Cholesterol Esters in Myelin Isolated from Cerebral White Matter of Patients with Multiple Sclerosis[1] (34566)

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In normal human adult whole brain or white matter, cholesterol esters have been reported as absent or present in small amounts (1–8). Variably increased quantities have been demonstrated in demyelinated (6, 9–12) and so-called normal areas (4, 10, 13) of cerebral white matter from cases of multiple sclerosis (MS), although such increases have not been found consistently in macroscopically normal white matter (9, 10) and plaques (10, 12, 13). Cholesterol esters were either not detected (9, 10, 13) or were slightly increased (9) in the gray matter from MS cases.

It is important to establish whether these changes in MS white matter occur in myelin or extramyelinic structures. This paper describes the isolation, quantitation, and fatty acid composition of cholesterol esters in CNS myelin from normal and MS subjects.

Materials and Methods. Patient material. The left cerebral hemisphere was sliced at room temperature into 3-mm coronal sections, frozen within 24 hr postmortem, and stored at −90°.

Patient 1, a "normal" control, was a 67-year-old male caucasian who died of congestive heart failure secondary to chronic obstructive lung disease and acute bronchopneumonia. Gross and microscopic examination of the central nervous system revealed no abnormalities.

Patient 2, a "normal" control, was a 77-year-old female caucasian who died with arteriosclerotic heart disease, severe anemia, and idiopathic thrombocytopenia purpura without any lesions of the central nervous system (CNS) on gross and microscopic examination.

Patient 3 was a 41-year-old caucasian female with a diagnosis of MS for 10–15 years. Gross inspection of coronal sections of the brain revealed multiple plaques of demyelination typical of MS. The three pooled plaques were classified histologically as an inactive chronic plaque of demyelination (14).

Patient 4 was a 41-year-old male caucasian with a diagnosis of MS for 7 years. Gross inspection of the coronal section of the brain revealed multiple plaques of demyelination on the edge of chronic plaques of demyelination.

Patient 5, a 62-year-old female caucasion had had MS for 26 years prior to death. Coronal sections of the brain revealed typical plaques of demyelination due to MS. Only normal-appearing white matter was studied.

Tissue dissection. The plaques of demyelination were dissected grossly so as not to contain any normal-appearing white matter. Normal-appearing white matter was obtained from an area approximately 1 cm from the plaque. A piece of each dissection was analyzed histologically with hematoxylin and eosin, as well as luxol fast blue with a Sudan IV counterstain.

Isolation of myelin and lipid extraction. Myelin was isolated from frozen white matter and plaques by ultracentrifugation (15). The isolation procedure was terminated at

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the "crude myelin" stage since the "light" and "heavy" myelin obtained by additional centrifugation steps were indistinguishable in electron micrographs and did not differ in lipid composition (15).

The solvents used were ACS reagent grade and were distilled and degassed with N_2 before use. Ethanol (1 %) was added to the chloroform as preservative. The N_2 used contained less than 5 ppm of O_2 (prepurified nitrogen, Liquid Carbonic Company, Los Angeles, Calif.). All solvent ratios were measured on a volume basis.

In the lipid-extraction procedure and subsequent operations, including chromatography, weighing, hydrolysis, and storage, a N_2 atmosphere was maintained to prevent oxidation of unsaturated fatty acids. Vacuum desiccators were routinely evacuated and flushed three times with N_2 after addition of lipid material, and the vacuum was released with N_2.

The lipids were extracted from the freeze-dried myelin with C-M 2:1 according to the procedure of Folch *et al.* (16) modified as described previously (17). Since the myelin dissolved completely, giving a very faint, opalescent solution, only a single extraction step was necessary, but protein was removed from the lipid extract (17).

Isolation of sterol ester. The neutral lipids were isolated by DEAE–cellulose column chromatography (17). Approximately 70 mg of C-M 2:1 extract was applied to a column 20 × 2.5 cm (id). The rate of elution was 3 ml/min, and 300 ml of chloroform was used as eluent. The eluate was evaporated to dryness on a rotary flash evaporator at 27°.

