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LIPID METABOLISM AND OXYGEN CONSUMPTION IN A HEREDITARY DEMYELINATING NEUROPATHY, THE TREMBLER MOUSE: AN IN VITRO STUDY

JEAN-MARIE MATTHIEU, MICHELE MARKERT, MARIE-T. VANIER, MARIANNE RUTTI, JACQUELINE REIGNER and JEAN-MARIE BOURRE

Laboratoire de Neurochimie et Laboratoire Central de Chimie clinique, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne (Switzerland) and (M-T.V.) Laboratoire de Neurochimie, INSERM U 189, Faculté de Médecine Lyon-Sud, F-69600 Oullins and (J.-M.B.) INSERM U.134, CNRS ERA 421, Hôpital de la Salpêtrière, F-75634 Paris Cedex 13 (France)

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SUMMARY

Sciatic nerves from 15-day-old trembler and control mice were maintained in vitro up to 53 h and the metabolism of myelin lipids and the oxygen consumption were investigated. [^{35}S]Sulfate was incorporated into sulfatides at a higher rate and turned over more rapidly in trembler nerves than in controls. [^{14}C]Galactose was incorporated into cerebrosides of trembler nerves at a lower rate and turned over like the controls. In contrast, synthesis of sulfatides labeled with [^{14}C]galactose was increased in mutants and no significant turnover was observed for both trembler and control nerves during the whole incubation period. Similar results were obtained using [^{3}H]serine as a precursor and no significant differences were observed in the turnover rates of sphingomyelin and phosphatidylcholine between trembler and control nerves. These data suggest the presence of two different pools of cerebrosides, a small one formed by the fast recycling of sulfatides and which does not mix with a second, larger one. The rate of oxygen consumption did not change significantly during the incubation period and was 2–3-fold higher in trembler nerves than in controls, reflecting, at least partly, the increased sulfatide metabolism.

INTRODUCTION

Trembler mutant mice are affected by a dominantly inherited neuropathy[9] secondary to an impairment of Schwann cell differentiation and also to an inability of

these cells to maintain myelin[2]. The protein and lipid composition of trembler sciatic nerves and of purified myelin is consistent with marked demyelination[13,16]. Recently, we reported increased activities of cerebroside sulfotransferase (CST, EC 2.8.2.11), and of radioactive sulfate incorporation into sulfatides (3-O-sulfate ester of cerebroside) of trembler nerves[17]. In the present investigation, we studied the turnover rate of cerebrosides (N-acyl-1-O-β-galactosyl sphingenine), sulfatides, sphingomyelin and phosphatidylcholine in trembler and control sciatic nerves incubated, in vitro, in the presence of different radioactive precursors. Lysosomal hydrolase activities were also measured extending our previous results[17]. A preliminary account of this work was presented at the twelfth meeting of the American Society for Neurochemistry[18].

MATERIALS AND METHODS

Animals and incubation techniques

Trembler and control mice on B6-CBA strain were obtained from the Salpêtrière Hospital in Paris. Actively myelinating animals, 15 days old, were used for this study. The sciatic nerves between the spinal origin and the knee were removed and carefully freed of surrounding connective tissues and incubated, under sterile conditions, in Dulbecco's modified medium containing high glucose, no pyruvate, GIBCO 320 1965, newborn calf serum (10%) and 20 μg/ml gentamycin. Nerves were kept for up to 53 h in Petriperm (Heraeus, Zürich, Switzerland) dishes at 36.5 °C in a humidified atmosphere of 8% CO_2–92% O_2. Several radioactive precursors were added to the incubation medium: $Na_2{}^{35}SO_4$ (10–1000 mCi/mmol sulfur), D-[^{14}C(U)]galactose (10–25 Ci/mmol), [methyl-^{3}H]thymidine (40–60 Ci/mmol) and L-[^{3}H(G)]serine (1–5 Ci/mmol).

Oxygen consumption

Oxygen consumption was measured polarographically with a Clark type electrode. For these determinations, two sciatic nerves were placed in a 0.42 ml glass chamber containing culture medium stirred and thermostatically controlled (37 °C)[15].

