European Neurology
Editor-in-Chief: H.E. Kaeser, Basel

Publishers: S. Karger, Basel
Reprint (Printed in Switzerland)

Eur. Neurol. *18:* 312–321 (1979)

Infantile Form of So-Called Neuronal Ceroid Lipofuscinosis: Lipid Biochemical Studies, Fatty Acid Analysis of Cerebroside Sulfatides and Sphingomyelin, Myelin Density Profile and Lipid Composition[1]

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Key Words. Ceroid lipofuscinosis · Neuronal ceroid lipofuscinosis · Fatty acids · Ethanolamine phosphoglycerides · Wallerian degeneration

Abstract. The biochemical analysis of a case of infantile neuronal ceroid lipofuscinosis, as determined by clinical and neuropathological findings, is presented. A diminished amount of solids is found, the amount of lipids is 30% of the normal as expressed in lyophilized tissue. The yield of myelin isolated by the density gradient is 1.8% of the normal. Phospholipid patterns show a reduction in ethanolamine phosphoglyceride, *N*-acetylneuraminic acid is extremely low and sphingolipids are largely reduced, cerebrosides being most affected (2.5% of the normal). In cerebrosides and sulfatides the decrease in very long chain fatty acids is important, but the deficiency in any type (including hydroxy compounds) is not too dramatic.

According to the aspect under electron microscopy, the density profile, and the biochemical composition of the subfractions, isolated myelin is close to normal. The loss of the myelin sheath appears to reflect a Wallerian degeneration in the CNS: myelin loss is a secondary effect. This disease, from a biochemical point of view, seems to be the ideal control for leukodystrophies.

A case was presented (*Hagberg et al.*, 1968) with severe progressive encephalopathy of late infantile onset. The biochemical changes indicated a disturbance in the metabolism of arachidonic acid (*Svennerholm*, 1976) or a loss of brain structures containing its derivatives. Indeed combined clinical, histological, histochemical and biochemical investigations prompted the assumption of a previously unknown generalized metabolic disorder affecting particularly the brain and retina. Recently, the so-called 'infantile neuronal ceroid lipofuscinosis' (INCLF) was delineated (*Haltia et al.*, 1972) and the name of polyunsaturated fatty acid lipidosis was proposed by *Hagberg et al.* (1974) and *Svennerholm et al.* (1974). They found that the brain content of all lipid classes, particularly the sphingolipids, was very low.

[1] This work was supported by Inserm and CNRS.

Journal of Neurochemistry, Vol. 32, pp. 85–90.
Pergamon Press Ltd. 1979. Printed in Great Britain.

0022-3042/79/0101-0085$02.00/0

THE ACTIVITY OF PARTIAL REACTIONS IN THE CHAIN ELONGATION OF PALMITOYL-CoA AND STEAROYL-CoA BY MOUSE BRAIN MICROSOMES

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(*Received* 13 *April* 1978. *Accepted* 19 *June* 1978)

Abstract—Partial reactions in the overall chain elongation of palmitoyl-CoA and stearoyl-CoA by mouse brain microsomes have been analyzed. The rate of the initial condensation reaction between palmitoyl-CoA and malonyl-CoA was more than 5 times greater than the rate obtained with stearoyl-CoA, and in both cases good agreement between condensation and overall chain elongation rates was observed.

By contrast, both β-hydroxyoctadecanoyl-CoA and β-hydroxyeicosanoyl-CoA were quite rapidly dehydrated by brain microsomes at similar rates. Similar results were obtained with 2-*trans*-octadecenoyl-CoA and 2-*trans*-eicosenoyl-CoA in which both substrates were rapidly reduced at nearly the same rate in the presence of NADPH. In all cases, intermediate reactions subsequent to condensation were much more rapid than overall chain elongation. These results suggest that the mechanism of malonyl-CoA-dependent fatty acid chain elongation in brain microsomes is similar to that observed in other tissues, and are consistent with an overall regulation of chain elongation mediated primarily by the initial condensation reaction.

