EVIDENCE OF ALKANE SYNTHESIS BY THE SCIATIC NERVE OF THE RABBIT

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1. Introduction

Several human diseases involve demyelination of the peripheral nerve [1]. Progress of our knowledge in treating or preventing these disorders implies a better understanding of myelin formation. The myelin of both central and peripheral nervous systems contains remarkably high levels of very long-chain fatty acids [2,3], particularly C_{24} and $C_{24:1}$; these acids may account for some physico-chemical properties of myelin [4–8], but very little is known about their biosynthesis in the peripheral nervous system. The myelin may be also characterized by another family of very long, aliphatic chains: alkanes. We have recently shown that the brain myelin of the normal mouse accumulated specifically high quantities of alkanes, while mitochondria, synaptosomes and microsomes contained only minor accounts of them. Aliphatic hydrocarbons are widely distributed in the internal organs of mammals [10] but their amounts are generally low (ca. 3–60 μg/g) [11-14]. Whatever the origin of the used system, no interpretation of the alkane occurrence or of their synthesis by mammals is available; the lack of the appropriate experimental results, has led to the widely accepted idea that alkanes found in mammalian tissues are almost certainly exogenous [10]. But an alternative possibility is that these very long, aliphatic chains are synthesized in situ by the animal cells, although this last hypothesis is just speculative. Supporting the latter idea is the observation that the alkane concentration in the brain myelin was reduced by 70%, in the Quaking mouse, which is a recessive autosomal mutant characterized by a defective myelination and a related decrease of the very long-chain fatty acid level [9].

We report here the results of experiments designed to test whether the sciatic nerve is able to synthesize a new class of neurolipids: the alkanes.

2. Materials and methods

The sciatic nerves were dissected immediately after the rabbits were killed; the epineurium was split off and the nerves were deposited in 10 vol. 0.05 M Tris–HCl, pH 7.5, 0.25 M sucrose. The sciatic nerves were homogenized at 4°C in a Potter Tissue grinder. The homogenate was spun at 20 000 × *g* for 20 min. The 20 000 × *g* pellet was stored and the 20 000 × *g* supernatant was then centrifuged at 150 000 × *g* for 2 h. The pellets were gently resuspended in 0.05 M Tris–HCl, pH 7.5, 0.25 M sucrose.

Alkane biosynthesis was measured by the incorporation of [1-^{14}C]stearate (1 μCi, 1 Ci/mol), or [1-^{14}C] acetate (5 μCi, 50 Ci/mol) or [1-^{14}C]stearoyl-CoA (0.1 μCi, 1 Ci/mol) or [1,3-^{14}C]malonyl CoA (0.1 μCi, 1 Ci/mol). With [1-^{14}C]stearate as labeled substrate, incubation mixtures contained in total vol. 0.5 ml: Tris–HCl, pH 7.5, 50 mM, sucrose 250 mM, NADPH 0.5 μmol, malonyl CoA 0.1 μmol, CoA 0.25 μmol, ATP 1 μmol, Mg^{2+} 1 μmol, sodium ascorbate 1 μmol. (NADH, 0.5 μmol and β mercaptoethanol, 25 μmol

were also used in some experiments.) Assays were started by the addition of enzyme (0.5 mg protein). Incubations were carried out at 37°C for 1 h. Assays were stopped by the addition of KOH 5 M (0.5 ml). The incubation mixtures were saponified for 1 h at 80°C. The alkane fraction was prepared as described previously [9]. Alkane radioactivity was analyzed by gas–liquid chromatography on an Intersmat I G 120 DFL gas chromatograph equipped with a flame ionization detector and an effluent splitter on a 1/8 in. × 6 ft column containing 3% OV 17 programmed from 150–280°C at 4°C/min, with N_2 as the carrier gas.

The fractions were collected with a Packard 852 fraction Collector on cartridges filled with anthracene. The radioactivity of alkanes was measured by liquid scintillation counting (Tri Carb Packard Model 3003 Spectrometer).

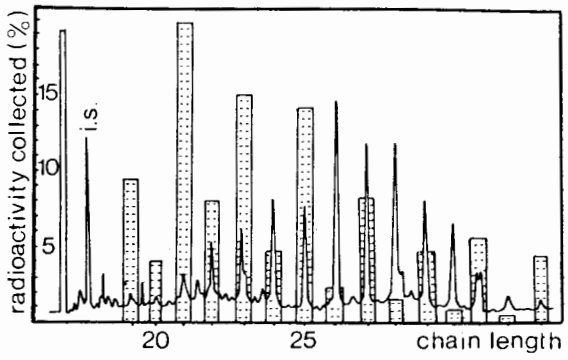

Fig.1. Gas-liquid chromatography of alkanes obtained from a homogenate incubated with [1-^{14}C]stearate. The height of the bars indicates the % radioactivity of each alkane collected during the time interval covered by the bar. The mass of the alkanes is indicated by the recorder tracing. IS, Internal Standard (hexadecane).

3. Results and discussion

The analysis of the lipids from rabbit sciatic nerve showed the presence of all the alkanes in the C_{19}–C_{33} range, with approximately equal amounts of odd and even chains. The quantity was about 2 mg/g fresh nerve, and thus resembled what we observed in the mouse sciatic nerve (unpublished observations) and in the brain myelin of normal mice [9]. In order to determine whether the sciatic nerve is able to synthesize very long, aliphatic chains, we studied the incorporation of various labeled substrates in the fatty acids and in the alkanes. In this paper we will present and briefly discuss only the results concerning alkane biosynthesis. Freshly-removed sciatic nerve allowed to incorporate [1-^{14}C]acetate (5 μCi, 10 Ci/mol), synthesized mostly very long-chain fatty acids, but all the label of the hydrocarbon fraction was due only to a squalene formation. On the other hand, [1-^{14}C]stearate (1 μCi, 1 Ci/mol) was readily incorporated in both very long-chain fatty acids and alkanes. In the light of these results, subsequent experiments used [1-^{14}C] stearate as labeled substrate.

