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Lignoceric Acid Biosynthesis in the Developing Brain. Activities of Mitochondrial Acetyl-CoA-Dependent Synthesis and Microsomal Malonyl-CoA Chain-Elongating System in Relation to Myelination

Comparison between Normal Mouse and Dysmyelinating Mutants (Quaking and Jimpy)

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Age-related changes in the activities of microsomal and mitochondrial elongating systems have been determined in mouse brain from birth to maturity.

In microsomes, the components necessary for behenyl-CoA (docosanoyl-CoA) elongation have been found to be NADPH and malonyl-CoA. In mitochondria, both NADH and NADPH are used and acetyl-CoA is the only donor of two-carbon-atoms unit. The synthesised fatty acids were identified by thin-layer and gas chromatography. The specific activity is higher in microsomes than in mitochondria.

In microsomes, the specific activity for malonyl-CoA incorporation reached a maximum at 15–20 days of age; this peak was not obtained in the Quaking and Jimpy mutants. The increase in enzyme activity (specific activity and total activity per brain) paralleled the myelin deposition. The activity of the mitochondrial system increases regularly during development: it is not correlated to myelination and it is not affected in the Quaking mutant. The interplay between microsomal and mitochondrial elongation systems is studied.

Changes in the fatty acid composition of individual lipids during brain development, particularly during the period of myelination, have been well documented [1–6]. Among various lipids, sphingolipids are very important: brain sphingolipid fatty acids are chiefly saturated, mono-unsaturated and α-hydroxylated long-chain fatty acids, predominantly with 24 carbon atoms [6–9]; they are concentrated in myelin galactolipids [10,11]. The presence of these fatty acids increases the transition temperature, so the lipids can less readily leave the membrane structure [12]. Moreover the high concentration of saturated and mono-unsaturated fatty acids preserves the integrity of the membrane (unlike poly-unsaturated fatty acids, they are not susceptible to break down by peroxidase and lipoxygenase). Other factors can also be important, as their chain length leads to interdigitated complexes [13]. Indeed their presence in the myelin sheath confers to this membrane increasing stability and their biosynthesis is a fundamental event during the maturation of the brain: alteration in the activities of enzymes involved in long-chain fatty acid synthesis could have a profound effect on the stability of the myelin membrane, as the defective myelination in the Quaking mutant is correlated with a defect in the biosynthesis of very long-chain fatty acids [14,15], especially those with 24 carbon atoms [16,17].

Cerebral fatty acids can be derived from endogenous fatty acids or from uptake of fatty acids from the circulation at all stages of development [18,19] and, so far, the endogenous biosynthesis is a very important pathway.

Whereas most enzymes involved in lipid synthesis show increased enzyme activity during the period of most active myelination and lipid deposition, in the cerebral nervous system, the activity of rat-brain soluble fatty acid synthetase has been found to decline a few days after birth [20–22]. Thus the relationship between this enzyme complex and lipogenesis in the brain is uncertain; moreover the reaction products are medium-chain fatty acids, mainly palmitic acid [23,24]. However, total fatty acid biosynthesis seems to be maximal at time of rapid myelination [25,26]

Trivial Names. Lignoceric, tetracosanoic; behenyl, docosanoyl.

Enzymes. Malate dehydrogenase (EC 1.1.1.37); monoamine oxidase (EC 1.4.3.4); glucose-6-phosphate dehydrogenase (EC 1.1.1.49); 5'-nucleotidase (EC 3.1.3.5); phosphotransacetylase (EC 2.3.1.8).

and the biosynthesis of lignoceric acid has not been studied during brain maturation. This acid is biosynthesised either in microsomes [14,15,16,21,22,27,28] or in mitochondria [28,29] but it is likely that the fatty acid moiety of myelin lipids must be synthesised in the microsomal fraction [30] where other enzymes involved in the formation of sphingolipids are present.

The purpose of this work is to study lignoceric acid biosynthesis in the developing brain, measuring the activities of mitochondrial acetyl-CoA-dependent synthesis and microsomal malonyl-CoA-dependent chain-elongation system in normal mice and dysmyelinating mutants (Quaking and Jimpy), in the presence of behenyl-CoA.

