A Microbial Sulfolipid. II. Structural Studies*

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ABSTRACT: A new sulfolipid, 1,14-docosyl disulfate, was isolated from the phytoflagellate, *Ochromonas danica*, by solvent extraction, chromatography, ion-exchange gel filtration, and crystallization. Acid hydrolysis of the sulfatide produced a diol. The structures of the sulfatide and the diol were determined by analysis, infrared spectroscopy, proton magnetic resonance spectrometry, and mass spectrometry.

The mass spectra of the analogous diols, eicosane-1,4-diol and octadecane-1,12-diol, were studied. Eicosane-1,4-diol was synthesized *via* 1-hydroxy-4-eicosanone.

The polar lipids isolated from biological tissues to date have had one structural characteristic in common. They generally consist of a long aliphatic chain of at least 14 methylene carbons with a polar functional group at *one end*. The polar group is commonly phosphate, glycerol, inositol, a glycoside, an amine, sulfate, or a combination of these. To date no lipids present in significant quantity have been reported which have such polar groups at *both ends* of the molecule. This generalized structure for lipids has formed a basis for the bimolecular leaflet theory of membrane structure originally proposed by Danielli and Davson (1935) and later modified and extended by Robertson (1958), Vandenheuvel (1963), Stoeckenius (1964), and others (*e.g.*, Kavanau, 1964). Benson and Singer (1965) have proposed an entirely different model for the molecular structure of biological membranes. They proposed a lipoprotein matrix in which lipids were attached to proteins by hydrophobic bonding and the properties of the membrane could be derived from the lipoprotein. This hypothesis was later expanded by Green *et al.* (1967), who proposed that the lipoproteins were actually enzymes and that they were grouped into macromolecular repeating units in which a coordinated series of chemical reactions could occur.

This report deals with the structure of a new sulfolipid. The substance appears to be 1,14-docosyl disulfate. It is the third sulfolipid to be characterized to date, and the first aliphatic sulfate. Mammalian sulfolipid, cerebroside sulfate, was first characterized by Blix (1933), but its structure was not completely elucidated until 1962 (Yamakawa *et al.*, 1962). A chloroplast sulfonolipid[1] (6-sulfo-α-D-quinovopyranosyl-(1–1′)-2′,3′-di-*O*-acyl-D-glycerol) was characterized by Benson *et al.* (1959). 1,14-Docosyl disulfate has been identified in a wide variety of microbes ranging from *Pseudomonas* sp. (sea water bacterium) to *Tetrahymena pyriformis* (Haines, 1965).

Experimental Procedure

Materials. All reagents were analytical grade. Solvents were distilled before use. Octadecane-1,12-diol (K & K Laboratories) was recrystallized first from hexane and then from methylene chloride before use (mp 72–72.5°, lit. (Shinozaki and Kagawa, 1937) mp 71.2°). Pure sodium dodecyl sulfate was the generous gift of Dr. H. L. Rosano. γ-Butyrolactone and 1-bromohexadecane (K & K Laboratories) were vacuum distilled before use. Pronase, a proteolytic enzyme excreted by *Streptomyces griseus* (Nomoto *et al.*, 1960), was obtained from Enzyme Development Corp.

Isolation of the Sulfatide. Ochromonas danica was cultured on defined medium as described earlier (Haines and Block, 1962). The procedure for isolation of the sulfatide from large batches of media (approximately 26 l.) was also described in a previous publication (Haines, 1965). A brief summary of this procedure follows.

Cells were collected by centrifugation and extracted three times with 20 volumes of chloroform–methanol (2:1, v/v). The solvent was removed *in vacuo* and the residue was saponified in 0.2 N potassium hydroxide in methanol for 45 min at 37°. The solution was diluted with an equal volume of water, acidified, and extracted with chloroform until no further pigment was extracted. The water layer was then diluted with an equal volume of 2 M potassium chloride and extracted three times with 1-butanol. The 1-butanol was removed *in vacuo*.

The product thus obtained was found to be a pro-

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[1] The term *sulfolipid* in this paper means a sulfur-containing lipid; *sulfatide* is a lipoid sulfate ester; *sulfonolipid* is a sulfolipid in which the sulfur is found as a sulfonic acid.

teolipid. The protein was removed by digestion with pronase as described earlier (Haines, 1965). The sulfatide was extracted from the digestion mixture with 1-butanol and the 1-butanol was removed *in vacuo*.

