Rho(D) factor (24,25) (Table III). However, investigations along these lines have not been pursued so far as those described in the earlier portions of this communication.

Finally, some attention has been directed towards the possibility that anti-ganglioside antibodies might occur in the serum of patients with various lesions of the central nervous system, particularly with regard to multiple sclerosis (26). Antiganglioside antibodies are not normally present in human serum. A low but demonstrable antiganglioside antibody titre does occur in about 20% of patients with multiple sclerosis, and amyotrophic lateral sclerosis (Table IV). The antibody in the sera from patients with multiple sclerosis appears to be specific for monosialoganglioside (27). However, the role of antiganglioside antibodies in the etiology of pathological conditions such as multiple sclerosis and amyotrophic lateral sclerosis remains obscure. As in the case of animals in which anticerebroside antibodies were induced, no signs of neurological disability could be attributed to the presence of circulating antiganglioside antibody (28).

In retrospect, one might conclude that serum anticerebroside or antiganglioside antibodies would not necessarily be found in patients with multiple sclerosis even if an autoimmune reaction plays a role in the pathogenesis of this disease. This concept is supported by the widely accepted view that delayed hypersensitivity is a cellularly-mediated phenomenon and that circulating antibodies seem relatively unimportant in reactions of this nature (29).

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The Structure and Chemistry of Sulfatides

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Abstract

Recent advances in the chemistry of cerebroside sulfates which have led to a precise formulation of their structure and to a detailed knowledge of their composition are reviewed.

Qualitative and quantitative analyses of the constituent fatty acids and sphingosine-type bases of cerebrosides and sulfatides have shown a close relationship between the cerebrosides and their sulfate esters.

Sulfatides were converted into cerebrosides in high yield by mild desulfation with methanolic HCl at room temperature. The products of partial degradation of cerebrosides—ceramide and psychosine—were found to be identical to the products obtained under similar conditions from desulfated sulfatides.

By methylation and periodic acid oxydation, the sulfate group has been located conclusively and apparently only at C-3 of the galactose moiety in the sulfatide molecule.

A β-configuration of the galactosidic linkage in cerebrosides and in sulfatides has been demonstrated by direct comparison of the products of periodate oxidation of dihydropsychosine obtained from cerebrosides and from sulfatides, with the products of oxidation of 1-D-glyceryl β-D-galactoside of known configuration.

Investigations of the composition and structure of sulfatides abnormally accumulated in the brain and kidneys in cases of metachromatic leukodystrophy, a demyelinating disorder, are reported.

Introduction

SEVEN YEARS AGO Jatzkewitz (1) and Austin (2) identified as sulfatides the metachromatic substance (3) which accumulates abnormally in the central nervous system (4) and the kidneys (5) of patients afflicted with metachromatic leukodystrophy, a diffuse demyelinating disorder. This finding, confirmed many times since (6–9), has undoubtedly been largely responsible for the growing interest shown recently in the chemistry and biochemistry of the cerebrosides sulfuric esters and their relationship to other complex lipids with which they are associated, notably as constituents of the myelin membrane.

The present article is intended to summarize the advances which have been made lately in the chemistry of sulfatides, and to discuss some of the experimental data which are leading to a more accurate and detailed knowledge of the composition, the structure and the chemical properties of these sulfolipids.

A general review entitled "The Sulfolipids" and covering the different aspects of earlier work in the field has been published by Goldberg in 1961 (10).

A chapter on Metachromatic Leukodystrophy by Moser and Lees is to be published soon (11).

Isolation

A great deal of effort has been devoted in many laboratories to the development of procedures for the isolation and the purification of the sulfatides present in lipid extracts of various tissues. The number of such contributions reflects undoubtedly the difficulty of the problem.