Lipid analysis

Sciatic nerves were homogenized with 10 vols of chloroform–methanol (2:1, v/v) and the total lipid extract was partitioned[10]. After separation by one-dimensional TLC on silica gel G plates developed in chloroform–methanol–water (70:30:4 by vol.), lipids were visualized in iodine vapor and compared to purified lipid standards. Spots were scraped; lipids were extracted, dried and counted for radioactivity in Aquasol (New England Nuclear, Boston) by scintillation spectrometry.

Protein and enzyme assays

The sciatic nerves were homogenized in 0.32 M sucrose and 10 μl was used to assay for protein[14]. Cerebroside sulfotransferase (EC 2.8.2.11, CST) was determined in sciatic nerve homogenates[7]. Arylsulfatase A (EC 3.1.6.1) was assayed with *p*-nitro-

catechol sulfate[3]. Cerebroside β-galactosidase (EC 3.2.1.46), cerebroside β-glucosidase (EC 3.2.1.45) and sphingomyelinase (EC 3.1.4.12) activities were determined using the corresponding labeled sphingolipid as substrate according to a micromodification[22] of the procedures of Svennerholm et al.[21]. Labeled [^{3}H]galactosylceramide was prepared in the laboratory. Labeled [^{14}C]sphingomyelin and [^{3}H]glucosylceramide was purchased from New England Nuclear (Dreieich, FRG). β-Glucosidase was also assayed with 4-methyl-umbelliferyl-glucoside as substrate[19]. N-acetyl-β-glucosaminidase (EC 3.2.1.30), β-galactosidase (EC 3.2.1.23) and α-fucosidase (EC 3.2.1.51) activities were measured using the appropriate 4-methyl-umbelliferyl-glycoside (Koch–Light Laboratories, Colnbrook, UK) as substrate according to published procedures[21].

RESULTS

Enzyme specific activities in trembler tissues

The specific activities of CST were measured in different organs from 15-day-old control and trembler mice (Table I). The only significant difference was observed in trembler sciatic nerves in which CST was 2.5 times higher than in controls. Lysosomal hydrolase activities were measured in control and an approximately 200% increase was observed for N-acetyl-β-glycosaminidase and arylsulfatase A, while most other enzymes studied showed a slight elevation (approx. 20%) over the control values (Table II).

Oxygen consumption

In both control and trembler nerves, the rate of oxygen consumption was practically constant during the whole incubation period (Table III). Trembler nerves presented 2–3-fold higher oxygen consumption rates than controls. The addition of 2-deoxy-D-glucose (70 μmol/liter) to nerves kept in vitro for 29 h decreased the rate of oxygen consumption by 62%.

In vitro lipid metabolism

The incorporation of [^{35}S]sulfate into sulfatides was 4 times higher in trembler than in control nerves (Fig. 1). Chase experiments showed no important loss of

TABLE I

CST specific activity in different organs of 15-day-old mice

Values are given as pmol/h/mg protein, mean ± S.E.M. (number of separate experiments).

	Control	*Trembler*
Brain	12.8 ± 0.2 (3)	9.5 ± 2.0 (3)
Sciatic nerve	26.5 ± 4.6 (5)*	68.1 ± 7.9 (5)*
Kidney	16.1 ± 2.1 (3)	17.9 ± 1.9 (3)
Liver	0.6 ± 0.2 (3)	0.4 ± 0.1 (3)

* $P < 0.001$. Student's t-test.

TABLE II

Specific activities of lysosomal enzymes in Trembler sciatic nerves

Results are means from two individual experiments performed in duplicate in 15-day-old animals.

	Percent of control values
Arylsulfatase A	218
Cerebroside β-galactosidase	117
Cerebroside β-glucosidase	124
Sphingomyelinase	125
N-acetyl-β-glucosaminidase	180
β-Galactosidase	103
α-Fucosidase	92

radioactivity in sulfatides from control nerves, while sulfatides turned over rapidly in trembler nerves. When nerves were pre-incubated for 20 h before pulse-labeling, the difference between control and trembler nerves was even more striking (Fig. 2). The lower rate of radioactive precursor incorporation observed in the latter experiment is caused by a decrease in CST specific activity observed in vitro as well as in vivo following Wallerian degeneration (Matthieu et al., unpublished results).

