme histochemical electron microscopy is a sensitive method in identifying cytochrome-c-oxidase. Even after a 12 months period of storage an intensive, even staining free of diffusion artefacts can be demonstrated in the mitochondria. The applied histochemical method allows at least in our cases a more differentiated view of the magnitude and the location of the enzyme defect than biochemical investigations. Our enzyme histochemical studies show first of all that the defect of cytochrome-c-oxidase is not confined to Type I fibres (cf. Di Mauro et al. 1980a, Fig. 5a). It involves fibres showing vacuolated degeneration as well as morphologically normal fibres. Therefore the enzyme defect is not due to degenerative changes. Secondly the results prove that the loss of cytochrome-c-oxidase in the skeletal muscle is incomplete. Activity remains not only in the mitochondria of the blood vessel wall, but also in some of the muscle fibres. The localization of the enzyme defect in muscle fibres of different types is the more remarkable as the structural abnormalities are seen above all in the predominantly oxidative Type I fibres, just as in primary carnitine deficiency. Finally it should be stressed that in our two cases with defective mitochondrial respiration there were none of those structural abnormalities of mitochondria commonly found in the so-called mitochondrial myopathies (Kamieniecka and Schmalbruch 1980; Walter 1981).

Concerning the origin of the mitochondrial defect and the associated deficiency of carnitine no firm conclusions can be drawn. Carnitine deficiency may be associated with an increase of free fatty acids (Di Donato et al. 1978). It is known that the latter interfere with oxidative phosphorylation (Borst et al. 1962) and cause swelling of mitochondria (Zborowsky and Wojtczak 1963). In rats brominated vegetable oils produce a lipid storage myopathy, probably through interference with β-oxidation, but with normal carnitine levels (Brownell and Engel 1978). The myopathological changes in the Type I fibres of our cases may also be due to a variety of factors acting singly or in combination. Thereby interference with mitochondrial respiration may play a vital part in the disturbance of the energy dependent carnitine transport in striated muscle.

In addition it is of interest that ethidiumbromide will interfere in vitro with cytochrome aa_3- and b-activity (Soslau and Nass 1971) by acting on mitochondrial DNA. Disturbed function of mitochondrial respiration is further known to occur in such fatal metabolic disorders as the Zellweger Syndrom (Goldfischer et al. 1973; Versmold et al. 1977; Endres et al. 1981). Therefore it appears reasonable to regard the defect of cytochrome-c-oxidase in our cases with their rapid but not immediately perinatal fatal outcome as an expression (consequence?) of a progressive metabolic disorder.

Irrespective of these considerations the presence of carnitine deficiency in newborn infants with fatal lipid storage myopathy, especially in association with a proximal tubular defect, should prompt to look for additional

defects in the respiratory chain. Besides the biochemical approach the cytochemical-finestructural demonstration of cytochrome-c-oxidase is a very sensitive method. Our experience shows that even after a 12 months period of storage this technique gives an intensive evenly distributed reaction without diffusion artefacts in the muscle fibre. Thereby a precise anatomical localization of the enzyme defect is possible.

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Note Added in Proof

Since submission of this manuscript two similar cases with a defect of cytochrome-c-oxidase have been published.

However carnitine levels either are not mentioned (Stansbie D. et al. (1982) J Inher Metab Dis 5:27–28), or in a normal range (Heiman-Patterson TD et al. (1982) Neurology 32:898–900. Moreover our finding that cytochrome-c-oxidase deficiency is not necessarily accompanied by structural alterations of the muscle fibres and their mitochondria is confirmed by a report of Rimoldi et al. (1982) J Neurology 227:201–207. The total carnitine content in the muscle of this case was low too. However the clinical course and the biochemical results differ from ours.