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Steryl esters and their relationship to normal and diseased human central nervous system

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Abstract The composition and distribution of steryl esters in human diseased or developing brain tissue has been studied. The abnormal brain conditions included sudanophilic leukodystrophy, multiple sclerosis plaque, subacute sclerosing panencephalitis, and an old cerebral infarction and two types of brain-derived tumors. In addition to the above abnormal tissue, steryl esters were also examined in developing and normal adult human brain. It was found upon subcellular fractionation that the steryl ester was localized mainly in the soluble nonparticulate material. A cholesteryl ester-rich fraction, floating on top of distilled water after centrifugation, was recovered only in the developing brain or in instances where there was myelin damage. The sterol portion of the steryl ester was largely cholesterol. The fatty acid moiety was mainly composed of C_{16}, C_{18}, and C_{20} fatty acids. The dominant fatty acid was oleic acid, and the proportion of this fatty acid increased in demyelination. Although there were great differences in the quantities of steryl ester found, the fatty acid profiles of normal developing and adult brain were quite similar. As has been noted by others, the fatty acid composition of brain steryl esters most closely resembles that of brain phosphatidylcholine.

Supplementary key words brain lipids · cholesterol · development · demyelination

Since sudanophilic lipid was first described in brain undergoing demyelination, the role of steryl esters in normal and diseased brain has been a matter of considerable speculation. Although it has been commonly accepted that steryl esters are present in brain during demyelination, their presence or absence in normal mature brain has been in doubt (1). Alling and Svennerholm (2) have, however, demonstrated a low but consistent quantity of steryl ester in normal human brain. Increased concentration of cholesteryl esters in sudanophilic demyelination has been reported in subacute sclerosing panencephalitis (3, 4), multiple sclerosis (5, 6), sudanophilic leukodystrophy (7), gangliosidoses (8), Schilder's disease (9), and adrenocortical atrophy with diffuse sclerosis (10). Steryl ester is also a large lipid component of tumors derived from brain tissue (11). Adams and Davison (12) found in developing human corpus callosum and spinal cord large amounts of cholesteryl ester, which dissipates during maturation. Eto and Suzuki (13), in their study of steryl ester in developing rat brain, contend that there is not a large increase of ester during development. The origin of cholesteryl esters in these various conditions is still a matter for speculation. It has been suggested that they may be formed by a brain lecithin:cholesterol acyltransferase (14), by infiltrating macrophages (15), or by direct acylation by free fatty acid released from phosphoglyceride by prior lipase activity. The first possibility may have been disproven (16) and the second would not necessarily apply if increased ester in normal developing brain could be established. The normal brain contains enzyme systems for the synthesis and slow catabolism of steryl ester (15, 17), and a small amount of ester may be formed from free fatty acid and cholesterol. In this paper we will show that there is a similarity of distribution and composition of steryl ester in several different conditions of the brain. Evidence for the origin of the fatty acid in steryl esters during demyelination will also be presented.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

MATERIALS AND METHODS

Brain tissue, with the exception of the tumors, was taken at autopsy at the Hospital for Sick Children, Great Ormond Street, or the National Hospital, Queen Square, London. The tumors studied were obtained from the neurosurgical services of the National Hospital and Maida Vale Hospital, London. Tissues were stored at −20°C before biochemical analysis and subcellular fractionation.

Subcellular fractionation

Subcellular fractionation was carried out on certain steryl ester-containing neural tissues. The tissue was first homogenized with a Teflon pestle in a glass homogenizer containing 0.32 M sucrose. This homogenate was then

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TABLE 1. Human sterol and steryl ester content of normal and diseased central nervous system tissue[a]

Brain or Derived Tissue	Total Sterol	Steryl Ester	Percentage of Total Sterol Esterified
	mg/g wet wt		%
Corpus callosum (3-wk-old infant)	9.8	0.5	5.3
Corpus callosum (8-wk-old infant)	8.2	1.60	19.5
White matter (normal adult)	34.8	1.4	4.0
Oligodendroglioma	14.6	5.0	34.0
Acoustic neurinoma	5.4	2.1	38.9
Degenerated white matter (cerebral infarction)	8.0	3.2	40.0
Plaque (multiple sclerosis)	6.5	0.013	0.2
Demyelinated white matter (subacute sclerosing panencephalitis)	34.8	15.8	45.3
Demyelinated white matter (sudanophilic leukodystrophy)	33.3	14.3	42.9
Degenerating gray matter (sudanophilic leukodystrophy)	8.3	2.4	28.7