When [^{14}C]galactose was used as precursor (Fig. 3), trembler nerves showed lower incorporation into cerebrosides but the turnover rate was not significantly different from controls. In contrast, the incorporation of [^{14}C]galactose into sulfatides was 4 times higher in trembler than in control nerves and no significant turnover rate was observed for mutants and controls during the whole incubation period.

In order to label the sphingosine residue of cerebroside and sulfatide molecules, [^{3}H]serine was used as a precursor. Since this precursor plays a key role in

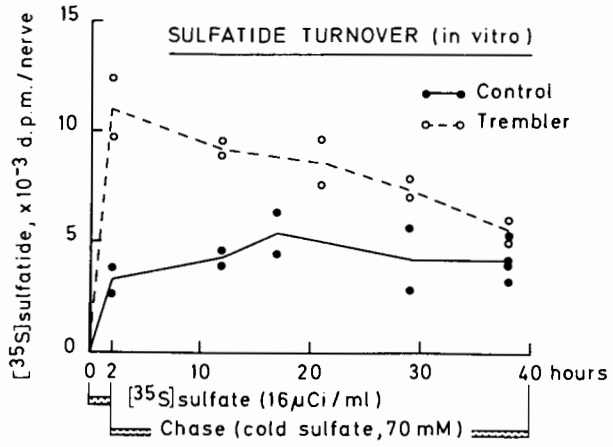

Fig. 1. Turnover of sulfatides. Control (●—●) and trembler ○—○) nerves were incubated with 16 μCi/ml of [^{35}S]sulfate. After 2 h, the nerves were rinsed and placed in a fresh medium containing 70 mmol/liter Na_2SO_4. Each point represents an individual value.

TABLE III

Oxygen consumption of sciatic nerves from 15-day-old mice

Values given as nmol O_2/min·mg dry weight, means from 2 nerves; the oxygen consumption was measured during 15 min periods.

Time in vitro (h)	*Control*	*Trembler*
2	1.56	3.32
29	1.44	5.27
53	1.49	4.98

phosphatidylcholine synthesis, incorporation of ^3H into this compound was also investigated. Cerebroside turnover in control and trembler nerves was similar to that observed using radioactive galactose: trembler showed lower incorporation of radioactive precursor and it turned over rapidly (Fig. 4). The labeling of sulfatides was less striking but, after 20 and 40 h of incubation, the amounts of radioactivity were higher in trembler than in control nerves.

For both [^3H]serine and [^{14}C]galactose, the incorporation of radioactivity into control nerves was 16 and 40 times higher for cerebrosides than for sulfatides, respectively. In trembler nerves, the difference was smaller, 8 and 7 times for [^3H]serine and [^{14}C]galactose, respectively. Expressing the results as specific radioactivity using published results for cerebroside and sulfatide contents in control and trembler nerves[13] does not modify this trend.

Phosphatidylcholine showed a fast turnover rate using [^3H]serine as a precursor (Fig. 5). No significant difference could be demonstrated between control and trembler nerves. Using the same radioactive precursor, sphingomyelin turned over slower than phosphatidylcholine and no important difference could be observed in either control or trembler nerves (not shown).

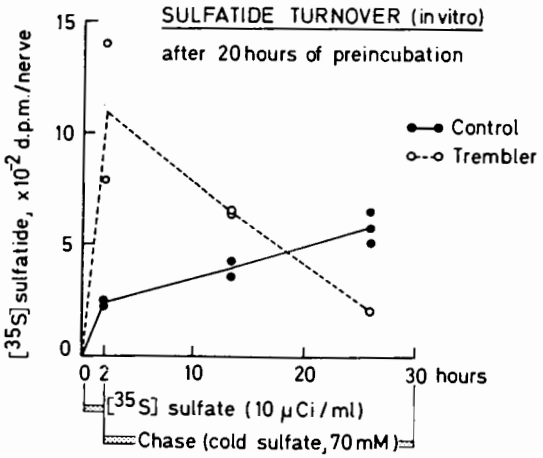

Fig. 2. Turnover of sulfatides. Sciatic nerves were placed first for 20 h in a normal medium, then incubated with 10 μCi/ml of [^{35}S]sulfate for 2 h. The rest of the experiment was as reported in Fig. 1.

