BIOSYNTHESIS OF LIGNOCERIC ACID FROM BEHENYL-COA IN MOUSE BRAIN MICROSOMES. COMPARISON BETWEEN NORMAL AND QUAKING MUTANT.

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SUMMARY : Enzymatic elongation of behenyl-CoA has been studied in mouse brain microsomes. Malonyl-CoA and reduced nicotine-adenine-dinucleotide phosphate are required. The effect of protein concentration and incubation time have been determined. Phosphotransacetylase was added to reduce the de novo microsomal system and the radioactive fatty acids synthesized were identified by thin layer chromatography and gas chromatography with automatic counting of the eluate. The elongation capacity of Quaking microsomes is reduced to 30% of the normal value.

Lignoceric acid is an important fatty acid of the central nervous system : its presence in the myelin sheath confers to this membrane increasing stability (1). Indeed its biosynthesis is a fundamental event during the maturation of the brain.

In vivo studies have shown that the brain is capable of synthesizing lignoceric acid by an elongation process (2,3,4,5,6). In vitro, evidence has been presented showing that brain mitochondrias can elongate behenyl-CoA (7). Using as a primer acetyl-CoA or malonyl-CoA instead of very long chain fatty acyl-CoA, long chain fatty acid biosynthesis has been studied during maturation in mitochondria (8,9) or microsomes (8,10). We have previously demonstrated that there are at least three different systems in mouse brain microsomes (11,12,13). One is a de novo system; the second one synthesizes fatty acids with eighteen carbon atoms from palmityl-CoA and the third one builds very long chain fatty acids from stearyl-CoA (reaction products were checked by radio-gas chromatography). We have postulated the existence of a fourth system involving arachidyl-CoA as a primer (13). These results have been confirmed (14). All these studies have been helped by the comparison between normal and Quaking mouse which is a recessive autosomal mutant characterized by defective myelination of

the central nervous system (15). The galactolipid content in myelin lipids of Quaking mice is much less than normal and the non hydroxylated very long chain fatty acids typical of these myelin lipids are drastically reduced (16,17,18,19). We have previously reported (13) that the microsomal enzyme system which converts palmityl-CoA to stearate was slightly below normal in Quaking mice, but the elongation products of stearyl-CoA were severely defective, especially the production of lignocerate.

The purpose of this work is to present further characterization of lignoceric acid biosynthesis in brain microsomes and to demonstrate that biosynthesis from behenyl-CoA is impaired in mutant mice.

MATERIAL AND METHODS : Microsomal preparation from 18 day-old mice and the purity of this fraction have been previously described (20). Briefly, tissue is homogenized in cold 0.32 M sucrose-0.1 M phosphate pH 7. It is first centrifuged at 17,500 g and the pellet containing cellular debris, mitochondria , synaptosomes and myelin is discarded. The supernatant is then spun at 100,000 g and the resuspended pellet is respun under the same conditions. Proteins were determined by Lowry's method (21). Behenyl-CoA was synthesized from behenic acid and CoA with slight modifications (22) of a technique (23) previously described. Its purity was checked by thin layer chromatography.

The assay for fatty acid synthesis was checked by determining the amount of [1 - 3 ^{14}C] malonyl-CoA incorporated. Routinely the assay mixture involved 15μM behenyl-CoA (2 mCi/mmol), 500μM NADPH, 1 mg microsomal protein ; 3μg of phosphotransacetylase was added to block de novo biosynthesis (12,13) and the final volume was made up to 1 ml with phosphate-sucrose buffer (12). Incubation lasted 60 min. at 37°C. The reaction was stopped by the addition of 0.5 ml 4.5 N alcoholic potassium hydroxide solution. The reaction mixture was saponified for 15 min. in a boiling water bath, followed by acidification with 0.5 ml 5.5 N HCl. This acidified mixture was extracted twice with petroleum-ether, the extracts were collected and the solvents evaporated to dryness. The residue was counted and eventually methylated (24).