[a] Duplicate analyses were performed on a single specimen in each case.

further separated into soluble, microsomal, mitochondrial, synaptosomal, and myelin fractions as described by Ramsey et al. (18–20). After the initial slow-speed spin of the homogenate, a layer of white material floating on the surface of the supernate was found in several of the samples. This layer was carefully removed with a Pasteur pipette. More of this material was encountered after the initial myelin isolation steps, near the top of the tube where the top layer was 0.32 M sucrose. This material was clearly separated from the myelin, which was localized at the 0.32–0.8 M sucrose interface. All of this floating layer was combined and diluted approximately 15-fold with distilled water and centrifuged at 112,000 *g* for 1 hr to give material floating on the surface of the water. This fraction hereafter will be referred to as the floating fraction. A pellet was also collected as a result of centrifugation of the "water-shocked" floating fraction. This pellet was resuspended in distilled water and centrifuged for another hour at 112,000 *g*. A pellet was again recovered with no evidence of floating material being generated from the original pelleted material. This fraction will hereafter be referred to as the pellet from the floating fraction. The myelin fraction and the floating fraction, as well as the pellet derived from it, were each found to be at least 90% free from contaminants from other recognizable subcellular fractions, as judged by electron microscopy (19).

Thin-layer chromatography

TLC was carried out on silica gel H plates that had been activated 1 hr prior to use. Phospholipids were separated in a solvent system consisting of chloroform–methanol–glacial acetic acid–water 50:30:8:4 (by vol). Free sterols and steryl esters were separated by means of a solvent system composed of petroleum ether–ethyl ether–acetic acid 82:18:1 (by vol). Total and free sterols were determined by the method of Sperry and Webb (21). In certain instances the steryl ester fraction was eluted and then saponified in 2 N KOH in ethanol–water 9:1 (v/v) by refluxing for 1 hr. The free sterols were then extracted with petroleum ether. The original free sterol fraction and the free sterols derived from the steryl ester fraction were then further fractionated into 4,4-dimethyl, 4α-methyl, and 4-desmethyl sterols by use of the solvent system of Rahman et al. (22). Prior to elution, the plate was exposed to iodine and the spots were marked out, and the iodine was then allowed to evaporate. Due to contaminants in the gel itself, which were found to interfere with lipid quantitation and fatty acid analysis, thin-layer plates were developed in chloroform–methanol 2:1 (v/v) prior to final plate activation and subsequent sample application.

Gas–liquid chromatography

GLC was performed using a Perkin-Elmer F-11 with hydrogen flame detector in conjunction with an Infotronics CRS-208 digital integrator. The carrier gas was nitrogen at 60 ml/min. The columns, 6 ft × ⅛ inch ID, were packed with either 3% OV-17 on Chromosorb W HP (80–100 mesh) or 10% DEGS Celite (80–100 mesh). The phases were acquired from Phase Separations Ltd., Queensferry, Lancashire, England.

The 3% OV-17 column was used for sterol separation and quantitation and was operated at 265°C. Flash heater and detector were operated at 280°C. Sterol peaks were identified by comparison with standard samples or calculated retention times of reference compounds, as has been described (23, 24).

After elution from the silica gel, samples for fatty acid composition studies were dried and then taken up in 14% (w/v) boron trifluoride in methanol (Phase Separations Ltd.) for methylation, following generally the procedures of Morrison and Smith (25). Samples were extracted from the methylating mixture with hexane. The methylated fatty acids were then chromatographed on the 10% DEGS column, which was operated at 180°C. The flash heater and detector were at 210°C. Chromatograms were compared with reference samples obtained from Sigma Chemical Co. Ltd., London.