THE MALONYL-CoA-dependent fatty acid chain elongation system in microsomes represents a potentially important means of modifying fatty acids obtained from *de novo* synthetic activity or the diet to meet the requirements of a particular tissue. Although early studies were primarily concerned with the hepatic system (ABRAHAM *et al.*, 1961; STOFFEL & ACH, 1964; NUGTEREN, 1965), fatty acid elongation in brain has proven to be of considerable interest due in part to the prominence of very long chain saturated and mono-unsaturated fatty acids in the sphingolipids of myelin (O'BRIEN, 1965) and to the availability of mutant mice defective in both myelination (SIDMAN *et al.*, 1964) and fatty acid biosynthetic activity (BOURRE *et al.*, 1973*a*; 1977).

In 1965, Nugteren established that β-keto, β-hydroxy and *trans*-α,β-unsaturated intermediates were formed during fatty acid chain elongation in rat liver microsomes. A recent investigation of partial reactions in the hepatic fatty acid chain elongation sequence employing representative saturated and polyunsaturated substrates has demonstrated that in each case the rates of the initial condensation reaction between fatty acyl-CoA and malonyl-CoA show good agreement with those of overall chain elongation, while subsequent partial reactions are much more rapid than chain elongation (BERNERT & SPRECHER, 1977). The object of this study was to establish whether the same malonyl-CoA-dependent chain elongation system is operative in mouse brain microsomes, and whether the considerably slower rate of stearoyl-CoA elongation relative to that of palmitoyl-CoA in this tissue may be attributed to a difference in condensation activity. Evidence for the existence of separate systems acting on these two substrates has been provided (BOURRE *et al.*, 1970; 1973*a*; GOLDBERG *et al.*, 1973).

MATERIALS AND METHODS

Materials. Malonyl-CoA, NADPH, palmitoyl-CoA and stearoyl-CoA were obtained from Pabst-Biochemical, while bovine serum albumin containing less than 0.005% free fatty acids was purchased from the Sigma Chemical Co., St. Louis, MO (U.S.A.). [1-^{14}C]Palmitoyl-CoA and [1-^{14}C]stearoyl-CoA were products of N.E.N. Chemicals (Dreieichenhain, West Germany) and were diluted to a specific activity of 2 Ci/mol with the unlabeled analogue. [3-^{14}C]DL-β-hydroxy-18:0-CoA (0.49 Ci/mol), [3-^{14}C]DL-β-hydroxy-20:0-CoA (0.15 Ci/mol), [3-^{14}C]2-*trans*-18:1-CoA (0.16 Ci/mol) and [3-^{14}C]2-*trans*-20:1-CoA (0.38 Ci/mol) were chemically synthesized using the procedures previously described (BERNERT & SPRECHER, 1977).

Microsome preparation. Normal 18-day-old C57-black mice of either sex were used. The pooled brains were washed and homogenized in cold 0.32 M-sucrose, 0.1 M-phosphate buffer, pH 7. Microsomes were then prepared as previously described by BOURRE *et al.* (1973*b*).

Protein concentration of the washed microsomal preparation was determined by the method of LOWRY *et al.* (1951) using bovine serum albumin as a standard. A total of 93 mice were required for these studies.

Incubations and analyses. All condensation reactions were carried out for 30 min at 37°C, during which time linearity was maintained. Unless otherwise noted, each tube contained 1 mg of microsomal protein, 100 nmol of malonyl-CoA and the indicated concentration of [1-^{14}C]-acyl-CoA with the total volume adjusted to 1 ml by the addition of phosphate-sucrose buffer. In all cases, albumin was included in the incubation at a molar ratio of 2 (acyl-CoA:albumin) assuming a molecular weight of 66,000 for the albumin.

β-Hydroxy acyl-CoA dehydrase reactions were conducted with 0.5 mg of microsomal protein and the indicated concentration of substrate in a total volume of 1.0 ml phosphate-sucrose buffer. Albumin was included in the same manner as described for the condensation reaction, and an incubation period of 3 min at 37°C was employed. 2-*Trans* Enoyl-CoA reductase reactions were conducted with the appropriate substrates in an identical fashion except that 2 μmol of NADPH were included in each tube.