Chain length	16	18	22	22	24	Total activity
	%	%	%	%	%	(cpm)
Without PTA	45	16	nd	nd	39	26720
With PTA	7	10	nd	nd	83	12790

Table I -Suppression by PTA of the activity of the de novo microsomal system from normal mouse.

µ substrate	0	1	3	6	10	15	20	30	50	70	100	150	200	300	500
NADPH	-	46	73	456	475	np	785	957	1005	1225	1352	1384	1491	1433	1478
Malonyl-CoA	-	312	710	1140	1438	np	2015	2499	3150	3250	3630	398	np	np	np
Behenyl-CoA	1105	1503	1780	2014	2195	2286	2233	1907	1687	1270	950	np	np	np	np

Table II -Effect of varying concentrations of NADPH, or malonyl-CoA or behenyl CoA, on the elongation process. Other conditions as in methods - np : not performed. The radioactivity (given in cpm) refers to the formation of total fatty acids (mainly C24 and C26, few activity is found in C16 and C18 : see Table I).

Identification of fatty acid methyl esters was accomplished by a combination of various analytical procedures that included thin layer and gas chromatographies. Separation of hydroxy and non hydroxy methyl esters was performed on silica gel thin layer chromatography using as solvent petroleum ether-ether (80/20 v/v) ; $AgNO_3$ impregnated plates separated saturated and unsaturated fatty acids using benzene-hexane (50/50 v/v). Gas liquid chromatography was performed on a 3% SE 30 column using Packard 5750 ; the radioactivity of the eluate was measured with a Panax counter. Each experiment was performed a minimum of three times.

RESULTS

Synthesis of saturated, unsaturated and hydroxylated fatty acids. Thin layer chromatographies show that few unsaturated and hydroxylated fatty acids are synthesized (1.8% and 2%respectively).

Effect of phosphotransacetylase (PTA). Adding PTA depresses the

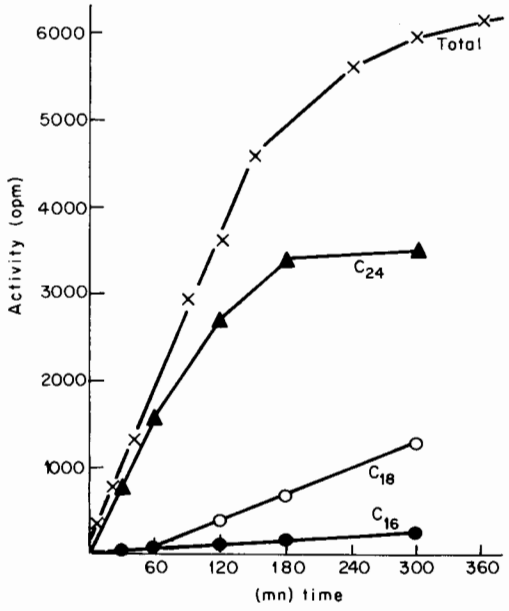

Figure I -Effect of incubation time on the elongation of fatty acids.

incorporation of malonyl-CoA by 50%, by impending de novo fatty acid biosynthesis. The addition of PTA into the incubation mixture permits the isolation of the elongation system. Table I shows the reaction products with and without PTA.

Components necessary for the elongation process. (Table II) The incorporation of malonyl-CoA increases linearly up to 200µM NADPH and then appears to plateau. The addition of NADH, FAD, FMN do not produce any increase in the incorporation without or with NADPH (200µM) (not shown here).

Increasing concentration of malonyl-CoA produces larger amount of substrate to be incorporated into fatty acids, but the slope is slightly decreased after 50µM.

Over 15uM behenyl-CoA there is an inhibition of malonyl-CoA incorporated into fatty acids.

Effect of incubation time on the elongation of fatty acids (endogenous fatty acids + behenyl-CoA added). The elongation reaction was measured at various time intervals by determining the amount of [1 - 3 -^{14}C] malonyl-CoA incorporated in total fatty acids as well as into each fatty acid. Figure I shows that maximum velocity of incorporation is found at about 2 hours, but that incorporation continues to increase up to 6 hours of incubation. At 2 hours the elongation of exogenous behenyl-CoA is nearly complete ; from 2 to 6 hours palmitate and especially stearate are synthesized from endogenous fatty acids.