RESULTS

Quantity of steryl ester in neural tissue

Since it has been uncertain as to whether or not steryl esters are an invariant constituent of neural tissue, a variety of normal and diseased tissues were examined for free and esterified sterol. Although ester is always present, its

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TABLE 2. Sterol and steryl ester subcellular distribution in multiple sclerosis plaque tissue[a]

Fraction	Free Sterol		Steryl Ester		Percentage of Total Sterol Esterified
	μg/g wet wt	% distribution	μg/g wet wt	% distribution	%
Homogenate	6480	100	13.2	100	0.2
Floating fraction	1.84	0.07	5.51	45.6	75.0
Pellet from floating fraction	90.8	3.4	1.76	14.6	1.9
Soluble	25.5	0.9	1.94	16.0	7.1
Microsomes	28.5	1.1	0.57	4.7	2.0
Mitochondria	8.90	0.3	0.97	8.0	9.8
Synaptosomes	23.2	0.9	0.69	5.7	2.9
Myelin	2520	93.4	0.65	5.4	0.02

[a] Duplicate analyses were performed on a single specimen. Original tissue wet wt was 5.70 g.

proportion of the total sterol varies widely (Table 1). Examination of numerous normal tissues over a period of years in this laboratory has shown that there is a wide range of ester content (0.2–5%). In the case of the developing brain, differences in ester content reflect changes due to maturation in various parts of the nervous system (see also Adams and Davison [12]). The tumors had widely differing total sterol content, but a high proportion of the sterol was esterified. The cerebral white matter infarction was an old, very scarred sample with diminished blood supply. The total sterol content was much below that of normal white matter but the steryl ester content was elevated. The multiple sclerosis plaque was also necrotic and of long duration. The low steryl ester content indicates that the active stage of demyelination had ceased, and the steryl ester content was even below that of normal mature brain. The brain of a 19-yr-old male with subacute sclerosing panencephalitis contained considerable proportions of cholesteryl ester. An abnormally high steryl ester content was also found both in the white matter and the gray matter of a 5-yr-old boy with sudanophilic leukodystrophy.

Subcellular distribution of steryl ester

Normal adult white matter, when it was examined for the subcellular localization of steryl ester, was found to have more than 50% of the total tissue ester in the soluble fraction, with the mitochondria being the particulate fraction with the most ester. No floating material was found in the normal mature white matter.

In several cases where there was evidence of active demyelination, the tissue was fractionated to allow examination of the myelin fraction. In doing so, a "floating fraction" that contained large amounts of steryl ester was also recovered. This fraction has been found consistently in tissues undergoing active demyelination. Since developing white matter is also rich in steryl ester (Table 1), we also fractionated samples of developing corpus callosa. Once again a floating fraction, rich in steryl ester, was recovered. A pellet could also be sedimented from this floating material upon water shocking, as has been described in Materials and Methods. It was low in steryl ester content. The particulars regarding the composition and morphology of these fractions are being reported elsewhere.[1] The multiple sclerosis plaque tissue contained very little ester, but on subcellular fractionation it was found to be selectively enriched in certain subcellular fractions (Table 2). The final floating material appeared as a yellowish scum, particularly rich in steryl ester, floating on top of the water. Some ester was present in the soluble fraction. Of the particulate fractions, mitochondria had the highest percentage of its total sterol complement esterified. The subcellular distribution of sterol and steryl ester in the tissue of the subacute sclerosing panencephalitis case was similar to the multiple sclerosis tissue just described. The floating material contained much more ester and was a bluish-white color.

On fractionation of white matter from the case of cerebral infarction, a floating fraction rich in steryl ester was found (Table 3). Unlike the other tissues examined, the pellet from the floating fraction had a considerable portion of its sterol in an ester form. Esterified sterol also accounted for a large portion of the total sterol in the soluble and synaptosomal fractions.

A floating fraction was not obtained on separation of tumor tissue, but again the steryl ester was of a nonparticulate nature. Half the total ester in an oligodendroglioma sample appeared in the soluble fraction (Table 4), and almost two-thirds of the sterol in this fraction was esterified. Mitochondria and synaptosomal fractions both contained considerable amounts of steryl ester. The exact nature of the synaptosomal fraction from these tumors is unknown, but may represent once-normal synaptic structures en-

[1] Ramsey, R. B., N. Banik, T. Scott, and A. N. Davison. Manuscript in preparation.