The separation of cholesterol ester from the remaining neutral lipids was carried out by silicic acid chromatography (18). Each column (5 mm id) was a pipette with the extension removed (Disposable Capillary Pipets, R. L. Scherer Company, Los Angeles, Calif.). A plug of glass wool was fitted in the lower constricted end and above this, ¼ inch of tightly packed celite (Johns-Mansville, Lompoc, Calif.). The column was then packed about three-quarters full with 140-mesh silicic acid (Mallinckrodt Chemical Company, St. Louis, Mo.). A rate of elution of approximately 1 drop/second was maintained by applying slight N_2 pressure to the top of the column. Quantitative TLC (20, 21) of the cholesterol ester fraction was carried out by a modified densitometric procedure (22) using 5% ether-*n*-pentane (19). A standard of cholesterol oleate (10 μg) was alternately spotted with volumes of sample previously shown to give spots of comparable density after charring.

The remainder of the cholesterol ester fraction was transesterified by heating at 80° for 4 hr in 5% methanolic H_2SO_4 in Teflon-lined screw-capped Pyrex tubes under an atmosphere of N_2 (17).

Gas-liquid chromatography. Fatty acid methyl esters were analyzed in a Barber-Colman Model 10 gas chromatograph with a 183-cm (6-ft) column of 12 % ethylene glycol succinate on chromosorb W. The column, detector, and flash heater temperatures were 184, 248, and 245°, respectively, and gas flow was 65 ml of N_2 per minute through the column. The hydrogen flame cell was calibrated with a standard containing equal weights of methyl palmitate, methyl stearate, methyl arachidate, and methyl behenate (Hormel Institute, Austin, Minn.). The peak areas were all within ± 3 % (relative) of known values. A mixture of methyl esters of fatty acids comprising 12:0, 14:0, 16:0, 16:1, 18:0, 18:1, 18:2, 20:4 (Hormel Institute, Austin, Minn.) and 20:1, 20:5, 22:6 (National Institutes of Health) was used as a reference standard for identification.

Results and Discussion. Determination of the content of cholesterol esters in normal myelin (0.23 g % dry weight) represents the first direct quantitative analysis of this lipid in the CNS (Table I). Cholesterol esters previously reported in normal white matter constituted 0.3 g/100 g dry weight calculated from the data of Cumings (9), and 0.3 and 1.0 g/100 g dry weight (23) when analyzed indirectly by difference between total and free cholesterol. Other investigators (1, 2, 4, 5) using this indirect procedure were unable to detect cholesterol esters in adult CNS nervous tissue.

TABLE I. Content of Total Lipids and Cholesterol Esters in MS Myelin.

Source of myelin		Total lipids (% dry wt of myelin)	Cholesterol esters	
			% By wt of total lipids	% Dry wt of myelin
Normal control white matter	Patient 1	78.0	0.3	0.2
	Patient 2	76.1	0.3	0.2
MS normal-appearing white matter	Patient 3	80.6	0.4	0.3
	Patient 4	78.6	—	—
	Patient 5	77.2	0.4	0.3
Plaques of demyelination	Patient 3	76.7	0.2	0.2
	Patient 4	77.2	0.2	0.2

Demyelinated formalin-fixed MS white matter contained 0.51 to 8.98 g/100 g dry weight of cholesterol esters in five samples analyzed by Cumings (9, 10). This exceeded values for fresh and formalin-fixed normal white matter (9). Plum and Hansen (4) found a rise of cholesterol esters in three cases of MS white matter taken mainly from sites showing macroscopic pathological lesions but detected none in normal white matter. If the average value of 80.3 % water content of demyelinated white matter of four MS cases (9, 10) is assumed, values recalculated from the data of Plum and Hansen (4) ranged from 0.48 to 0.93 g of cholesterol ester per 100 g dry weight white matter. All these MS samples (4, 9, 10) exhibited a higher content of cholesterol ester than the average value of 0.10 g/100 g dry weight of myelin isolated from demyelinated MS white matter. Honegger's TLC data (12) cannot be compared because of inherent errors ranging from 10 % for pale spots to 100 % for spots of greater intensity.