MATERIALS AND METHODS

Acetyl-CoA, malonyl-CoA, NADPH, NADH, CoA were obtained from Pabst-Biochemical, [1-^{14}C]acetyl-CoA and [1,3-^{14}C]malonyl-CoA from N.E.N., behenic acid from Fluka, scintillation products (PPO and POPOP) and Triton X-100 from Packard, all other chemicals from Merck. The radioactivity was counted in a Packard liquid scintillator (with PPO and POPOP in toluene). Behenyl-CoA was synthesised with modifications [16] of a previously described technique [31], then purity was checked by thin-layer chromatography on silica gel plates visualised under ultraviolet light (350 nm); the eluting solvent was butanol/water/acetic acid (50/30/20, v/v/v). The gas-liquid chromatographies were made on SE-30 column in a Hewlett-Packard 5750 with automatic counting of the eluate (Panax). During gas-liquid chromatography (150 – 250 °C, 2 °C/min) 40 % of the effluent gas passed through the flame ionization detector for determination of relative retention time of esters. The other 60 % passed through a radioactivity monitoring system with a combustion oven at 650 °C.

C57-black mice of either sex were used. Microsomal preparation from 18-day-old mice have been previously described [30]. Briefly tissue is washed and homogenised in cold 0.32 M sucrose, phosphate 0.1 M pH 7, NaCl 0.9 %. It is first centrifuged at 17500 × *g* and the pellet is discarded. The supernatant is then spun at 100000 × *g* and the resuspended pellet is respun under the same conditions. Mitochondria were prepared with modifications [32] of a previously described technique [33]. The brain tissue was homogenised in ice-cold 0.32 M sucrose, 10 mM phosphate pH 7 to give a tissue concentration of 10 % (v/v). Nuclei, large myelin fragments and tissue debris were sedimented at 2000 × *g* for 5 min. The supernatant was respun under the same conditions. Microsomes and soluble proteins were eliminated at 12500 × *g* for 30 min. The pellet contains impure mitochondria. This pellet was gently homogenised in the medium (1 ml/g of fresh tissue) and centrifuged into a discontinuous sucrose density gradient formed by pipetting a layer of 0.8 M sucrose (13 ml) on an equal volume of 1.2 M sucrose. After centrifuging at 53000 × *g* for 2 h, a small gelatinous pellet was obtained containing pure mitochondria. The purity of microsomal and mitochondrial preparations was checked by electron microscopy and marker enzymes [30, 32]. As determined by marker enzymes, no mitochondrial (malate dehydrogenase and monoamine oxidase) or soluble (glucose-6-phosphate dehydrogenase) were detected in microsomes [30]; microsomal (glucose 6-phosphatase), soluble (glucose-6-phosphate dehydrogenase) or membranous (5′-nucleotidase) were also hardly detectable in mitochondria [32]. The proteins were determined by Lowry's method [34].

Behenyl-CoA elongation was measured by determining the amount of malonyl-CoA or acetyl-CoA incorporated in fatty acids. Except when indicated otherwise, incubation mixture contained routinely behenyl-CoA (15 µM for microsomes, 50 µM for mitochondria), 50 µM [1,3-^{14}C]malonyl-CoA (2 mCi/mmol) for microsomes or 50 µM [1-^{14}C]acetyl-CoA (2 mCi/mmol) for mitochondria, 500 µM NADPH (plus 500 µM NADH for mitochondrial assay), 1 mg proteins and buffer up to 1 ml. When using microsomal proteins, 2 µg of phosphotransacetylase was added to block *de novo* biosynthesis [15, 16, 27]. Triton X-100 (1 mg) was added when mitochondria were tested. Extraction and identification of the biosynthesised fatty acids have been previously described [16] (identification of saturated, unsaturated and hydroxy fatty acids was accomplished by a combination of thin-layer and gas liquid chromatographies).

Each experiment was performed a minimum of three times. All these experiments required about 500 normal mice, 120 Quaking mutants and 28 Jimpy mice.

RESULTS AND DISCUSSION

Optimal Conditions for Elongation of Behenyl-CoA in Microsomes and Mitochondria

In both organelles this acyl-CoA is capable of being elongated (Fig. 1). In mitochondria, there is no acetyl-CoA incorporated when behenyl-CoA is omitted from the incubation mixture; but with microsomes, malonyl-CoA is incorporated into fatty acids when the acyl-CoA is absent (mainly providing palmitic and stearic acid through *de novo* mechanism and elongation of endogenous fatty acids). It is noteworthy that the inhibition (above 17 µM) shown in microsomes is not present in mitochondria at any concentration. The omission of either NADH or NADPH decreased drastically the amount of ligno-

