

## BIOSYNTHESIS OF VERY LONG CHAIN FATTY ACIDS BY THE SCIATIC NERVE OF THE RABBIT

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### 1. Introduction

The alkanes, a new class of neurolipids, are accumulated in the brain myelin when compared to the other brain subfractions [1–4]. The role of these hydrophobic substances in the myelin formation is far from clear. It appears that alkanes are required for a correct myelination, since the remaining myelin of the Quaking mutant, which bears a defect of myelin formation, presents only 30% total alkanes found in the wild type [1]. The peripheral nervous system contains also large amounts of alkanes [5].

By using a microsomal fraction from rabbit sciatic nerve, we established that the alkanes can be synthesized by mammals, contrary to the widely accepted idea that these substances are due to an exogenous contamination [5]. The nature of the cofactor requirements and of the substrates leading to alkane synthesis in subcellular fractions from rabbit sciatic nerve suggested that alkanes are biosynthesized by decarboxylation of very long chain fatty acids [5]. That hypothesis would remain speculative until a synthesis of these acids in the peripheral nervous system has been demonstrated. In fact saturated very long chain fatty acids are well-known components of the lipids of the brain myelin [6–17], and they are assumed to play a great role in its stability and cohesiveness [6,7,18–22]. Their biosynthesis has been studied in brain subfractions [23–29].

Although the occurrence of very long chain fatty acids has been demonstrated in the peripheral nervous system [7,22,30–32], their origin or the conditions of their formation have not been examined so far [33].

We report here that very long chain fatty acids can be synthesized in sciatic nerve and we discuss their probable role as precursors of myelin alkanes.

### 2. Materials and methods

The sciatic nerves were dissected immediately after the rabbits were sacrificed, as in [5]. They were either used for *in vivo* experiments or used for the preparation of microsomal fractions.

In the second case, the sciatic nerves were homogenized at 4°C with a Potter tissue grinder in 10 vol. 0.05 M Tris–HCl, pH 7.5, 0.25 M sucrose. The homogenate was spun at 20 000 × *g* for 20 min and the resulting supernatant was centrifuged at 150 000 × *g* for 2 h. The pellets were resuspended in 0.05 M Tris–HCl, pH 7.5, 0.25 M sucrose and homogenized. The proteins were estimated as in [34].

The biosynthesis of the very long chain fatty acids in the C<sub>20</sub>–C<sub>30</sub> range was measured by the incorporation of [1-<sup>14</sup>C]acetate (5 μCi, 10 Ci/mol), [1-<sup>14</sup>C]-stearate (1 μCi, 1 Ci/mol) or [1-<sup>14</sup>C]stearoyl CoA (0.1 μCi, 1 Ci/mol). [1-<sup>14</sup>C]Acetate was used for *in vivo* experiments.

With [ $1-^{14}\text{C}$ ]stearate as the labelled substrate, incubation mixtures contained in final vol. 0.5 ml: Tris-HCl, pH 7.5, 50 mM, sucrose 250 mM, NADPH 0.5  $\mu\text{mol}$ , malonyl-CoA 0.1  $\mu\text{mol}$ , CoA 0.25  $\mu\text{mol}$ , ATP 1  $\mu\text{mol}$ ,  $\text{Mg}^{2+}$  1  $\mu\text{mol}$ , sodium ascorbate 1  $\mu\text{mol}$ , and when used: NADH 0.5  $\mu\text{mol}$  and  $\beta$ -mercaptoethanol 25  $\mu\text{mol}$ .

With [ $1-^{14}\text{C}$ ]stearoyl CoA as the substrate, CoA, ATP and  $\text{Mg}^{2+}$  were omitted from the reaction mixture. The assays started by the addition of enzyme (0.3–0.6 mg protein). The incubations were carried out at 37°C for 1 h, stopped by the addition of 0.5 M KOH (0.5 ml), and saponified at 80°C for 1 h.

The non-saponifiable matter was extracted and the fatty acid methyl esters were prepared as in [5,32,34–36].