As is the case with most, if not all, complex lipids possessing polar groups together with long aliphatic chains in their molecules, the quantitative isolation from biological sources of pure sulfatides is a difficult task. In general, polar complex lipids are so prone to enter into more or less stable associations with other substances—a feature which, by the way, is probably essential to their function as membrane constituents in the living cell—that it is extremely difficult to discriminate between true molecular species and aggregates. For example, the existence of phosphorus-containing sulfatides has been a long-debated question which may not be fully settled yet. Most procedures of isolation of sulfatides are based on their separation from other lipids present in tissue extracts by chromatography. A great variety of supports have been used, such as: cellulose (12,13), silicic acid (14,15), Florisil (16–18), ion exchange resins (16), DEAE cellulose (15,19,20) and charcoal (21,22). Generally, repeated chromatography on several different supports used successively is necessary to achieve adequate purification especially when the starting material contains a very small proportion of sulfatides. Worth special mention is the use of silica gel in the form of thin layers on glass plates (23–25). This technique gives usually remarkably neat separations. It affords not only a very elegant means of qualitative analysis of mixtures of lipids, but it can also be used for preparative purposes when the silica containing the desired lipid is scratched off the plate and the lipid is recovered by solvent extraction. Such procedures, singly or in combination, yield usually a large proportion of phosphorus-free sulfatides. Frequently, however, fractions containing variable amounts of phosphorus have been reported. The proportion of phosphorus in those fractions does not seem sufficient, however, to account for the existence of molecular species. In the very mild procedure of linked distributions of Lees et al. (17), in which no chromatography on strongly adsorbing supports is used, a first fraction of phosphorus-free sulfatides is obtained simply by crystallization. From the mother liquors a phosphorus-rich fraction is then collected. Chromatography of this fraction on Florisil results in the isolation of a second crop of pure sulfatides, the phospholipids, mainly phosphatidyl serine, being retained on the column. It may be hard to decide, of course, if the phosphatidosulfatide fraction is composed, at least in part, of a true combination that is split by the strongly adsorbing properties of Florisil, or, if the chromatography simply separates the components of a mixture. In this connection, it is interesting to recall the striking and still unexplained increase of the stability of the sulfate group in sulfatides toward hot acetic acid when phosphatides are added to the reaction mixture, pointing out the strong influence of the phospholipid on the chemical reactivity of sulfatides in a case where, apparently, no covalent bond exists between the two types of lipids (17). Sulfatide fractions containing hexosamine and inositol have also been reported (26,27). Here again, however, it has not been proven that these are real complex molecules. Thus, it appears that the phosphorus content of sulfatides decreases as more refined procedures of purification are proposed. This achievement may be the ideal of the analytical chemist. It leaves open the whole fascinating domain of study of associative and binding properties which might explain the architecture of functional membranes.

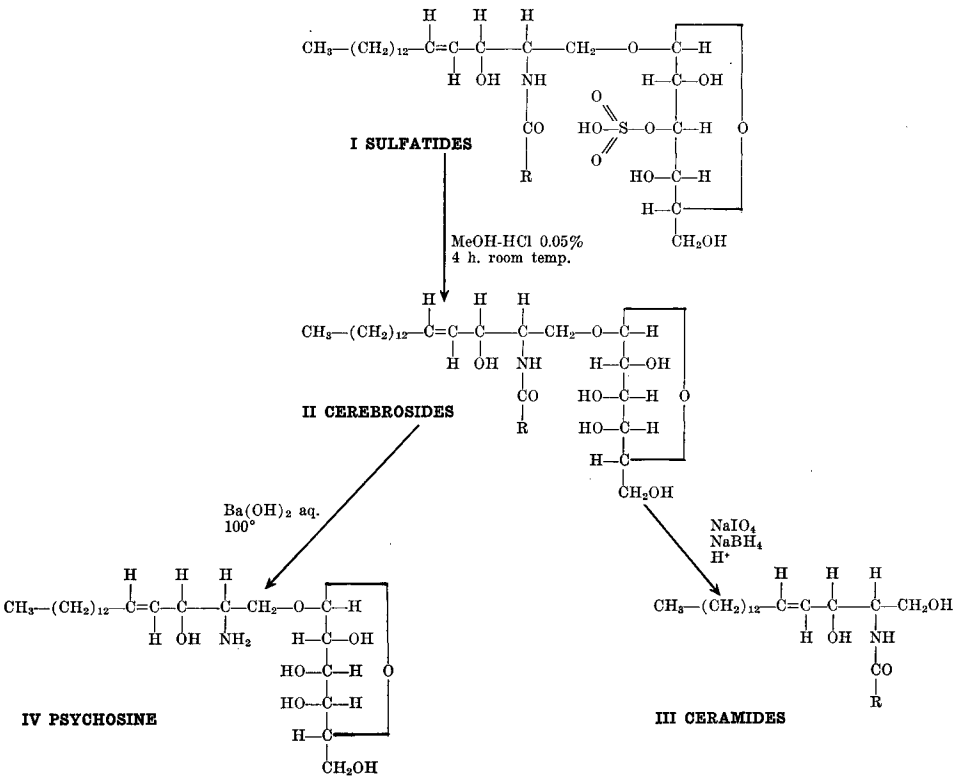

FIG. 1.