Condensation reactions were terminated and analyzed with internal standards as previously described (BERNERT & SPRECHER, 1977) except that precoated TLC plates were employed (Merck, Darmstadt, Germany) and the solvent system was *n*-hexane–ethyl ether–acetic acid (75:25:1). Following an initial development, the plates were allowed to air dry for 30 min and then subjected to a second development in the same direction. These conditions provided for the separation of the substrate fatty acid and its methyl ester from β-keto acid and its corresponding methyl ester and methyl ketone. The origin of the methyl ester and methyl ketone derivatives, and their characterization, has previously been described (BERNERT & SPRECHER, 1977). The separated components were located by spraying the plates with a 0.2% alcoholic solution of 2′,7′-dichlorofluorescein, the appropriate regions of the chromatogram were scraped into vials and 10 ml of scintillation fluid was added (BRAY, 1960). Radioactivity of the fractions was determined in a model 3320 Packard liquid scintillation spectrometer.

Dehydrase and reductase reactions were also analyzed exactly as previously described (BERNERT & SPRECHER, 1977) except that methyl esters were formed with methanolic boron trifluoride (MORRISON & SMITH, 1964). In each case, hydroxy and nonhydroxy methyl esters were separated by TLC using hexane–ethyl ether–acetic acid (70:30:1) as solvent. When reductase reactions were analyzed, a portion of the nonhydroxy methyl ester band was eluted and further fractionated by radioactive GLC to separate unreacted substrate from the α,β-saturated product. These analyses were conducted using a 3% SE 30 column in a Packard model 800 gas chromatograph equipped with a flame ionization detector and a cartridge fraction collector. A splitter ratio of 9:1 was employed, and all analyses were carried out with a temperature program of 155–248°C at 1°/min.

RESULTS

v/s Curves for the condensation reaction at a fixed malonyl-CoA concentration are given in Fig. 1. It is apparent that under the conditions employed, the rate of formation of β-keto 18:0 from 16:0-CoA and malonyl-CoA was always much greater than the activity observed when 16:0-CoA was replaced with 18:0-CoA. Although the condensation rate with 16:0-CoA continued to increase throughout the substrate range examined, both reaction curves appeared to approach saturation at an acyl-CoA concentration of 35–40 μM. At a substrate concentration of 50 μM the condensation rate with 16:0-CoA as substrate was more than 5.5-fold greater than the rate obtained with 18:0-CoA.

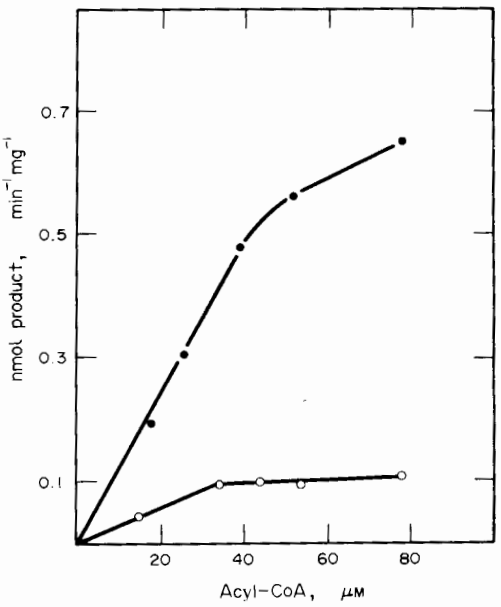

FIG. 1. Kinetics of condensation with [1-^{14}C]16:0-CoA (●) or [1-^{14}C]18:0-CoA (○) at a fixed concentration (100 μM) of malonyl-CoA. Conditions of assay were as described in Materials and Methods.

A similar result was obtained when condensation activity as a function of malonyl-CoA concentration was examined (Fig. 2). In both cases a malonyl-CoA dependency for the reaction could be demonstrated, although a small amount of activity could be detected with either substrate in the absence of exogenous malonyl-CoA. Whether this represented low levels of endogenous malonyl-CoA provided by the microsomes or reflected a side reaction is not clear. While this residual activity was of little consequence in the palmitoyl-CoA condensation reaction, it represented nearly one-third of the maximum rate observed with stearoyl-CoA. Since, as noted below, there appeared to be low levels of endogenous NADPH in these microsomal preparations, it is possible that some malonyl-CoA may have been present as well. Again, as in Fig. 1, a marked difference in condensation rate was observed with the two acyl-CoA primers.