Apparently normal MS white matter freshly extracted from five cases gave values for cholesterol esters calculated from the data of Davison and Wajda (13) of 0.93, 0.93, 0.94, 1.33, and 0.20 g/100 g dry weight white matter, corresponding to 2.90, 1.94, 1.72, 2.23, and 0.56 percentage of the total lipids respectively. No cholesterol esters were detected in comparable normal adult tissue. In formalin-fixed MS white matter, Cumings (9, 10) reported an increase in content of cholesterol esters in apparently normal areas of two out of five cases. The amounts of cholesterol ester (0.90 and 4.69 g/100 g dry weight white matter) like the values of Davison and Wajda (13) considerably exceed the average value of 0.21 g/100 g dry weight of myelin isolated from apparently normal MS white matter.

The major components in CNS white matter are vascular elements, true extracellular space, glia (oligodendroglia and astrocytes), myelin, and axons. In MS, plaques of demyelination and to a lesser extent normal-appearing white matter also include reactive cells such as microglia, macrophages, hypertrophied and fibrous astrocytes, myelin-forming cells, and perivascular inflammatory cuffs comprised mostly of lymphocytes (24). Since the cholesterol esters increase in MS white matter in both apparently normal and demyelinated areas (4, 9, 10, 13) but not in myelin isolated from corresponding areas (Table I) this rise must be in the extramyelinic portion. Implication of the axons or blood can be eliminated since cholesterol esters were not detected in fresh gray matter despite their presence in the white matter of each of 5 MS cases investigated by Davison and Wajda (13). The increase of cholesterol esters, therefore, apparently occurs in the reactive glial cells, possibly the macropahges.

Consideration of the percentage of cholesterol present in the esterified form supports this probability. Proportions reported include 28.3 and 63.7 % in demyelinated areas of the midbrain (25), 5.3 % for one case and 33.5–62.7 % for four cases in demyelinated cerebral white matter (9, 10), and 26.5 % for three cases including eight samples of white matter showing macroscopic pathologi-

TABLE II. Fatty Acid Composition of Cholesterol Esters in MS Myelin and Glial Cells.

Fatty acid[a]	Relative retention ratio[b]	Normal cases		Multiple sclerosis: Myelin from normal-appearing white matter			Multiple sclerosis: Myelin from plaques of demyelination		Glial cells[c]	
		1	2	3	4	5	3	4	I	II
12:0	0.15	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
14:0	0.27	7.3	7.3	4.7	11.8	5.8	6.5	7.2	7.1	11.6
14:1	0.32	4.6	3.6	4.6	6.7	4.6	1.9	6.5	5.7	6.4
15:0	0.37	2.3	1.2	1.2	3.2	3.4	1.0	3.7	4.4	3.2
	0.44	4.3	2.3	3.2	8.5	4.0	1.9	3.3	3.8	1.8
16:0	0.53	15.4	21.7	11.0	17.4	12.0	26.9	17.3	19.2	26.9
16:1	0.61	19.4	18.1	14.6	12.8	15.3	13.0	11.4	18.7	14.8
17:0	0.81	7.3	1.9	7.0	6.1	7.8	4.9	3.2	4.2	2.5
18:0	1.00	11.5	11.2	9.0	11.8	8.0	8.8	11.0	7.4	9.5
18:1	1.15	13.1	14.3	12.0	9.8	20.8	13.1	14.1	10.5	6.1
18:2	1.48	8.1	5.0	5.2	5.9	5.5	7.8	2.9	4.2	2.8
18:3	1.95	1.9	—	—	—	0.3	2.9	—	2.0	1.8
	2.16	2.0	—	—	—	0.7	3.4	—	—	—
	3.20	—	7.6	18.0	—	3.9	—	12.1	12.5	12.4
20:4	3.56	2.1	5.9	—	—	6.2	3.2	6.4	—	—
Saturated		43.8	43.3	32.9	50.3	37.9	48.1	42.4	42.3	53.7
Monoenoic		37.1	36.0	31.2	29.3	40.7	28.0	32.0	34.9	27.3
Dienoic		8.0	5.0	5.2	5.9	5.5	7.8	2.9	4.2	2.8
Tri-tetraenoic		3.9	5.9	9.1	5.2	6.5	6.1	6.4	2.0	1.8
Unidentified		6.3	4.9	21.2	14.7	8.6	5.5	15.4	16.3	14.2

[a] Amount of each fatty acid expressed as a weight percentage of the total fatty acids in each cholesterol ester.

[b] Relative retention ratio of the fatty acid (methyl stearate as 1.00).