Ion-Exchange Gel Filtration. A column (6 × 2 cm) of Sephadex SE-25 (3 g) (Pharmacia Fine Chemicals) suspended in 0.16 M potassium phosphate buffer (pH 7.5) was prepared. Crude sulfolipid (1.75 g) in 7 ml of water was placed on the column. Water (3 ml) and buffer (6 ml, pH 7.5) were used successively to wash the material onto the column. The sulfolipid was eluted with further addition of phosphate buffer (pH 7.5). Fractions were monitored with thin layer chromatography (tlc). Those containing the sulfatide were combined. The sulfolipid was extracted with 1-butanol and the 1-butanol was removed *in vacuo*. The product (2.5 g) was contaminated with sodium phosphate.

Thin Layer Chromatography. Chromatograms, consisting of thin layers (250 μ) of magnesium silicate with 10% calcium sulfate binder (Adsorbosil M-1, Applied Science Laboratories, State College, Pa.), were *not* activated. The developing solvent was chloroform–methanol–water (65:33:2, v/v). Plates were run for 45 min (7 cm) and visualized by spraying with 2,7-dichlorofluorescein (0.2% in methanol). Before pronase digestion the sulfolipid chromatographed as a single spot (R_F 0.95). After digestion with pronase the R_F was 0.9.

Chromatography on Magnesium Silicate. A column (13.5 × 2 cm) of magnesium silicate (Adsorbosil M-2) (15 g) in chloroform–methanol (2:1, v/v) was charged with the sulfatide from the gel filtration. The sulfatide was eluted with the same solvent and collected in 5-ml fractions. The fractions were monitored with tlc. Fractions containing pure sulfatide were combined and the solvent was removed *in vacuo*.

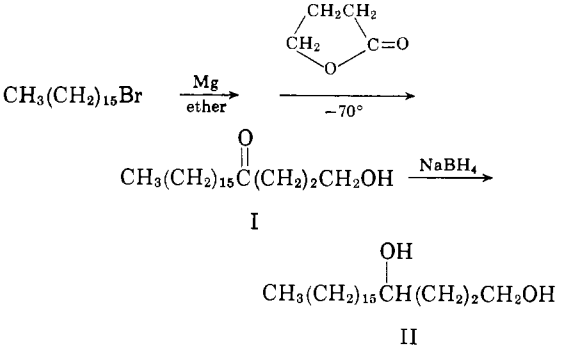
The material was dried *in vacuo* at 60°. The substance was a faint green and weighed 594 mg. This green contaminant was removed by dissolving the sulfatide in a minimum volume of absolute ethanol and then precipitating it by the addition of 20 volumes of anhydrous diethyl ether. This precipitation was repeated twice. The product, an off-white fine powder, was dried *in vacuo* at 60° (weight, 176 mg).

Hydrolysis of the Sulfatide. The sulfatide was dissolved in 1 ml of 1 N hydrochloric acid. The solution was maintained at 100° for 2 hr. The brown oil which formed was extracted with diethyl ether, dried over sodium sulfate, and evaporated *in vacuo*. The residue was chromatographed on tlc using diethyl ether–petroleum ether (bp 30–60°, 30:70, v/v) as the developing solvent. At least six spots were obtained. The bulk of the material, however, chromatographed as a single compound in this system. The system does not generally separate compounds of varying chain length or distinguish between compounds which differ in the position of a functional group on the chain. The major component cochromatographed with 1,12-octadecanediol, which suggested that it, too, was a long-chain aliphatic diol. This did not, of course, indicate the chain length or position of the hydroxyls, nor did it prove the compound was a diol. It did, however, permit us to tentatively assume that the compound was a diol. The appearance of unknown spots in the tlc of the hydrolysate suggested either that the sulfatide preparation was not pure or that the hydrolysis procedure caused breakdown of the organic moiety.

Column Chromatography of the Sulfolipid Diol. A column (18 × 2 cm) of magnesium silicate (Adsorbosil M-2) (15 g) in diethyl ether–petroleum ether (3:7, v/v) was charged with crude diol (150 mg) from the sulfatide hydrolysis. The column was eluted with the same solvent. The eluent was collected in 3-ml fractions which were monitored with tlc. Fractions containing pure diol were combined and evaporated *in vacuo*. The product was recrystallized from hexane and then from carbon tetrachloride. The crystals were fine white feathers (25 mg), mp 87–87.5°.

Analyses and Spectra. The elemental analyses were carried out by Schwartzkopf Microanalytical Laboratories, New York, N. Y. The infrared spectra were determined on potassium bromide wafers using an Infracord Model 137. Samples were prepared by drying at 60° in a vacuum oven. The nuclear magnetic resonance (nmr) spectrum was determined with a Varian A-60 spectrometer on a deuterium oxide solution. Mass spectra were obtained by Morgan Schaffer Corp. on an Hitachi mass spectrometer using the indirect inlet at 250°. Melting points are corrected.