By contrast, the rates of dehydration of either β-hydroxy 18:0-CoA or β-hydroxy 20:0-CoA were not greatly dissimilar (Fig. 3). With either substrate the dehydration rate was more than 10 times greater than that observed in the condensation reaction

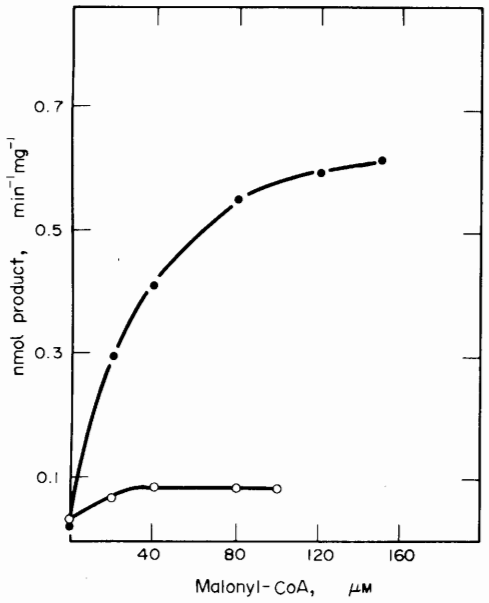

FIG. 2. Kinetics of condensation as a function of malonyl-CoA concentration. Conditions of assay were the same as in Fig. 1 using 51.7 μM-[1-^{14}C]16:0-CoA (●) or 53.4 μM-[1-^{14}C]18:0-CoA (○).

when dehydrase rates were estimated in the vicinity of the *v/s* curve plateau (i.e. 100 μM). Furthermore, both *v/s* curves displayed biphasic kinetics as has previously been observed in the hepatic system (BERNERT & SPRECHER, 1977). The reason for this behavior remains unclear but could conceivably reflect an interaction of the enzyme with the membrane, since treatment of the liver enzyme with a bile salt both solubilizes the activity and converts the *v/s* curve to

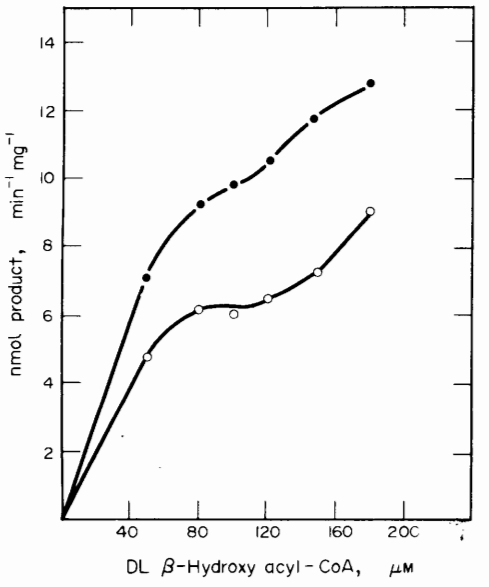

FIG. 3. Kinetics of the dehydrase reaction using [3-^{14}C]DL-β-hydroxy 18:0-CoA (●) or [3-^{14}C]DL-β-hydroxy 20:0-CoA (○) as substrate. Conditions of assay were as described in Materials and Methods.

one approximating normal hyperbolic saturation kinetics (BERNERT & SPRECHER, 1977).

In order to establish the identity of the non-hydroxy product produced when β-hydroxy 18:0-CoA was the substrate, the appropriate zone from the thin-layer plate was eluted with ether and fractionated by GLC. When analyzed in this fashion, about 72% of the total radioactivity was in a compound with a retention time identical to the 2-*trans* 18:1 internal standard. An additional 15% of the radioactivity could be recovered in a component tentatively identified as the 3-18:1 isomer based on its retention time relative to methyl stearate (BARRON & MOONEY, 1970). This is similar to results previously obtained with liver microsomes (BERNERT & SPRECHER, 1977) and probably represents an artifact of the analysis since DAVIDOFF & KORN (1964) have reported that Δ^2 methyl esters may add methanol across the conjugated double bond during saponification to form the 3-methoxy derivative. Subsequent β,γ-elimination then leads to the formation of the Δ^3 isomer as an artifact. The direct equilibration of 2- and 3-alkenoic esters by heating in 10% KOH solution has also been observed (CASON *et al.*, 1953). It might be noted that similar artifacts have been reported to occur with non-conjugated unsaturated acids when a BF_3-methanol system is employed for methylation (FULK & SHOUB, 1970; COOK & SPENCE, 1974). It was also determined that approximately 10% of the total counts could be recovered in 18:0 and 18:1. This could only have resulted if the microsome preparation contained small amounts of endogenous reducing equivalents since NADPH was not added to the incubations.