Estimation

The quantitative determination of sulfatides in the presence of other complex lipids in tissues or in tissue extracts is a problem intimately linked to their successful isolation. Indeed, none of the constituents of the sulfatides—fatty acids, sphingosine, galactose and sulfate—is specific to these compounds, and, if their estimation is based on the quantitation of one of their constituents, careful previous purification is required. Radin (28,29) has described a procedure for the determination of sulfatides, as well as of other glycolipids, which is based on the estimation of the sugar content with anthrone sulfuric acid. The sulfatides are first separated by selective retention on ion exchange resin from which they are subsequently eluted with suitable solvent. Witmer (30) has developed a method for measuring the sulfatides by their absorption at 8.02 μ in the infrared (IR) spectrum. More recently, Jatzkewitz (31) has developed a procedure for the ultramicrodetermination of brain lipids, including the sulfatides, which is based on their separation by chromatography on thin layers of silica gel followed by the determination of the hexose content with anthrone-sulfuric acid reagent directly on the silica powder scratched off the plate. Svennerholm (32) has proposed a simple method based on the estimation of the intensity of the metachromatic staining with cresyl violet of sulfatides, separated on paper chromatograms.

Rouser et al. (20) have recently described a relatively rapid method for the determination of sulfatides and other brain lipids utilizing DEAE cellulose column chromatography followed by thin-layer chromatography (TLC) and transmission densitometry of charred spots produced by heating with a sulfuric acid-potassium dichromate reagent.

Chemical Structure

Examination of Degradation Products

Since Blix (33) found cerebron-sulfuric acid to be composed of equimolar amounts of fatty acids, sphingosine, galactose and sulfuric acid, it has been assumed that sulfatides are sulfate esters of cerebrosides. All data accumulated since have confirmed this proposition. Lees (17) showed that sulfatides, like cerebrosides, contain no free amino nor reducing group, indicating that the constituent fatty acids and sphingosine are linked by an amide bond and that the galactose is attached by a glycosidic linkage. Jatzkewitz (1,12) was able to separate two types of sulfatides from brain by chromatography on paper, on cellulose columns and on thin layers of silica gel. Paper chromatographic examination of the constituent fatty acids and sphingosine bases, indicated that one of the sulfatides is a sulfate ester of phrenosine-type cerebrosides, containing hydroxylated fatty acids; the other one was recognized as a cerasine sulfate containing normal fatty acids. By desulfation with dilute methanolic HCl, Stoffyn (34) converted the two sulfatides (Fig. 1, I) in high yield into the corresponding cerebrosides (Fig. 1, II), phrenosine and cerasine, showing directly the correlation between the two groups of substances. The cerebrosides thus obtained from the sulfatides could then be degraded, on the one hand, into ceramide (35) (Fig. 1, III) by the procedure of Carter (36) involving periodic acid oxidation, reduction, and weak acid hydrolysis. On the other hand, alkaline hydrolysis of these desulfated sulfatides gave psychosine (35) (Fig. 1, IV).

TABLE I

Specific Optical Rotations

Specific optical rotations in water of the aldehydes from periodate oxidation of dihydropsychosine obtained from phrenosine and from desulfated sulfatides as compared to 1-glyceryl α- and β-D-galactosides.

Starting material	$[\alpha]_D$ of Aldehydes	
Dihydropsychosine from phrenosine	−83°	±4°
Dihydropsychosine from sulfatides	−81°	
1-glyceryl β-D-galactoside	−82°	
1-glyceryl α-D-galactoside	+51°	

Both ceramide and psychosine, derived from sulfatides in this way, were indistinguishable from the products of similar degradation of authentic cerebrosides. With the availability of gas-liquid chromatography (GLC), detailed qualititative and quantitative analysis of the fatty acid composition of sulfatides, and other lipids in general, became readily feasible with great accuracy. In this respect also, the close relationship of sulfatides to cerebrosides was indicated (37–40). The sphingosine bases present in the sulfatides have also been analyzed by TLC and by GLC according to the method of Sweeley (41), and were shown to be identical to those of cerebrosides (39) (Fig. 2).