SCHEME I



Synthesis of 1,4-Dihydroxyeicosane. 1-HYDROXY-4-EICOSANONE (I) (SCHEME I). Hexadecyl bromide (101 g, 0.33 mole) dissolved in 100 ml of anhydrous diethyl ether was added dropwise at room temperature to 8.0 g (0.33 g-atom) of magnesium in 200 ml of anhydrous ether. The suspension was stirred vigorously under nitrogen for 2 hr. Upon completion of the reaction, the mixture was diluted with 250 ml of anhydrous ether. The temperature was lowered to −65° and maintained at that temperature throughout the addition (2 hr) of 57 g (0.66 mole) of freshly distilled butyrolactone in 100 ml of anhydrous diethyl ether. The temperature was permitted to rise over 16 hr to −10°. A white precipitate formed when water (100 ml) was added with vigorous stirring. After 30 min the supernatant was decanted and the white precipitate was

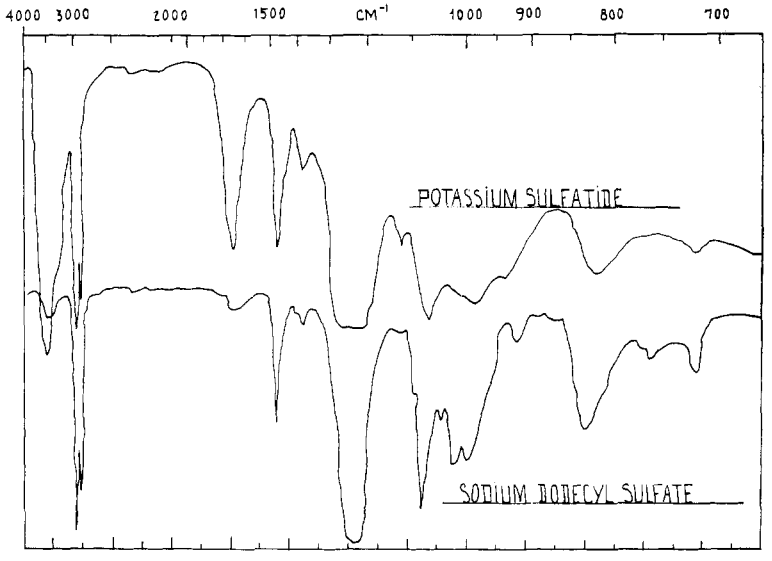


FIGURE 1: Infrared spectrum of the potassium salt of the sulfolipid and of sodium dodecyl sulfate. Each spectrum was taken of a potassium bromide wafer with an Infracord Model 137.

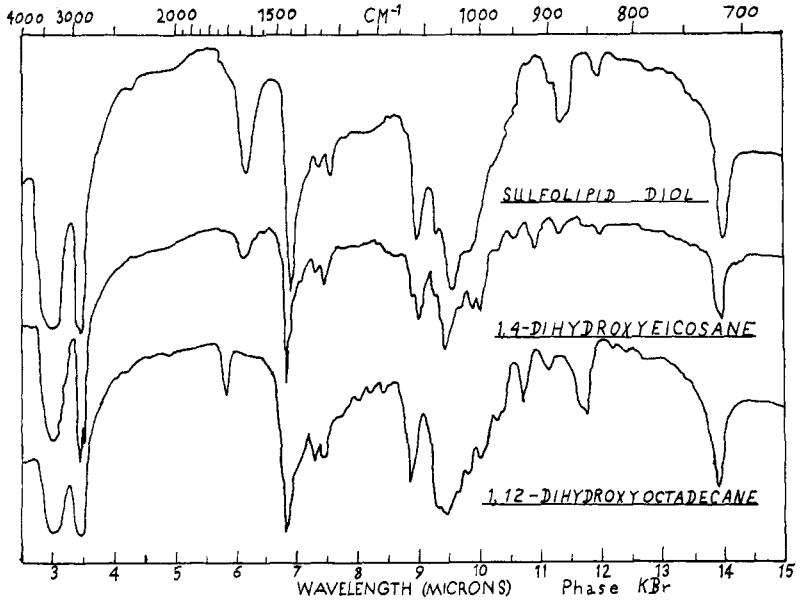


FIGURE 2: Infrared spectra of the sulfolipid diol. The spectra of 1,4-eicosanediol and 1,12-octadecanediol are shown for comparison. The spectra were taken of potassium bromide wafers with an Infracord Model 137.

washed with 100 ml of diethyl ether. The combined ether extracts were evaporated *in vacuo*. To the residue was added 250 ml of methanol and 250 ml of 6 N potassium hydroxide. The suspension was stirred at 37° for 90 min. After the addition of 250 ml of water the solution was extracted twice with 500 ml of chloroform. The chloroform was dried over sodium sulfate and the solvent was removed *in vacuo*. The residue was crystallized from hexane (600 ml) at 5° yielding 45.0 g (43% based on hexadecyl bromide) of crystalline 1-hydroxy-4-eicosanone (I) (mp 70–71°).