v/s Curves for the enoyl-CoA reductase reaction are presented in Fig. 4. Both [3-^{14}C]2-*trans* 18:1-CoA and [3-^{14}C]2-*trans* 20:1-CoA were rapidly converted to their respective saturated products at very similar rates. In addition, a partial conversion of either substrate to a hydroxy fatty acid could be detected. This probably represented a direct hydration of the 2-*trans* enoyl-CoA substrate to the β-hydroxy derivative since this conversion is known to occur quite readily in liver microsomes (NUGTEREN, 1965; BERNERT & SPRECHER, 1977). This conversion with brain microsomes was more extensive than that observed in liver however, since in the latter, hydration activity remained low until the reductase was saturated. It may be that the difference in relative K_m for 2-*trans* enoyl-CoA of the reductase and (reverse) dehydrase is not as great in brain as it is in liver microsomes. Although the identity of this secondary reaction product was not further investigated, it is unlikely that it represented 2-hydroxy acid derived from the α,β-saturated product since endogenous 2-hydroxy acids are mainly longer chain compounds of 22–26 carbon atoms, and stearic acid was not converted by the *in vitro* α-hydroxylating system of HOSHI & KISHIMOTO (1973).

Analysis of the rates of conversion of the three par-

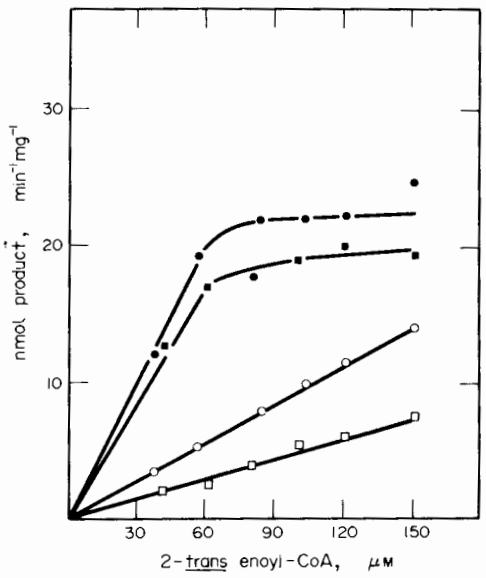

FIG. 4. Kinetics of the enoyl-CoA reductase reaction indicating the simultaneous formation of stearic acid (●) and β-hydroxy 18:0 (○) when [3-^{14}C]2-*trans* 18:1-CoA was the substrate, and of arachidic acid (■) and β-hydroxy 20:0 (□) when [3-^{14}C]2-*trans* 20:1-CoA was employed as substrate. Conditions of assay were as described in Materials and Methods.

tial reactions investigated in this study confirmed that the slowest by far was that of condensation (Table 1). Such an observation is consistent with an overall regulation of the chain elongation process mediated by this first committed step. Further support for this hypothesis is provided by a comparison of the partial reaction rates obtained in this study with overall chain elongation rates calculated from the data of BOURRE *et al.* (1975*b*) (Table 1), which were obtained under very similar reaction conditions. While a close relationship between chain elongation and condensation activities could be discerned, both β-hydroxy acyl-CoA dehydrase and 2-*trans* enoyl-CoA reductase reactions occurred at rates at least 10–20 times greater than chain elongation. Both the dehydrase and enoyl reductase reactions displayed some selectivity in that the 20 carbon substrates were converted somewhat more slowly than were the 18 carbon analogs. However, in neither case were the differences as marked as they were in the condensation reaction.