Anomeric Configuration

A β-configuration is generally attributed to the glycosidic linkage in cerebrosides. The proof given is based on their hydrolysis by β-galactosidase (42), on the presence in the IR spectrum of a peak at 11.22 μ (43) also found in the spectrum of simpler β-galactosides (44,45), and on their synthesis by a Königs-Knorr type of condensation which usually yields the β-anomer (46). Contradictory results have been obtained, however, by their hydrolysis with α-galactosidase (47,48). In sulfatides, except for the presence of a peak at 11.22 μ in the infrared spectrum, no proof has been given supporting a β-

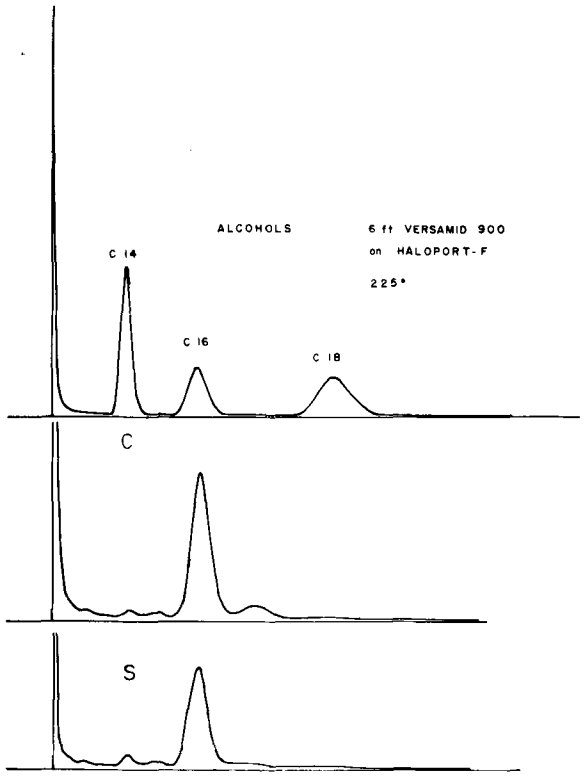

FIG. 2. Identification of the sphingosine bases of cerebrosides (C) and of sulfatides (S) by gas-liquid chromatography of the alcohols obtained by reduction with $LiAlH_4$ of the aldehydes resulting from the periodate oxidation of the long chain bases. References of C_{14}, C_{16} and C_{18} normal alcohols.

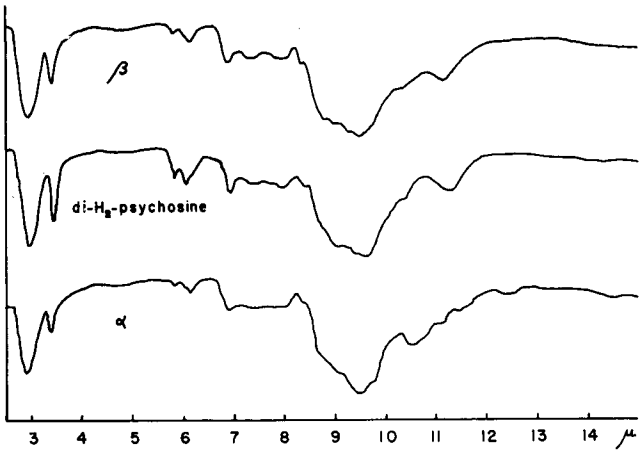

FIG. 3. Infrared spectra of periodate oxidation products of 1-glyceryl α- and β-D-galactosides compared to the oxidation product of dihydropsychosine.

configuration of the galactosidic linkage. Recently, a general method has been developed for the determination of the configuration of the anomeric center in cerebrosides (49). It is based on the comparison by specific optical rotation values (Table I), IR spectra (Fig. 3) and chromatographic behaviour (Fig. 4) of the products of periodate oxidation of dihydropsychosine (Fig. 5, I) with the trialdehyde (Fig. 5, II) obtained under identical conditions from 1-D-glyceryl α- and β-D-galactosides (Fig. 5, III) of known configurations (50). Thus, a β-configuration of the galactosidic linkage in phrenosine has been confirmed. When the method was applied to the cerebrosides obtained by desulfation of sulfatides (34), identical results were obtained, demonstrating the existence of the β-configuration in sulfatides as well (51).