Unless otherwise stated the fatty acid methyl esters were subjected to thin-layer chromatography on Silicagel G/AgNO<sub>3</sub>, 80/20 (w/w) with benzene-hexane 70/30 (v/v) as the solvent system. The saturated fatty acid methyl esters were then analyzed by gas-liquid chromatography on an Intersmat IGC 120 DFL gas chromatograph equipped with a flame ionization detector on a 1/8 in.  $\times$  6 ft column containing 10% SE 30 or 3% OV 17, programmed from 150–280°C at 4°C/min, or on a 1/8 in.  $\times$  6 ft column containing 5% DEGS programmed from 120–220°C at 4°C/min.

The labelled fractions were collected with a Packard 852 fraction collector on cartridges filled with anthracene. Alternatively the fatty acid methyl esters were analyzed with a Barber-Colman Radiochromatograph 5000, equipped with the same columns in the same conditions.

The radioactivity was measured by liquid scintillation counting on a Tri carb 3003 Packard spectrometer.

### 3. Results and discussion

The analysis of the lipids from rabbit sciatic nerve showed the presence of the C<sub>20</sub>–C<sub>28</sub> saturated fatty acids. We studied the incorporation of various labelled substrates, to test whether the rabbit sciatic nerve is able to synthesize very long chain fatty acids.

Freshly removed sciatic nerves were allowed to incorporate [ $1-^{14}\text{C}$ ]acetate. Figure 1 shows that the excised sciatic nerve is able to synthesize very long chain fatty acids from [ $1-^{14}\text{C}$ ]acetate. The total fatty acid methyl esters including unsaturated and  $\alpha$ -hydroxy

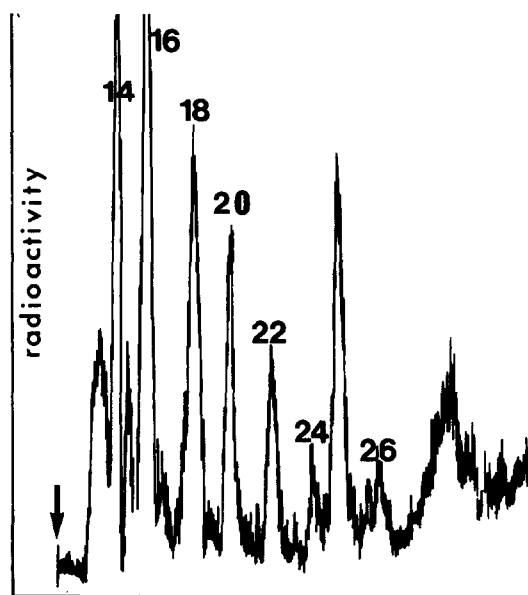


Fig.1. Total fatty acid synthesis from [ $1-^{14}\text{C}$ ]acetate by excised rabbit sciatic nerve. The numbers refer to fatty acid chain length. The arrow indicates the start of the radiochromatography.

fatty acids were subjected to thin-layer chromatography. The radioactivity in saturated fatty acids was 55% total. The label distribution within the various saturated fatty acids synthesized from [ $1-^{14}\text{C}$ ]acetate is given in fig.2. Myristic, palmitic and stearic acids accounted for 17%, 37% and 25% radioactivity of the saturated fatty acids, respectively; C<sub>20</sub>–C<sub>26</sub> fatty acids were also labelled and incorporated about 18% total radioactivity. The label decreased from C<sub>20</sub> (6.1–6.7%) to C<sub>26</sub> (0.5–1.2%) and lignoceric acid accounted for about 5% total label.

These results demonstrate for the first time the biosynthesis of saturated very long chain fatty acids by the peripheral nervous system.

[ $1-^{14}\text{C}$ ]Stearate is a good substrate for alkane synthesis by the rabbit sciatic nerve [5]. Experiments with excised sciatic nerves showed that [ $1-^{14}\text{C}$ ]stearate is elongated in C<sub>20</sub>–C<sub>30</sub> fatty acids. Subcellular fractions of the rabbit sciatic nerve were obtained and the elongating activity of the subfractions was tested in the presence of [ $1-^{14}\text{C}$ ]stearate, CoA, ATP, malonyl CoA and NADPH, in the same experimental conditions giving rise to alkane formation [5]. The results