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TABLE 3. Sterol and steryl ester subcellular distribution in cerebral infarction[a]

Fraction	Free Sterol		Steryl Ester		Percentage of Total Sterol Esterified
	μg/g wet wt	*% distribution*	*μg/g wet wt*	*% distribution*	%
Homogenate	4800	100	3200	100	40.0
Floating fraction	65.3	1.4	2704	84.5	97.6
Pellet from floating fraction	262	5.5	72.3	2.3	21.6
Soluble	53.8	1.1	72.3	2.3	57.3
Microsomes	744	15.5	65.9	2.1	8.1
Mitochondria	869	18.1	94.7	3.0	9.8
Synaptosomes	19.2	0.4	147.5	4.6	88.5
Myelin	2765	57.6	41.9	1.3	1.5

[a] Duplicate analyses were performed on a single specimen. Original tissue wet wt was 3.12 g.

gulfed by the tumor's growth. The acoustic neurinoma had 85% of the steryl ester localized in nonparticulate material. The synaptosomal fraction, although low in total sterol, was high in ester content (Table 4).

The possibility that increased amounts of steryl ester might be related to increased sterol synthesis was examined. In the free and steryl ester fractions of the demyelinating tissue from the subacute sclerosing panencephalitis brain, cholesterol was the only sterol measurable by our GLC methods. The developing corpus callosa contained small amounts of sterols other than cholesterol in both the free and ester fractions, but there was no marked difference in the distribution of these sterols between the free and esterified fractions. In the tumor, sufficient sterol was available for analyses, and cholesterol precursors were detected (Table 5). The steryl ester fraction was very poor in cholesterol precursors. The free sterol fraction contained significant amounts of desmosterol and other cholesterol intermediates. The fatty acid composition of the steryl esters exhibited considerably more variety than the sterol moieties (Table 6). Generally, oleic acid ($C_{18:1}$) was the dominant fatty acid; an exception to this were the fatty acids of cholesteryl ester from the 3-wk-old corpus callosum. Specimens contained different proportions of palmitoleic acid ($C_{16:1}$), unusual for brain lipids, and some contained large amounts of linoleic acid ($C_{18:2}$). Where linoleic acid was present in considerable quantity, it could reflect a contribution by blood steryl ester. There seemed to be a relative increase of oleic acid in instances of demyelination. In nondemyelinating tissue the oleic acid content was 40–45% of the total but in demyelinating tissue the oleic acid represented 60–75% of the total fatty acid steryl ester. This was not true of the steryl ester found in the sudanophilic leukodystrophy gray matter. Its origins may, however, be different from that of the degenerated white matter. A search was made to determine

TABLE 4. Sterol and steryl ester subcellular distribution in oligodendroglioma and acoustic neurinoma tumors[a]

Fraction	Free Sterol		Steryl Ester		Percentage of Total Sterol Esterified
	μg/g wet wt	*% distribution*	*μg/g wet wt*	*% distribution*	%
Acoustic neurinoma					
Homogenate	3300	100	2100	100	38.9
Soluble	419	12.7	1785	85.0	81.0
Microsomes	1650	50.0	116	5.5	6.6
Mitochondria	828	25.1	73.3	3.5	8.1
Synaptosomes	24.1	0.7	122	5.8	83.4
Oligodendroglioma					
Homogenate	14,650	100	4980	100	34.0
Soluble	1217	9.8	2247	50.0	64.9
Microsomes	5309	42.9	275	6.1	0.05
Mitochondria	4512	36.4	1232	27.4	21.4
Synaptosomes	1343	10.8	736	16.4	35.4

[a] Duplicate analyses were performed on each sample. Original tissue wet weights of acoustic neurinoma and oligodendroglioma were 0.280 and 0.466 g, respectively.

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TABLE 5. Sterol composition of oligodendroglioma tumor[a]

Sterol	Free Sterol		Steryl Ester	
	µg/g wet wt	%	*µg/g wet wt*	%
Cholesterol	14,490	98.9	4,973	99.8
Desmosterol	153.5	1.05	2.18	
4α-Methyl-5α-cholest-8-en-3β-ol	0.0364			
4α-Methyl-5α-cholest-7-en-3β-ol	0.298		5.08	
4α-Methyl-5α-cholesta-8,24-dien-3β-ol	3.68			
4,4-Dimethyl-5α-cholest-8-en-3β-ol	0.131		3.04	
4,4-Dimethyl-5α-cholesta-8,24-dien-3β-ol	3.47			
Lanosterol	2.95			

[a] Duplicate analyses of the tissue were performed.