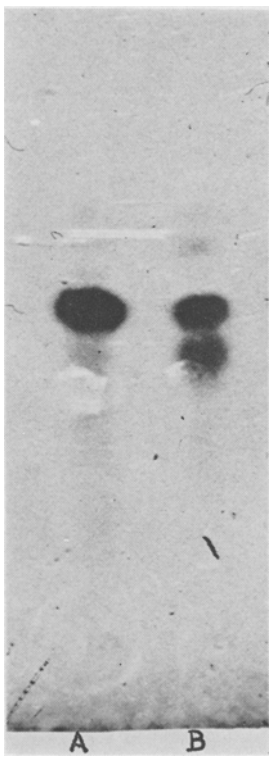

FIG. 4. Chromatogram on thin layer of silica gel of the aldehydic products of periodate oxidation of dihydropsychosine (A) and of 1-glyceryl β-D-galactoside (B). (Detection with Fuchsin-SO_2).

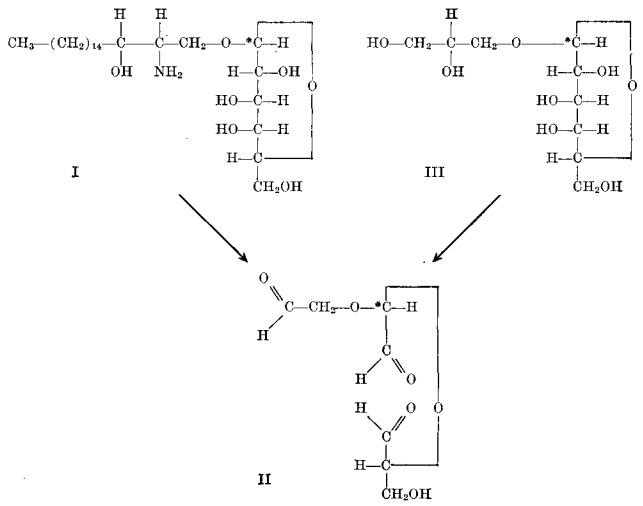

FIG. 5.

Position of the Sulfate Group

The position of the sulfate group in sulfatides, which has been assumed for many years to be at C-6 of the galactose moiety (33,52–54), has been recently reexamined. Yamakawa (55) found that, contrary to phrenosine, sulfatides are resistant to periodic acid oxidation. After methylation of sulfatides and subsequent methanolysis, Yamakawa characterized the resulting methylated galactoside fraction as methyl 2,4,6-trimethyl galactoside by GLC, thus showing that the sulfate group is attached at C-3 of the galactopyranose moiety of sulfatides. Stoffyn (56) observed that sulfatides are very stable under strongly alkaline conditions. This behaviour was unexpected since C-6 linked sulfate esters of galactopyranosides are known to be unstable under alkaline conditions and to form readily 3,6-anhydro derivatives (57). Methylation was also performed by Stoffyn (56), and after methanolysis and hydrolysis of the galactoside fractions, 2,4,6-trimethyl galactose was identified by chromatography on paper impregnated with dimethyl sulfoxide (58), by the formation of its crystalline anilide, and more recently, by a new TLC procedure giving a very neat separation of isomeric trimethyl galactoses (59) (Fig. 6). It is interesting to note that in all preparations of sulfatides which have been studied by methylation up to the present time, the sulfate group appears to be located only at C-3 of the galactose moiety. Indeed, 2,4,6-trimethyl galactose only has been detected after methylation and no trace of 3,6-anhydro galactose dimethyl acetal could be detected in methanolysates of sulfatides previously treated with hot sodium methoxide.

Hakomori (13), however, has observed the separation by paper chromatography of a sulfatide preparation into two isomeric fractions. Although these two fractions, I and II, have similar fatty acid composition, give identical IR spectra and are indistinguishable by chromatography on thin layers of silica, they were found to differ appreciably in their hexose to sulfate ratio. Sulfatide II appeared to contain a significant proportion of sulfate not titrated with alkali or with toluidine blue. Moreover, a large proportion of 2,4-dimethyl galactose was found in the hydrolysate of permethylated sulfatide II. These facts led Hakomori to propose for sulfatide II a hybrid structure containing probably sulfate diester bridges