ceric acid produced: when neither of these nucleotides were present, there was no lignocerate production. The effect of both NADH and NADPH is illustrated on Table 1. The optimal level for each nucleotide was measured; the effect of increasing the amount of one of the nucleotides was studied both in the absence of the other nucleotide or with a constant amount. In microsomes NADPH is only used (at relatively high concentration of NADH alone, there is a significant amount of elongation); adding NADH does not increase the biosynthesis of lignoceric acid and neither changes the small amount of hydroxy fatty acids. In mitochondria there is a requirement for both reduced pyridine nucleotides: with NADH alone or NADH + NADPH the same quantity of acetyl-CoA is incorporated, but there is a large incorporation rate of radioactive acetate into hydroxy derivatives when NADH is the sole hydrogen donor. In the presence of both NADH and NADPH, the preferential reaction products are saturated fatty acids with only small amount of hydroxy derivatives: according to these results, the second reduction step of chain-elongating process in brain depends on the presence of NADPH. Thus the mitochondrial enoyl-CoA reductase in brain is working with NADPH, as in other organs for medium-chain fatty acid biosynthesis [35]. In the presence of NADH alone, mitochondria provides mainly hydroxy and saturated fatty acids: thus keto-reductase is working with NADH (and not NADPH) and probably the specificity of enoyl-CoA reductase for NADPH is not total.

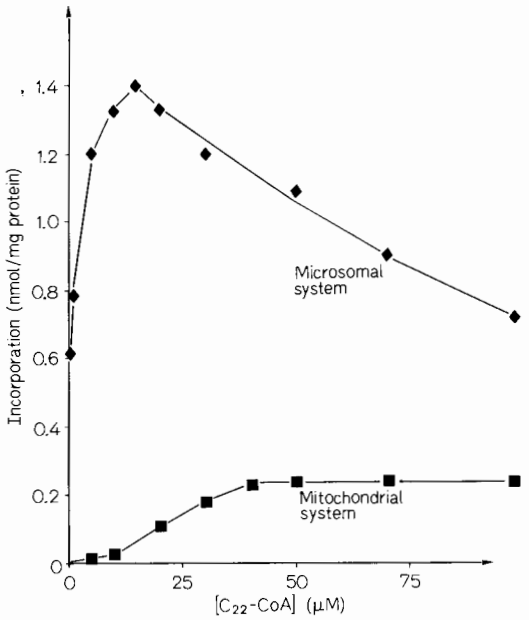

Fig. 1. *Microsomal and mitochondrial elongation of behenyl-CoA.* The specific activity of the elongating enzymes was calculated from curves drawn with varying amount of protein (0.05, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 1.5, 2 mg). At each age, the same curve as the one in Fig. 4 was drawn. Data are mean values of three experiments. The following systems were used. (◆) Microsomal system: 50 μM [1,3-^{14}C]malonyl-CoA (2 mCi/mmol), 500 μM NADPH, 0.08 M PO_4^{3-} pH 6.9, 0.30 M sucrose, 0.7% NaCl, 2 μg phosphotransacetylase and 1 mg microsomal proteins; all tubes are made up to a total volume of 1 ml and incubated 1 h at 37 °C. (■) Mitochondrial system: 50 μM [1-^{14}C]acetyl-CoA (2 mCi/mmol), 500 μM NADPH, 500 μM NADH, 1 mg Triton X-100, 8 mM PO_4^{3-} pH 7, 0.3 M sucrose, 1 mg mitochondrial proteins; all tubes are made up to a total volume of 1 ml and incubated 30 min at 37 °C. In microsomes, the radioactivity refers to the formation of total fatty acids: malonyl-CoA is mainly incorporated in C_{24}, little activity is found in C_{26}, C_{16} and C_{18}: see Table 2. The results are expressed as nanomoles of radioactive precursor incorporated per milligram of proteins

Changes in Activity during Development in Normal and Quaking

Changes in the specific activity of malonyl-CoA elongation of behenyl-CoA by the microsomal fraction from mouse brain, from birth to the adult, are shown in Fig. 2 and compared with changes in the specific activity of behenyl-CoA elongation by acetyl-CoA in mitochondria. Changes in the activity of the mitochondrial fatty acid synthesis is similar in normal and mutant: radioactive acetyl-CoA incorporation slightly and regularly increases during development. The temporal change in the activity of fatty acid bio-