[c] Data of Fewster and Mead (28).

cal lesions (4). Even in apparently normal areas of cerebral white matter, except for one report of 0.93 % (13) a range of 7.5 to 29.5 % cholesterol was esterified (9, 10, 13). These reports contrast markedly with data from the present study yielding averages of 0.45 and 1.1 % of cholesterol present in the esterified form in myelin isolated from plaques and apparently normal areas of white matter respectively. The latter values are the same as the values for normal myelin.

The distribution pattern for fatty acids in cholesterol esters of myelin (Table II) was remarkably similar for all the samples analyzed. Major fatty acids present included palmitic and stearic acids and their corresponding monounsaturated derivatives. The average saturated (43.6 %), monoenoic (36.6 %), and dienoic (6.5 %) fatty acids in cholesterol esters of normal myelin are similar to the corresponding percentages of 38.2, 38.3, and 11.5 reported by Tichy (26) for adult human white matter. The average combined proportion of saturated and monounsaturated fatty acids (80.1 %) approximates the value of 85.1 % reported by Young and Hulcher (27) for bovine myelin, but the distribution patterns of the fatty acids differed. It is possible that some of their unidentified peaks were artifacts (28). Cumings *et al.* (29) reported an average of 44.3 % saturated fatty acids in the cholesterol esters of plaques from four MS cases, which is essentially the same as 45.3 % present in myelin isolated from plaques. The distribution patterns of fatty acids were also similar except

for a higher percentage (27.2) of oleic acid in their sterol ester samples. Since there is a considerable difference between the distribution patterns of fatty acids in the present study and those of fatty acids from plasma cholesterol esters (29), the possibility of these cholesterol esters being derived from plasma can be rejected.

A decrease in the proportion of unsaturated fatty acids of phosphatidyl choline in apparently normal MS white matter has been reported (30). In addition, Cumings (31) suggested that an early feature of MS may be the splitting off of free fatty acids from the sheath lipids which could then unite with free cholesterol to form cholesterol esters. If such esterification were to account for the reported increases of cholesterol esters (4, 9, 10, 13), it must take place in the extramyelinic portion of white matter since there was no change in the distribution patterns of fatty acids in cholesterol esters of myelin isolated from normal-appearing MS white matter or plaques from those for corresponding normal tissue.

The fatty acid composition of human cerebral cholesterol esters correlates well with the corresponding data for bovine glial cell preparations (28), especially since preliminary TLC separation of the glial cholesterol esters, including concomitant oxidation hazards, probably explains their apparent lack of arachidonic acid. CNS myelin is formed by extension of the oligodendroglial cell perikaryon, so that the probability that the cholesterol esters in myelin originate in the glial cells is strengthened.

In certain pathological and experimental conditions resulting in myelin degradation, fragments of myelin have been observed by electron microscopy within oligodendrocytes (32) and "apparently activated oligodendrocytes" (33). The increases in cholesterol ester in MS white matter (4, 9, 10, 13) may reflect the extent of breakdown of phagocytosed myelin fragments in glial cells. Alternatively, since in the developing human nervous system, the ratio of cholesterol ester to free cholesterol reaches a peak at the onset of myelination (34), the increase could reflect myelin regeneration. The onset of myelination is probably also the period of most active fatty acid synthesis by the *de novo* and chain elongation pathways. One of the main functions of cholesterol esters in glial cells of normal CNS white matter may be to transport long-chain fatty acids in a soluble form from their site of *de novo* synthesis in the microsomes (34) to the mitochondria for elongation to very long-chain (>C18) fatty acids (34, 35) and to the site of sphingolipid synthesis.

The fatty acids in cholesterol esters of human glioblastomas (36–38) include not only the range present in cholesterol esters of normal bovine glial cells but also longer chain fatty acids (38). Approximately one-half of the total cholesterol is present in the esterified form. Since glial tumors do not produce myelin, Slagel *et al.* (39) have suggested that production of cholesterol esters may not be associated with myelin formation but with other cellular activities during myelination.

Summary. Cholesterol esters were detected in myelin isolated from normal human white matter, histologically normal multiple sclerosis (MS) white matter and plaques. No differences from normal samples were found for the amount of cholesterol esters in myelin from MS tissue. The distribution patterns of fatty acids in the cholesterol esters were similar for all the samples analyzed.

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