Effect of amount of enzyme, in normal and Quaking mouse. We have shown that the components necessary for the elongation process are the same for normal and Quaking mouse. More over the maximal concentrations for the various substrates are the same (unpublished results). Figure II shows that the specific activity in Quaking is nearly 50% of normal. Also (Table III) the reaction products are slightly modified in the mutant. More over the mutant brain has only 75% of the normal amount of microsomes (normal : 2.5 mg/g of brain, Quaking : 1.95 mg/g). Therefore the synthesis of lignoceric acid from behenyl-CoA is 30% normal in Quaking mice.

DISCUSSION : A de novo system exists in mouse brain microsomes (12,13). It needs acetyl-CoA and malonyl-CoA to function. When malonyl-CoA is the only substrate available, the de novo sys-

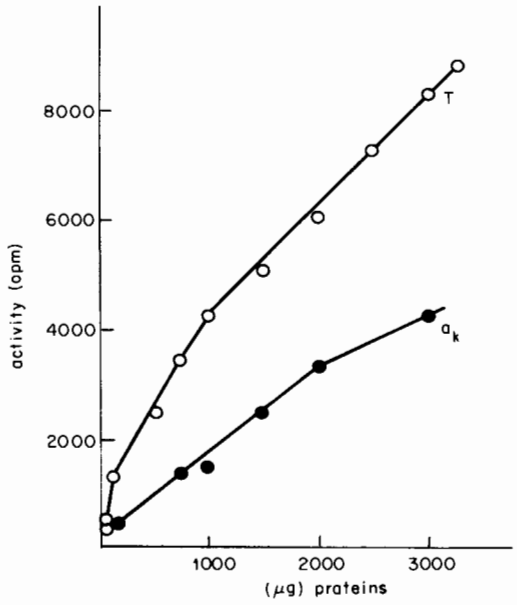

Figure II -Effect of variation of microsomal protein concentration in normal (T) and Quaking (Qk) mice.

Chain length	16 %	18 %	24 %	26 %	Total activity (cpm)
Normal	4	9	75	8	55310
Quaking	5	15	70	4	23840

Table III -Reaction products in normal and Quaking mice.

tem operates through a decarboxylase which provides acetyl-CoA from malonyl-CoA. This unwanted reaction is destroyed by phosphotransacetylase which converts acetyl-CoA into inactive substrate for the de novo system. The presence of phosphate in the medium is part of the reaction, thus acetyl-CoA is converted into acetyl-phosphate : the enzyme PTA catalyses the transfer of acetyl between phosphate and CoA. Malonyl-CoA is found to be the direct precursor of the active two carbon

unit in the elongation process in microsomes. In mitochondria it seems that acetyl-CoA is used (7). NADPH alone is indispensable, under our conditions of incubation. So it is not effective to add FMN or FAD, as it has been done (14) by others.

Table II shows that the activity of the elongation enzyme complex is not strictly dependent on the presence of behenyl-CoA. The activity found in its absence can be explained by elongation of endogenous behenate. But malonyl-CoA alone does not elongate only endogenous behenate, but also other fatty acids (not shown here). 83% of the activity is found in lignocerate in presence of 15µM of behenyl-CoA and less when behenyl-CoA is in minor concentration. We believe that increasing concentration of behenyl-CoA has an inhibitory effect on some elongating systems ; the detergent effect of behenyl-CoA cannot be excluded, for it is known that acyl-CoA can act as detergent (25) : over a given concentration the substrate inactivates its own enzyme. With 18µM behenyl-CoA, 17% of radioactive malonyl-CoA is incorporated in both palmitate and stearate. And no radioactivity is detected in arachidate or behenate.

The Quaking mice synthesize three time less lignocerate in brain microsomes ; these results are in agreement with the datas obtained from analysis of brain lipids.

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