Table 1. *Effect of NADH and NADPH on the elongation of behenyl-CoA*
The yield of the reaction was calculated with the complete system (see Fig. 1 and 2). HFA: hydroxy fatty acids, NHFA: non-hydroxy fatty acids (saturated + unsaturated) 100% refers to the maximal activity with the complete incubation medium (with mitochondria: C_{22}-CoA + acetyl-CoA + NADH + NADPH, with microsomes: C_{22}-CoA + malonyl-CoA + NADPH). Data are mean values of three experiments. Beh-CoA, behenyl-CoA; Mal-CoA, malonyl-CoA; Ac-CoA, acetyl-CoA

Fraction	Behenyl-CoA elongation with			Hydroxy fatty acids synthesised with		Non-hydroxy fatty acids synthesised with		Yield based on		
	NADPH	NADH	NADPH + NADH	NADH	NADPH + NADH	NADH	NADPH + NADH	Beh-CoA	Mal-CoA	Ac-CoA
	%									
Microsomes	100	24	100	–	3	–	97	10	2.8	0
Mitochondria	17	100	100	23	5	77	95	2.5	0	0.7

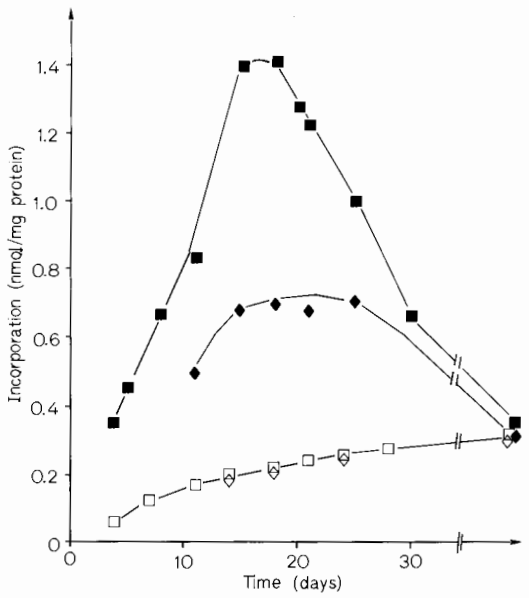

Fig. 2. *Changes in behenyl-CoA elongation during development.* Same remarks as in Fig. 1; behenyl-CoA was 17 μM and 40 μM in microsomes and mitochondria, respectively. Control microsomes (■); microsomes from Quaking mutant (◆); control mitochondria (□); mitochondria from Quaking mutant (◇)

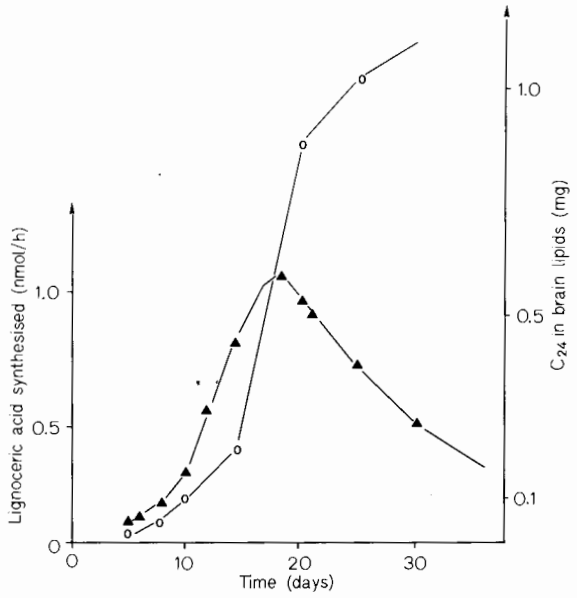

Fig. 3. *Lignoceric acid biosynthesis in total mice-brain microsomes during development.* Comparison with content of C_{24} non-hydroxylated fatty acids in brain lipids as a function of age. Same remarks as in Fig. 1 and 2; (▲) lignoceric acid synthesised per brain in microsomes (○) total content of C_{24} non-hydroxylated fatty acids in brain lipids

synthesis in microsomes is quite different. There is a period of maximum activity by 15 – 18 days where the specific activity is multiplied by 4 when compared to 4-day-old or adult animals (but the total synthesis of lignoceric acid per brain is multiplied by 11: see Fig. 3 and Table 2). However, in Quaking mutant, this peak is much less prominent. In controls, the specific activity of the microsomal elongating complex was 5 times (at 4 days) to 7 times (at 15 – 20 days of age) higher than the specific activity of the enzyme in the mitochondrial fraction. But at adult age, this activity is nearly the same in both organelles.