TABLE 6. Major fatty acid composition of steryl esters in normal and diseased central nervous system tissue[a]

Fatty Acid	Corpus Callosum (3-wk-old infant)	Corpus Callosum (8-wk-old infant)	White Matter (normal adult)	Oligodendro-glioma	Acoustic Neurinoma	White Matter (cerebral infarction)	Plaque-containing Tissue (multiple sclerosis)	White Matter (subacute sclerosing panen-cephalitis)	White Matter	Gray Matter
									(sudanophilic leukodystrophy)	
					% of total fatty acids					
16:0	37.6	28.8	27.6	15.0	33.0	20.3	16.0	17.7	15.3	21.1
16:1	21.1	14.1	8.7	6.4	10.9	9.0	6.1	7.4	9.2	7.1
18:0	17.7	7.4	16.0	10.6	2.4	8.5	2.2	3.0	3.3	4.8
18:1	17.6	42.2	46.4	38.7	39.5	39.4	73.0	68.6	62.8	43.5
18:2	1.5	4.8	1.7	22.8	12.2	7.9	tr	tr	tr	5.4
20:3							tr	tr	3.6	7.5
20:4	4.5	2.8	tr	6.2	1.9	14.8	2.2	3.2	5.5	8.5

[a] Duplicate analyses were performed on duplicate specimens.

TABLE 7. Major fatty acid composition of ethanolamine phosphoglyceride from steryl ester in normal and diseased central nervous system tissue[a]

Fatty Acid	Corpus Callosum (3-wk-old infant	Corpus Callosum (8-wk-old infant)	White Matter (normal adult)	Oligodendro-glioma	Acoustic Neurinoma	White Matter (cerebral infarction)	Plaque-containing Tissue (multiple sclerosis)	White Matter (subacute sclerosing panen-cephalitis)	White Matter	Gray Matter
									(sudanophilic leukodystrophy)	
					% of total fatty acids					
16:0	8.7	1.2	7.6	4.9	21.5	6.9	7.9	6.6	15.7	17.0
16:1	tr	tr	tr	tr	8.1	tr	tr	tr	1.3	tr
18:0	35.1	32.0	20.3	16.9	15.3	29.3	24.2	23.2	16.3	30.2
18:1	9.0	7.4	53.5	19.3	24.5	47.1	52.7	40.9	45.7	23.6
20:1	1.1	tr	1.0	tr		4.0	4.0	4.4	tr	
20:2	1.0	tr	1.0				tr			
20:4	15.4	15.6	4.1	22.8	16.7	5.7	4.9	8.8	6.5	12.7
22:5 (n − 6)	13.6	24.6	11.9	11.9	3.2	6.9	5.5	11.6	14.4	6.2
22:5 (n − 3)	2.1	tr	tr	tr	5.4		tr	tr		tr
22:6	13.9	17.9	1.7	24.1	5.2	tr	1.2	4.4	tr	10.1

[a] Duplicate analyses were performed on duplicate specimens.

whether or not the fatty acid compositions of other fatty acid-containing compounds of these tissues were similar to that of the steryl ester, because under given circumstances this fatty acid-containing compound might serve as the fatty acid source for steryl ester formation. Two of the more abundant phospholipids were examined: ethanolamine phosphoglyceride and choline phosphoglyceride. The ethanolamine phospholipids, while often diminished during demyelination, did not have a fatty acid composition in any of the tissues examined that would make it a potential source of fatty acid for steryl ester formation (Table 7). The choline phospholipid, which has a comparatively rapid turnover in brain, had a fatty acid profile similar to that of steryl ester (Table 8). However, the palmitoleic acid content was lower, and in the demyelinating tissue the concentration of oleic acid was not increased.