DISCUSSION

When compared to control levels, the total lipidic content of trembler nerves is reduced by 66% but each class of lipid is decreased in a similar way, except for cholesterol esters which show a 5-fold increase[13]. These results are consistent with morphological reports showing recurring demyelination with remyelination attempts

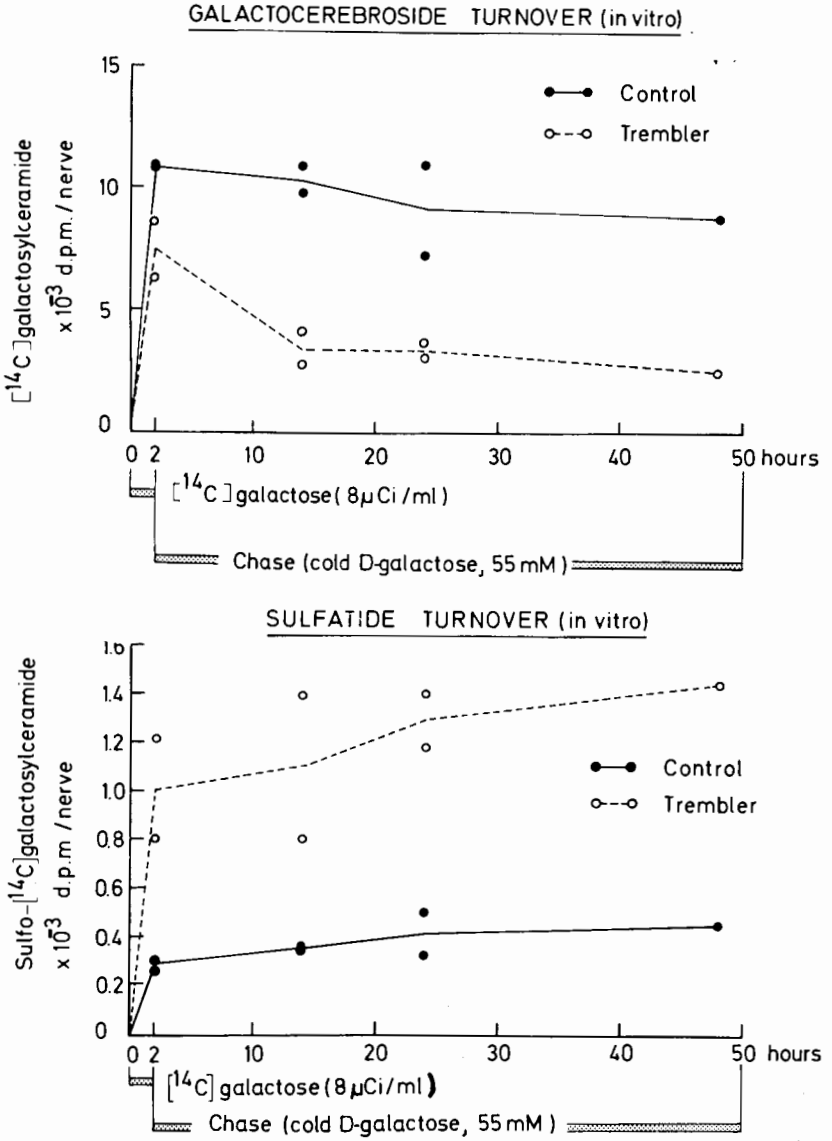

Fig. 3. Upper panel: cerebroside turnover. The experimental plan was similar to that in Fig. 1. The concentrations of [^{14}C]galactose and unlabeled galactose are given in the figure ([^{14}C]galactosylceramide = [^{14}C]galactocerebroside = [^{14}C]cerebroside). Each point represents individual values. Lower panel: same experiment, sulfatide turnover (sulfo-[^{14}C]galactosylceramide = [^{14}C]sulfatide).