linking several units together between C-3 and C-6. In connection with these challenging observations, it is interesting to recall the striking influence of the nature of the solvents on the course of the methylation of sulfatides with methyl iodide and silver oxide (56). With these reagents alone, methylation was favored at C-6 and 6-methyl galactose could be isolated in high yield from hydrolysates of partially methylated sulfatides. In the presence of dimethyl formamide (60), on the other hand, 2-methyl galactose was the main product in the hydrolysate. This change of preferential orientation of the methylation under the influence of the solvent is not observed with simple α- or β-methyl galactosides. At any rate, the complete methylation of sulfatides could not be achieved by many repetitions and combination of these two procedures. Recently, sulfatides were methylated by Stoffyn (61) and Malone (62) with methyl iodide and sodium hydride in dimethyl sulfoxide, according to a new and remarkable method of methylation proposed by Hakomori (63). Complete methylation was achieved in one short operation and 2,4,6-trimethyl galactose was the only methylated carbohydrate detectable in the hydrolysate. The absence of dimethyl galactoses indicated complete methylation and the absence of tetramethyl galactose showed that there was no loss of the sulfate group during the methylation. The existence of C-3 to C-6 diester bridges could be compatible with the methylation results only by assuming that the link at C-6 is very labile toward alkali (all methylations are done under alkaline conditions) while the link at C-3 is very stable. It may be that small amounts of impurities are responsible for the behavior of sulfatide II of Hakomori. These might be phosphatides which have been shown to alter so markedly the reactivity of sulfatides (17). Clarification of these interesting properties of sulfatides will require more experimental work.

To terminate this discussion of the chemical structure of sulfatides, a point of great interest which must be mentioned is the recent report by Mårtensson (64) describing the isolation of a ceramide dihexoside sulfate containing equimolar amounts of glucose and galactose from kidney. This sulfatide is thus apparently closely related to a number of glycolipids containing several hexose units which have been characterized recently in kidney by Mårtensson (65) and by Makita (66).

Sulfatides in Metachromatic Leukodystrophy

In search for a metabolic defect responsible for this demyelinating condition, analysis of the fatty acids from the sulfatides, the cerebrosides and the sphingomyelins isolated from MLD brain were carried out by O'Brien (67). He found that the sulfatides, accumulated in the diseased brain white matter, retain their normal fatty acid distribution (with C_{24} being preponderant), whereas in cerebrosides and sphingomyelins a much larger proportion of short chain fatty acids was found (C_{18}). Malone (62) has established by methylation studies that the sulfate group in brain sulfatides obtained from MLD cases is located only at C-3 of the galactose moiety showing that, in this respect also, the sulfatides have a normal structure in this disease. More recently, similar studies have been carried out by Malone (68) on sulfatides extracted from the kidneys of patients with MLD. Again, the sulfate group was found to be located only at C-3 of the galactose in the diseased as well as in the normal human kidneys (66,68). The distribution of fatty acids in MLD kidney sulfatides was also found to be similar to that of normal kidney (68) which, interestingly enough, is characterized by a very large proportion of C_{22} acids and thus differs in that respect from brain sulfatides. A large proportion of C_{22} acids has been previously reported by Mårtensson (65) in normal kidney neutral glycolipids and by Sweeley in a case of Fabry's disease (69). In conclusion, in MLD, the sulfatides accumulated in brain and in kidney retain both the structure and the organ-specific distribution in fatty acids they have in normal tissues (Fig. 7).

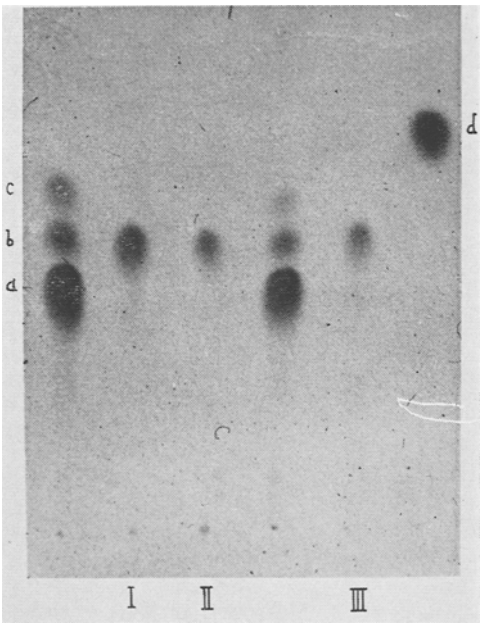

FIG. 6. Chromatogram on thin layer of silica gel of the methylated galactose from permethylated sulfatides from normal brain (I), MLD brain (II) and MLD kidney (III). Compared to 2,3,4 (a), 2,4,6* (b), 2,3,6** (c) trimethyl galactoses and 2,3,4,6-tetramethyl galactose.[4] Solvent system acetone-water-concentrated ammonium hydroxide (250-3-1.5 v/v). * Gift of Dr. F. Smith; ** gift of Dr. R. W. Jeanloz.