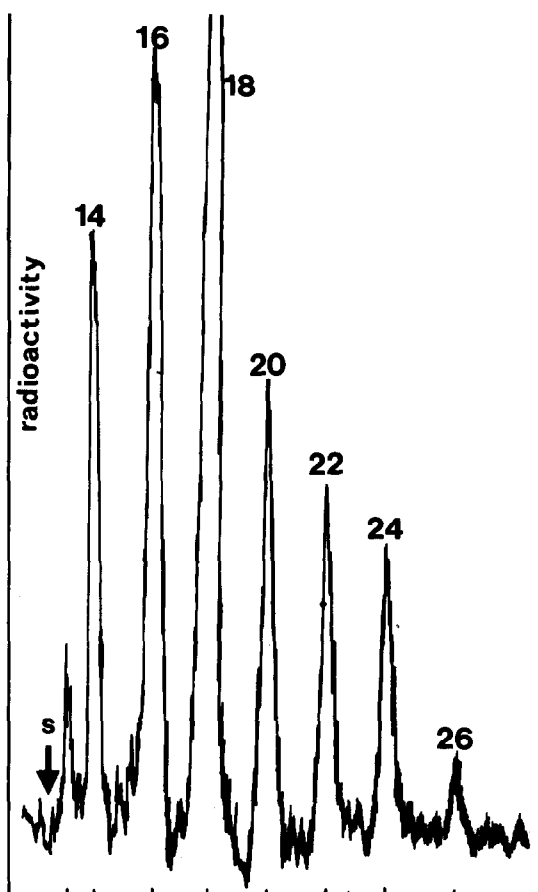


Fig.2. Saturated fatty acid synthesis from  $[1-^{14}\text{C}]$ acetate by excised rabbit sciatic nerve. The numbers refer to the fatty acid chain length. The arrow indicates the start of the radiochromatography. Radioactivity was monitored with a relative attenuation 1 K except for  $\text{C}_{14}$  and  $\text{C}_{16}$  (3 K).

are given in table 1. All the fractions incorporated stearate into saturated fatty acids; most of the synthesis occurred in the microsomal pellet which contained nearly 60% total elongating activity. The measured specific activity was about 15 nmol/mg protein/h from 1  $\mu\text{mol}$  stearate. Both alkanes and saturated very long chain fatty acids are synthesized chiefly in that membrane fraction.

The analysis of the labelling of each fatty acid synthesized from  $[1-^{14}\text{C}]$ stearate by the various subfractions is given in table 2. As expected the very long chain fatty acids were very poorly synthesized by the 150 000  $\times$  g supernatant, whereas the microsomal pellet was, by far, the most active subfraction. The formation of  $\text{C}_{20}$ – $\text{C}_{30}$  fatty acids was observed; the label incorporation decreased from eicosanoic to triacontanoic acid. The formation of eicosanoic acid was about 7 nmol/mg protein/h, accounting for 50% of the label found in the saturated very long chain fatty acids. The synthesis of lignoceric acid was less than 10% total fatty acids. These results are very similar to those observed from *in vivo* experiments.

Table 3 shows the effect of various cofactors on the synthesis of the saturated very long chain fatty acids from  $[1-^{14}\text{C}]$ stearate by a microsomal pellet from sciatic nerve.  $\beta$ -Mercaptoethanol which inhibited completely the alkane synthesis [5] had little effect on the stearate elongation; replacement of NADPH by NADH reduced the very long chain formation by 50%, indicating that NADPH was the preferred reducing agent. The omission of CoA or ATP led to a marked decrease of the label incorporation into saturated very long chain fatty acids, suggesting that stearate is not

Table 1  
Biosynthesis of saturated very long chain fatty acids by rabbit sciatic nerve

	Specific activity (nmol/mg protein/h)	% Total activity
Homogenate	4.9	100
20 000 $\times$ g pellet	3.3	21.2
150 000 $\times$ g pellet	14.9	58
150 000 $\times$ g supernatant	0.6	20.8

Incorporation of  $[1-^{14}\text{C}]$ stearate into very long chain fatty acids. For experimental details see materials and methods. Results are given as nmol fatty acids synthesized/mg protein/h

Table 2  
Saturated fatty acid synthesis from [ $1\text{-}^{14}\text{C}$ ]stearate by rabbit sciatic nerve fractions

Saturated fatty acid chain length	Homogenate	20 000 × g pellet	150 000 × g pellet	150 000 × g supernatant
20	2.5	1.5	6.9	0.26
22	0.9	0.8	4.7	0.2
24	0.8	0.4	1.4	0.1
26	0.3	0.3	0.9	0.05
28	0.3	0.2	0.7	n.d.
30	n.d.	n.d.	0.2	n.d.