A homogenate of the sciatic nerve (0.5 mg protein) incorporated [1-^{14}C]stearate into alkanes, in the presence of CoA, ATP, Mg^{2+}, malonyl CoA and NADPH as described under Materials and methods. The distribution of the label in the various alkanes is given in fig.1. All the alkanes from C_{19}–C_{33} were synthesized from radioactive stearate, but the odd chains contained more than 80% of the label, unlike the alkane distribution pattern which exhibits equal amounts of odd and even chains.

The alkane formation from [1-^{14}C]stearate by the various subcellular fractions is given in table 1. The specific activity in the homogenate was about 0.2 nmol/mg protein, in the protein concentration range we used (0.5 mg). The 150 000 × *g* supernatant. contained most of the soluble cytoplasmic material, it had very little, if any, alkane-synthesizing activity. The same is observed with the 20 000 × *g* pellet. In fact the most abundant activity was found in the 150 000 × *g* pellet which contained about 80% of the

Table 1
Alkane synthesis by rabbit siatie nerve fractions

	Specific activity (nmol/mg protein/h)	%
Homogenate	0.19	100
20 000 × *g* pellet	0.09	5.4
150 000 × *g* pellet	2.4	78.4
150 000 × *g* supernatant	0.05	16.2

Incorporation of [1-^{14}C]stearate into alkanes. For experimental details see Materials and methods. Results are given as nmol alkane synthesized/mg protein/h. % represents the total activity recovered in each fraction

Table 2
Effect of various cofactors on the alkane synthesis by a 150 000 × *g* pellet of rabbit sciatic nerve

	Remaining activity (%)		Remaining activity (%)
Complete	100	– Malonyl CoA	15
+ NADH – NADPH	49	– CoA	6
– NADPH	15	– Ascorbic acid	67
+ NADH	78	+ β mercaptoethanol	5
– ATP, Mg^{2+}	15		

The complete assay contained NADPH (0.5 μmol), ATP (1 μmol), Mg^{2+} (1 μmol), CoA (0.25 μmol), malonyl CoA (0.1 μmol), [1-^{14}C]stearate (1 μmol), sodium ascorbate (1 μmol) and enzyme (0.5 mg). The relative activity 100% (2.4 ± 0.3 nmol/mg protein/h) was given to the activity obtained with the complete assay. Results are given as the remaining activity when cofactors were omitted, or added.

total activity; the specific activity (ca. 2 nmol/mg protein/h) is, to our knowledge, the highest reported.

All subsequent experiments were done using the crude 150 000 × *g* pellet as the source of enzyme activity. Table 2 shows the effect of various cofactors on the synthesis of alkanes from [1-^{14}C]stearate. Suppression of ATP, Mg^{2+} or CoA led to a marked decrease of radioactive incorporation into alkanes, suggesting that stearate had to be activated before its incorporation. Malonyl CoA greatly stimulated the alkane synthesis, so that the stearyl CoA, formed from stearate in the presence of ATP, Mg^{2+} and CoA, could be elongated by malonyl CoA giving rise to very long-chain fatty acids, which in turn could be decarboxylated. The replacement of NADPH by NADH reduced the synthesis by more than 50%, indicating that NADPH is the preferred reductant. The effect of increasing amounts of NADPH was checked: the alkane synthesis increased linearly up to 4 μmol NADPH. A mercaptoethanol addition led to an almost total inhibition of alkane biosynthesis. That inhibition by thiols is the same as that observed in plants [15–17] algae [18] yeast [19]. In the light of these results the experiments were done in the presence of NADPH, malonyl CoA, ascorbic acid and [1-^{14}C]-stearyl CoA or [1-^{14}C]stearate plus CoA and ATP, Mg^{2+}.

Table 3 shows the label distribution pattern of alkanes from the 150 000 × *g* pellet. All the alkanes from C_{19}–C_{33} were synthesized and the odd chains incorporated 90% of the label.

Table 3
Alkane biosynthesis by a membrane fraction (150 000 × *g* pellet) from [1-^{14}C]stearate

Chain length	Synthesis (nmol/mg protein)
19	0.16
20	0.06
21	0.52
22	0.11
23	0.33
24	0.06
25	0.37
26	0.06
27	0.31
28	0.04
29	0.37
30	0.01
31	0.25
32	0.06
33	0.13

Same experimental conditions as in table 2 (complete assay). The results are given as nmol each alkane synthesized/mg protein/h

These results clearly demonstrate, for the first time, that alkanes may be synthesized by mammalians. That relatively high synthesizing activity may be related to the high alkane amounts we found in brain myelin [7] or in the peripheral nerve. On the basis of analysis done with brain myelin from normal and Quaking mice we postulated recently a possible relationship between very long-chain fatty acids and alkanes [9]. The results we present here are in good agreement with that proposal. The very long, aliphatic chains including acids and alkanes may play a great role in the myelin formation, stability and cohesion; this is attested to by their decrease in defective myelination [9] leading to abnormal myelin structures. Work is under progress in our laboratory to study the synthesis of these very long chains and their insertion in myelin of peripheral nervous system.

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