Conclusions

Sulfatides occupy a particular place among polar complex lipids by the presence in their molecule of a sulfate ester group attached to a carbohydrate moiety. They share this characteristic with the widely distributed mucopolysaccharides of connective tissue and with a number of sulfated polysaccharides present in large amounts in seaweeds. It is interesting to note that, at least on the basis of work done to the present time, the sulfate group has been found to be located only at the C-3 position of galactose in sulfatides.

From the information discussed above, the picture emerges of sulfatides having a chemical composition and structure indeed identical to the cerebrosides except for the sulfate ester group. The fact that the differences observed in the fatty acid composition of brain and kidney cerebrosides are paralleled by identical differences in the sulfatides of those organs, tends to emphasize even more the close relationship between the two groups of substances. The pathways of biosynthesis and degradation of sulfatides are, however, not yet completely elucidated (70–72). It is not definitively known if sulfatides are synthesized by sulfation of cerebrosides or if both cerebrosides and sulfatides derive from a common precursor.

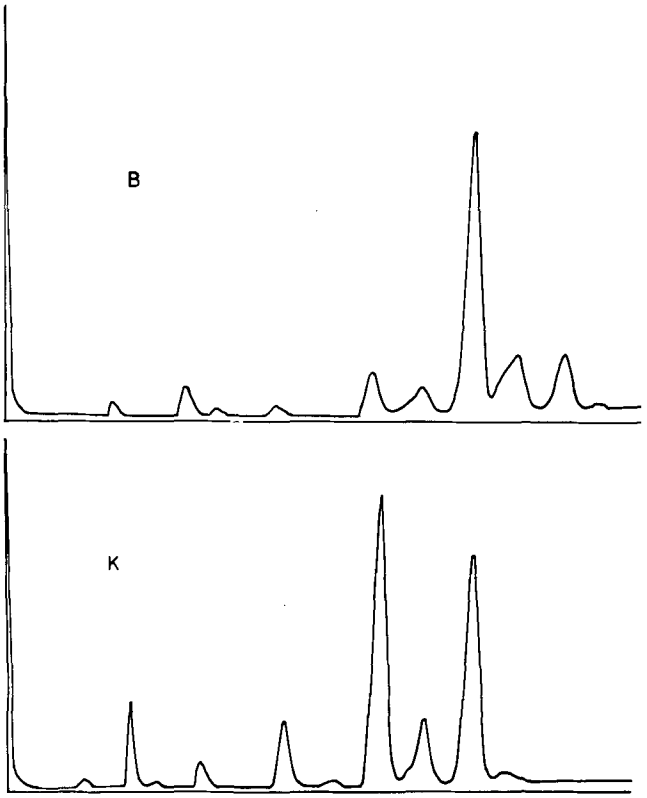

FIG. 7. Patterns of fatty esters of sulfatides from MLD brain (B) and kidney (K). Similar patterns are obtained from normal material.

O'Brien has hypothesized the lack of a system for the elongation of the fatty acid chains in the cerebrosides as a possible explanation for the breakdown of myelin in metachromatic leukodystrophy. The presence of a larger proportion of shorter chain fatty acids in cerebrosides in metachromatic leukodystrophy has recently, however, been attributed by Ställberg-Stenhagen (73) to the changed ratio between axoplasmic tissue and myelin in a demyelinating condition. Of considerable interest is the recent isolation by Mehl (74) of a cerebroside sulfatase. The deficiency of this enzyme appears to be related to the accumulation of sulfatides in metachromatic leukodystrophy and could be the cause of the disease.

Thus, in the last few years, noticeable progress in the chemistry of sulfatides has been made. Their structure seems now to be firmly established. Great recognition must be given to the recently developed microtechniques of TLC and GLC which have made possible the rapid progress in this particular field, as in many others. The detailed analysis of the constituent fatty acids has revealed the similarities in the composition of glycolipids, suggesting their metabolic relationship. On the other hand, attention has been attracted to differences occurring in disease states. This has opened the way for new investigations.

ACKNOWLEDGMENTS

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