in trembler nerves (for a review see ref. 6). High specific activities of CST, arylsulfatase A and increased incorporation of radioactive sulfate into sulfatides and non-lipidic material indicate an abnormal sulfate metabolism in trembler sciatic nerves[17]. The increased activities of several lysosomal enzymes observed in trembler nerves reflect unspecific demyelination processes since similar findings were observed in nerves

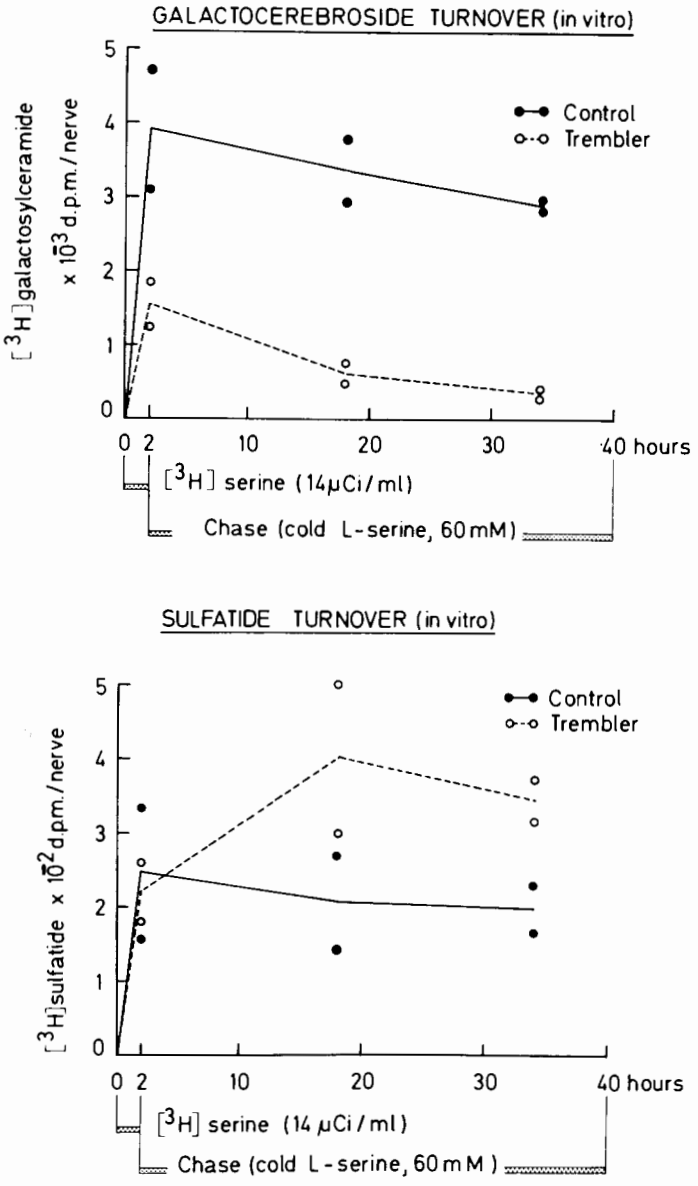

Fig. 4. Upper panel: cerebroside turnover using [^{3}H]serine as a precursor. Lower panel, sulfatide turnover from the same experiment. Other explanations as mentioned in the preceding figures.

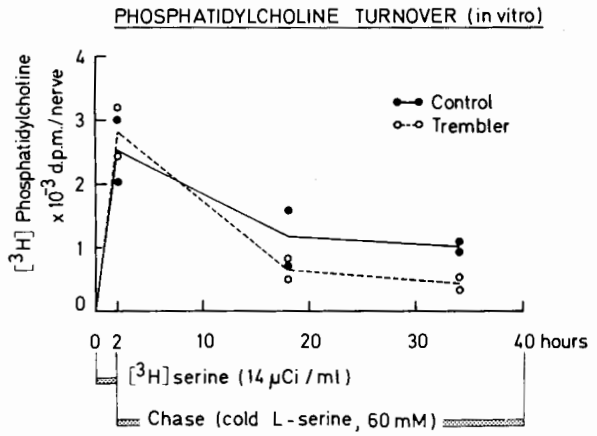

Fig. 5. Phosphatidylcholine turnover using [^{3}H]serine as a precursor. Same experiment as in Fig. 4.

undergoing Wallerian degeneration by us (Reigner et al., unpublished results) and others[12,20,23]. Nevertheless, in contrast to our findings in trembler nerves[17], in demyelination secondary to Wallerian degeneration, CST activities are low (Reigner et al., unpublished results).