DISCUSSION

In vivo studies have established that the essential fatty acid pattern in brain lipids may be altered by dietary manipulation (HOLMAN & MOHRHAUER, 1963; SUN, 1972); and it has been shown that non-essential fatty acids also may be taken up intact by the brain when either fed or injected (DHOPESHWARKAR & MEAD, 1969; GOZLAN-DEVILLIERRE *et al.*, 1976). Nevertheless, endogenous synthesis is probably of considerable importance in this tissue, especially in the production of the very long chain and α-hydroxylated acids characteristic of myelin. It is well-established that brain microsomes are capable of elongating a variety of long chain fatty acids by a malonyl-CoA-dependent process (AEBERHARD & MENKES, 1968; BOURRE *et al.*, 1970, 1973*a*, 1975*a*, 1976*a*, 1977; GOLDBERG *et al.*, 1973; BROPHY & VANCE, 1975). However, whether the mechanism of this reaction is similar to that occurring in liver which involves the production of at least three distinct intermediates in the overall process (NUGTEREN, 1965) has not previously been determined. The results of this study indicate that chain elongation intermediates may be recovered as partial reaction products, and are active as substrates when in their CoA thioester form, as is the case in the hepatic system.

We have previously reported that the inclusion of albumin in the incubation is essential in assays of both condensation and enoyl-CoA reductase reactions with rat liver microsomes, while neither the hydroxy acyl-CoA dehydrase nor overall chain elongation rates are greatly affected by its presence (BERNERT & SPRECHER, 1977). The albumin probably is involved in product protection during condensation assays, while the effect on enoyl-CoA reductase reactions is more complex (BERNERT & SPRECHER, in press). Although the effect of albumin on partial reactions in the brain chain elongation system has not been investigated, it was routinely included at a constant molar ratio of 2 relative to substrate concentration

TABLE 1. COMPARISON OF REACTION RATES IN THE CHAIN ELONGATION OF PALMITIC AND STEARIC ACID

Initial substrate	Condensation*	β-Hydroxyacyl-CoA dehydrase†	Enoyl-CoA reductase	Overall chain elongation‡
16:0	0.57 ± 0.01 2 (48)	9.8 ± 0.8 2 (45)	22.0 1 (30)	0.60 ± 0.08
18:0	0.09 ± 0.01 2 (48)	6.1 ± 0.2 2 (45)	19.5 1 (30)	0.04 ± 0.01

Results are expressed as nmols of product formed min^{-1} (mg protein)$^{-1}$ ± S.E.M. The number of microsomal preparations for each value is indicated immediately below the rate, with the total number of pooled brains given in parentheses.

* Determined at a substrate concentration of 50 μM.

† Determined at a substrate concentration of 100 μM.

‡ Calculated from the data of BOURRE *et al.*, 1975*b*.

in all assays. Since the total albumin concentration in the incubation was thus variable when *v/s* curves were analyzed, the gradual rise in rate observed in the condensation reaction with palmitoyl-CoA at substrate concentrations above 35–40 μM (Fig. 1) may have reflected a less than optimum acyl-CoA:albumin molar ratio for this reaction. It might be noted that an essentially normal *v/s* curve was obtained for this reaction when malonyl-CoA was varied at a constant palmitoyl-CoA (and albumin) concentration (Fig. 2). The inclusion of albumin in all reaction assays also reduces the possibility that substrate deacylation influenced reaction rates since BROPHY & VANCE (1976) noted that the addition of albumin to their incubations essentially eliminated acyl-CoA hydrolase activity.

There is good evidence that two different chain elongation systems are involved in the elongation of palmitoyl-CoA and stearoyl-CoA by mouse brain microsomes (BOURRE *et al.*, 1970; POLLET *et al.*, 1973; GOLDBERG *et al.*, 1973). The results of this study suggest that this dichotomy may involve the condensation reaction, in which one or more separate enzymes acting on these two substrates might serve to regulate their respective chain elongation activities. It would be interesting to determine whether a variation in condensation activity towards palmitoyl-CoA and stearoyl-CoA during early myelogenesis could account for the selective increase in overall chain elongation activity observed with these two substrates during this period (BOURRE *et al.*, 1976*b*).

Acknowledgements—This work was supported by grants from the Institut National de la Sante et de la Recherche Medicale (France) and by Research Grant AM20387 from the United States Public Health Service. The support provided J.T.B. by INSERM during part of this study is also gratefully acknowledged. The authors wish to thank F. LACHAPELLE for breeding the animals.

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