The complete assay contained NADPH (0.5  $\mu\text{mol}$ ), ATP (1  $\mu\text{mol}$ ),  $\text{Mg}^{2+}$  (1  $\mu\text{mol}$ ), CoA (0.25  $\mu\text{mol}$ ), malonyl CoA (0.1  $\mu\text{mol}$ ), [ $1\text{-}^{14}\text{C}$ ]stearate (1  $\mu\text{Ci}$ , 1  $\mu\text{Ci}/\mu\text{mol}$ ), sodium ascorbate (1  $\mu\text{mol}$ ) and enzyme (0.5 mg). Results are given as nmol/mg protein/h. n.d., not detected

Table 3  
Effect of various cofactors on the biosynthesis of saturated very long chain fatty acids by a 150 000 × g pellet of rabbit sciatic nerve

	Remaining act. (%)
Complete assay	100
+ $\beta$ -Mercaptoethanol	71
+ NADH	50
– NADPH	50
– ATP	35
– CoA	32
– malCoA	54

Same experimental conditions as in table 2 (complete assay) and when used: NADH (0.5  $\mu\text{mol}$ ),  $\beta$ -mercaptoethanol (25  $\mu\text{mol}$ ). The relative activity 100% was given to the activity obtained with the complete assay  $14.9 \pm 0.5$  nmol/mg protein/h. Results are given as the remaining activity when cofactors were omitted, or added

the true substrate of the microsomal elongase: probably stearate could be activated to stearyl CoA, and then elongated in the presence of malonyl CoA and NADPH. In order to check this hypothesis, experiments were run with [ $1\text{-}^{14}\text{C}$ ]stearyl CoA as the labeled substrate, without CoA and ATP.

Table 4 shows that stearyl CoA is a good substrate of the elongase. The main synthesis (about 60%) was observed in the microsomal pellet; the elongation rate of the stearyl CoA was more important than that observed with stearate in the presence of CoA and ATP. From 100 nmol stearyl CoA, about 13.3 nmol/mg protein/h of saturated very long chain fatty acids were synthesized. The radioactivity distribution pattern within the various fatty acids was quite similar to that observed by using [ $1\text{-}^{14}\text{C}$ ]stearate. Eicosanoic and docosanoic acids contained, respectively, 53% and 22% total radioactivity incorporated, and lignoceric acid 12.4%.

Table 4  
Biosynthesis of saturated very long chain fatty acids from [ $1\text{-}^{14}\text{C}$ ]stearyl CoA by rabbit sciatic nerve fractions

Saturated fatty acid synthesized (chain length)	homogenate	20 000 × g pellet	150 000 × g pellet	150 000 × g supernatant
20	0.84	0.74	7.07	1.42
22	0.42	0.95	2.98	0.56
24	0.13	0.27	1.65	0.21
26	0.11	0.22	1.08	0.19
28	0.10	0.02	0.52	0.32

The complete assay contained NADPH (0.5  $\mu\text{mol}$ ), malonylCoA (0.1  $\mu\text{mol}$ ), [ $1\text{-}^{14}\text{C}$ ]stearyl CoA (0.1  $\mu\text{Ci}$ , 1  $\mu\text{Ci}/\mu\text{mol}$ ), sodium ascorbate (1  $\mu\text{mol}$ ) and enzyme (0.3–0.6 mg). Results are given as nmol/mg protein/h

These results demonstrate that the rabbit sciatic nerve, able to synthesize alkanes [5] can also synthesize saturated very long chain fatty acids; the total elongating activity is somewhat comparable to that observed in the central nervous system. The biosynthesis occurs in the microsomal fraction, in the presence of stearoyl CoA, malonyl CoA and NADPH. From analytical and biochemical data [1–5,34–36] we postulated recently a plausible relationship between very long chain acids and alkanes. These results are in good agreement with that proposal.

Since alkanes can be considered as characteristic of a normal myelination, a possible approach of the myelin formation may be the study of the different steps – including fatty acids – involved in alkane biosynthesis as well as a further investigation of their insertion in both normal and abnormal (mutant) myelin. That work is under progress in our laboratory.

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