Since the characteristic abnormality of myelin formation present in trembler mutant peripheral nerve in vivo is directly expressed in organotypic cultures in vitro[6], we used an in vitro system. In this system, no myelin is synthesized and axons, severed from their perikarya, degenerate. Fibroblasts and Schwann cells are maintained and we observed an active incorporation of [^{3}H]thymidine into Schwann cells indicating that, similarly to Wallerian degeneration in vivo, substantial cell proliferation occurred under the experimental conditions used for this study (Matthieu et al., in preparation). The viability of the system was further demonstrated by the oxygen consumption rates which did not change significantly during the period of incubation. Furthermore, no important alteration of Schwann cells could be demonstrated by conventional histological techniques (Matthieu and Kraus-Ruppert, unpublished results).

The metabolism of [^{35}S]sulfatides in trembler nerves showed a high turnover rate with increased incorporation of radioactive precursor and a steady loss of radioactivity, whereas, in control nerves, labeled sulfatides did not turn over significantly. The increased rate of sulfatide synthesis was confirmed using [^{14}C]galactose and [^{3}H]serine as precursors. These results are supported by the high activities of CST and arylsulfatase A[17]. In contrast to sulfatide synthesis, different results were obtained for its turnover when radioactive sulfate or galactose and serine were used as precursors. Using radioactive galactose or serine, no loss of radioactivity could be observed during the period studied. This indicates that the sulfate group of sulfatides turns over rapidly in trembler nerves, whereas cerebrosides, generated by the removal of the sulfate group (labeled by radioactive galactose or serine), are actively recycled into sulfatides. The high oxygen consumption rates measured in trembler nerves reflect, at least partly, the increased sulfatide metabolism.

In controls, the amount of radioactivity incorporated into cerebrosides was more than an order of magnitude higher than in sulfatides, while cerebroside concentration was approximateiy twice that of sulfatides. This and the sulfatide metabolism already discussed strongly suggest the presence of two different pools of cerebrosides, a small pool available for sulfation and in which most molecules are recycled into sulfatides, and which does not mix significantly with the larger and more active precursor pool (ceramide $\rightleftharpoons$ cerebroside). The existence of two pools of cerebrosides was indicated in a previous in vitro study[5]. In trembler nerves, cerebroside synthesis was reduced, in agreement with reduced ceramide galactosyl-transferase activities[17].

Using [^{3}H]serine as a precursor, no significant difference could be demonstrated in the metabolism of phosphatidylcholine and sphingomyelin between trembler and control nerves. In controls, phosphatidylcholine, an ubiquitous membranous component, had a faster turnover rate than the other lipids studied. In trembler nerves, its metabolism was similar to that of controls. These results obtained in control nerves are in agreement with other reports showing that cerebrosides and sulfatides are relatively stable whereas phosphatidylcholine turns over more rapidly (for a review see ref. 4).

In the trembler mutant, deficient myelin formation is due to an abnormality expressed in the Schwann cell triggered to myelinate axons[1,6]. The populations of Schwann cells in trembler nerves increase rapidly during the first month of life to nearly 10 times that of controls but in older animals, due to a high rate of cell death, numbers of Schwann cells remain stable[2]. During development of normal nerves, CST activation[17] precedes that of ceramide galactosyltransferase[8]. Therefore, one would expect that, in a cell population where differentiation is impaired[1], there would be an imbalance in cerebroside–sulfatide metabolism. However, this abnormal sulfatide metabolism seems specific to trembler myelinating peripheral nerves since, as mentioned above, in Wallerian degeneration, Schwann cells proliferate actively but CST activity is low.

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