The fatty acid profiles have been performed at various ages: in agreement with previous results [29], mitochondria synthesise lignoceric acid. In microsomes however (Table 2), besides lignoceric acid, some medium-chain fatty acids are synthesised. As phosphotransacetylase is added to impede *de novo* mechanism [16], palmitic and stearic acids are synthesised by elongation of endogenous acids. These results are confirmed by decarboxylation data [39] as the ratio total activity/activity in carboxyl is 1.02. In microsomes, in the presence of behenyl-CoA, the ratio elongation of endogenous fatty acids/elongation of exogenous behenyl-CoA is more important at young ages: as behenyl-CoA possibly inhibits endogenous medium-chain fatty acid elongation [16], this inhibition may be less important in young animals. Moreover, the reaction products are not modified in the mutant. Thus the specific activity in Quaking

Table 2. *Reaction products in microsomes during development: gas chromatographic analysis of an aliquot of the methyl esters of the fatty acids synthesised*
Previously to this analysis, thin-layer chromatographies eliminated unsaturated and hydroxylated fatty acids. The latter were not identified; the former were mainly $C_{22:6}$. Data are mean values of three experiments

Age	Reaction products with chain length					
	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	C_{26}
days	%					
6	17	27	1	1	56	1
12	4	16	1	1	73	7
18	4	10	1	1	78	8
30	3	10	1	1	78	9
Adult	3	9	1	1	79	8

at 14 – 18 days is 50% of normal, but the mutant brain has only 75% of the normal amount of microsomes [16]; therefore, *in vitro*, the synthesis of lignoceric acid from behenyl-CoA is 30% normal in Quaking mice. These results are in agreement with the data obtained from analysis of brain lipids: in the normal total lipid extract, lignoceric acid represents 1.9 ± 0.2% of total saturated fatty acids, 0.6 ± 0.1% in the Quaking mutant. At adult age the synthesis of lignoceric acid is much less affected in the mutant, but the once-and-for-all opportunity for myelination

has gone and myelination cannot develop normally. The fate of this lignoceric acid synthesised at adult age is unknown: it is probably incorporated in other membranes than myelin or it is involved in myelin turn-over.

The temporal change in the elongating complex activity in the microsomal fraction implicates this enzyme in myelin lipid formation: the period of maximum activity of biosynthesis in the whole brain corresponds to the highest rate of lignoceric acid deposition (Fig. 3). This normal rise of lignoceric acid correlated with myelination is not found in the Quaking mice brain. In this mutant, the specific activity of lignoceric acid biosynthesis is 50% normal and brain total activity 30% normal at 18 days. Moreover (Fig. 4) in the Jimpy mice (a mutant without any myelination [36,37]), the specific activity of the microsomal enzyme is 6% normal and brain total activity of biosynthesis of lignoceric acid 3% normal (Table 3).

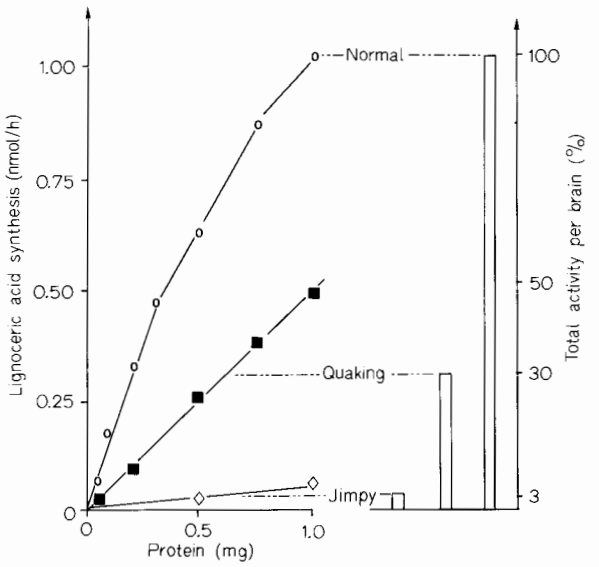

Fig. 4. *Lignoceric acid biosynthesis in control, Quaking and Jimpy mice.* Same remarks as in Fig. 3

The curve for lignoceric acid is drawn from results previously published [38] and is dealing with all fatty acids with 24 carbon atoms, saturated and mono-unsaturated, but mono-unsaturated and saturated fatty acids are biosynthesised through the same enzymatic complex [17]. At 18 days of age, 1.4 nmol of lignoceric acid is synthesised per hour per brain. This activity is nearly constant between 15–20 days. During this period, about 250 μg of lignoceric acid is incorporated into membrane and endogenous microsomal biosynthesis provides about 65 μg (80 μg taking into account mitochondrial system). Assuming that 50% of organelles are lost during preparation (as tested by marker enzymes) the total endogenous biosynthesis provides about 160 μg of lignoceric acid. These results show either that the optimum condition for enzymatic activity is not reached in the test tube or that the developing brain is actually dependent on exogenous fatty acids during the period of myelination.