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TABLE 8. Major fatty acid composition of choline phosphoglyceride from normal and diseased tissue[a]

Fatty Acid	Corpus Callosum (3-wk-old infant)	Corpus Callosum (8-wk-old infant)	White Matter (normal adult)	Oligodendro-glioma	Acoustic Neurinoma	White Matter (cerebral infarction)	Plaque-containing Tissue (multiple sclerosis)	White Matter (subacute sclerosing panen-cephalitis)	White Matter (Sudanophilic leukodystrophy)	Gray Matter (Sudanophilic leukodystrophy)
					% of total fatty acids					
16:0	66.2	59.3	43.8	41.2	42.8	45.4	35.6	44.3	34.1	47.8
16:1	4.8	5.5	1.0	6.1	2.1	5.3	tr	2.6	3.5	tr
18:0	8.3	11.7	11.4	12.7	18.3	7.7	13.9	10.0	19.1	40.6
18:1	17.3	21.9	43.1	27.6	28.3	28.6	50.5	42.7	38.1	10.1
20:1	3.3	1.6	tr	8.3			tr	tr		
20:4	tr	tr	tr	4.2	8.4	13.0	tr	tr	5.2	1.4

[a] Duplicate analyses were performed on duplicate specimens.

DISCUSSION

The role played by neutral fat in the nervous system has long been under question. It has been established for a number of years that the neutral fat seen in the central nervous system is mainly composed of sterol esterified to fatty acid. Pathologists have felt that in diseased brain the ester is generated by phagocytic cells. Werb and Cohn (16), working with isolated macrophages, have suggested that these cells do not have the ability to make sterol esters but can ingest and hydrolyze them. The fatty acid profiles of demyelinating brain esters are also unlike blood sterol esters (7), ruling out simple uptake of blood constituents. The question is, do all the steryl esters of the central nervous system have a common neural origin, and, if so, why do the steryl ester concentration and fatty acid composition vary?

There are many similarities among the steryl ester-containing tissues. In all instances the major part of the esterified sterol was found floating on or within the soluble material, although the presence of other lipids and some protein contributed to the low density of the layer. No floating material was found in fractionated normal adult white matter, although it is possible that traces of ester floating on top of the gradient were missed. In brain cell-derived tumors there was no floating material, reinforcing the idea that the floating fraction was not generated by the mere presence of the neutral fatty steryl ester.

The mitochondrial fraction was generally quite high in steryl ester and had a low ratio of ester to total sterol. This would be consistent with brain mitochondria being the fraction in which steryl ester is synthesized, as Eto and Suzuki have shown to be the case in rat brain (15).

Whether from developing, demyelinating, or neoplastic tissue, the steryl ester fraction contained mainly cholesterol, but with minor variations in the fatty acid composition.

Eto and Suzuki (15) have suggested that a lecithin:cholesterol acyltransferase may be operating in the brain, but there is little evidence of this from their own work. They have shown, however, that cholesterol esterification in the rat brain can be stimulated by free fatty acid, and particularly by oleic acid (15). Free fatty acid and cholesterol are the only components shown to be needed for steryl esters to be generated by neural tissue. The cholesterol may come from a variety of sources, but its availability appears not to be rate limiting. The important unknown is the origin of the fatty acid. In human whole brain tissue, however, steryl ester concentration changes during development and maturation (26) but there is no evidence of an increase of free fatty acid during development or a decrease after maturation (27). Biosynthesis of fatty acid is very rapid in developing brain for utilization in myelination, and quite probably excess fatty acid could be produced.

A basic principle of detoxification of free fatty acid may be operative in demyelination. Free fatty acid has been shown to be damaging to mitochondria (28). Other membrane structures may also be affected. It is known that under ischemic conditions or electroconvulsive shock, free fatty acid is increased in the brain (29). The increase in free fatty acid has been attributed to lytic action of an activated brain phospholipase A (29). We have also been able to show that under in vitro conditions added snake venom phospholipase A not only liberates free fatty acids but induces the formation of small amounts of steryl ester.[2] This is presumably happening by a previously described mechanism (15). This same series of reactions could be activated during human brain demyelination. Only small changes in phospholipase A activity, leading to increased breakdown of phospholipid, and release of free fatty acid pool would be sufficient to account for the slow accumulation of sudanophilic material.

We, therefore, propose that the general purpose of steryl ester is to serve as a carrier and storage point for otherwise toxic free fatty acid. In development, the increased steryl ester may result from elevated fatty acid biosynthe-

[2] Ramsey, R. B., and A. N. Davison. Unpublished observations.

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sis. In demyelination, steryl ester is actively generated because of the abundance of free fatty acid being produced by activated lytic enzymes such as phospholipase A (30). ■

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