Conclusions

The microsomal fraction of brain contains enzyme systems for the elongation of preformed fatty acids by malonyl-CoA (there are at least three different systems in mouse brain microsomes: one is a *de novo* system, the second one synthesises C_{18} fatty acids from palmityl-CoA and the third one very long chain fatty acids from stearyl-CoA [14,15,27,39]). The maximum activity occurs at the period of most active myelination. Moreover several microsomes enzymes involved in lipid synthesis show similar temporal changes in specific activity [40–44]. These enzymes are implicated in myelin lipid formation. The maximum synthesis of lignoceric acid and stearyl-CoA elongating system [22] occurs at time of maximum deposition of very long chain fatty acids in cerebral lipids. The steep rise in content of C_{24} fatty acids in normal mouse brain 18–20 days old after birth, which coincides with the active phase of myelination,

Table 3. *Reaction products in Jimpy and Quaking mutants and their respective control littermate*
The values, in counts/min, are referring to the fatty acid biosynthesis in microsomes from a whole brain. The incubation medium contains buffer, behenyl-CoA, malonyl-CoA and NADPH. No phosphotransacetylase was added in these experiments; thus the activity found in lignoceric acid is due to elongation of behenyl-CoA; the activity in myristic, palmitic and stearic acids is due to elongation of endogenous fatty acids and *de novo* mechanism. These values are the mean values of three experiments. tr: activity <150 counts/min

Animal	Reaction products with chain length						
	C_{14}	C_{16}	C_{18}	C_{20}	C_{22}	C_{24}	C_{26}
	counts/min						
Jimpy	175	5934	2150	tr	tr	454	tr
Control Jimpy	430	9350	3575	tr	tr	13150	1650
Control Quaking	527	9962	3516	tr	tr	13771	2051
Quaking	175	3150	1150	tr	tr	4480	570

is highly depressed in the Quaking mouse and fails to occur in the Jimpy mutant. This abnormality is due to a deficiency in lignoceric acid biosynthesis in microsomes. These studies in the developing brain support the concept that there is a close time relation between maximal lignoceric acid biosynthesis by microsomal malonyl-CoA system, and the period of rapid myelination: one of the possible subcellular levels for control of myelin synthesis may be localized to the brain elongation pathway, responsible for the formation of lignoceric acid.

These results confirm that microsomes contain enzymes for myelin lipid synthesis (possibly derived from glial cells) [30]. Striking is the difference between the components necessary for acyl-CoA elongation according to the organelle: in mitochondria, acetyl-CoA is the immediate precursor of the two carbon elongation units. Both NADH and NADPH are necessary for synthesis of saturated fatty acids. On the contrary microsomal enzyme need NADPH alone and malonyl-CoA. The physiological and genetic significance of these results is not yet known. The mitochondrial acetyl-CoA system provides also lignoceric acid and its synthesis slightly increases during maturation without any peak at myelination time. This latter result shows that mitochondria are not involved in myelin lipid biosynthesis. Mitochondrial elongation is not related to myelination. Mitochondria contain galactolipids [45] and non-myelin membranes contain lignoceric acid [46] possibly synthesised in mitochondria. But the interplay between the two elongation pathways (microsomal and mitochondrial) within brain cells is not known at present. Elongating complex contains at least four enzymes [47]: thiolase (condensing enzyme), β-hydroxyacyl dehydrogenase, enoyl-CoA hydratase and enoyl-CoA reductase; at least the first and the last one must be different in microsomes and mitochondria (because of the difference in the two-carbon elongation unit and in the nucleotides).

Changes in soluble synthetase activity during brain development are related to changes in content of enzyme [48]. So it is possible to speculate that the developmental change in very long chain synthesising enzymes is accompanied by alteration in the rate of synthesis and degradation of enzyme complex possibly related to the differenciation of the oligodendrocyte through brain specific regulatory mechanism [49].

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