Anal. Calcd for $C_{20}H_{40}O_2$ (312): C, 76.92; H, 12.82. Found: C, 77.01; H, 12.79.

The infrared spectrum of I (in carbon tetrachloride) showed the expected absorption bands for OH (3100–3500 cm^{-1}), CH_2 and CH_3 (2940, 2870, 1465, 1380, 720 cm^{-1}), C=O (1720 cm^{-1}), and alcohol CO (1060 cm^{-1}).

EICOSANE-1,4-DIOL (II). Into 100 ml of tetrahydrofuran was placed 0.6 g (0.016 mole) of sodium boro-

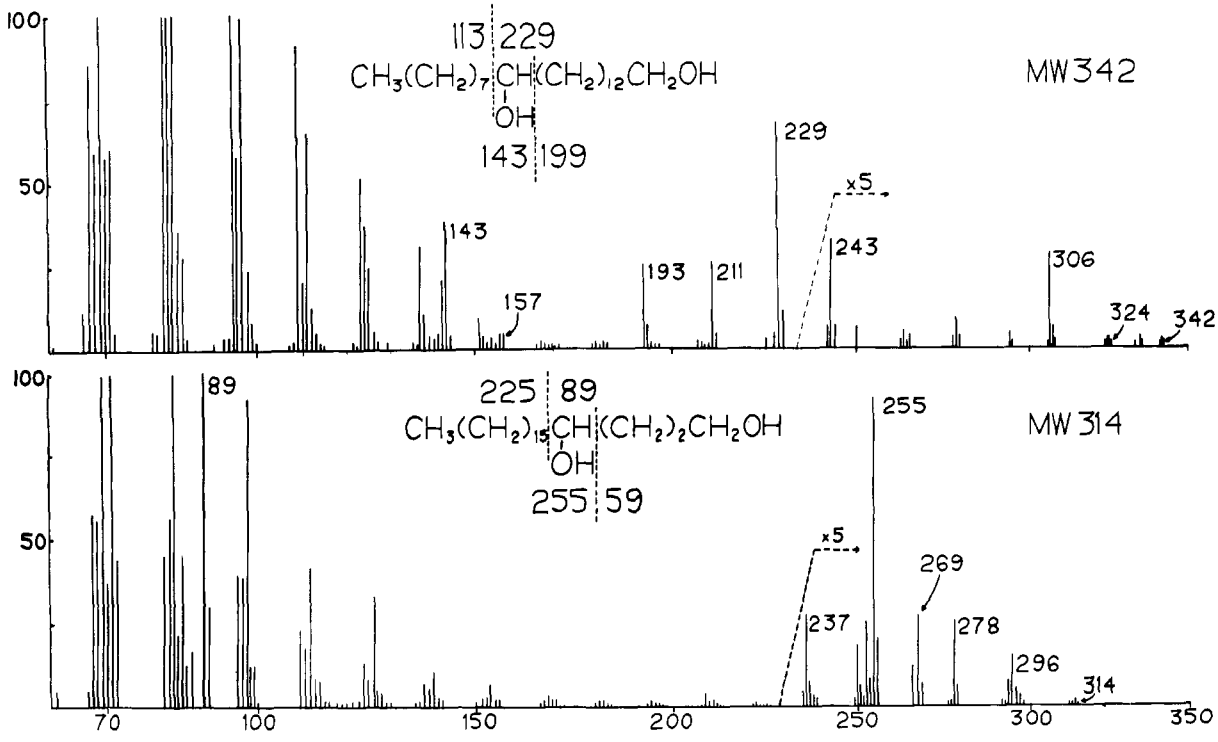


FIGURE 3: Mass spectrum of the sulfolipid diol. Spectrum of 1,4-eicosanediol is shown below for comparison. For an explanation of the numbered peaks see text and Table I. Spectrum was taken by Morgan–Schaffer Corp., Montreal, Canada, on an Hitachi mass spectrometer using the indirect inlet at 250°.

hydride and 5.0 g (0.016 mole) of I. After refluxing for 3 hr the solution was diluted with 250 ml of water and acidified with 2 N sulfuric acid. The aqueous solution was extracted twice with 100 ml of diethyl ether. The combined ether extracts were dried over magnesium sulfate and concentrated *in vacuo* to dryness. The residual oil was crystallized from hexane at 5° and then from carbon tetrachloride. The diol II (3.9 g) was obtained as needles, mp 78–79°. The product chromatographed as a single spot on tlc using diethyl ether–petroleum ether (30:70, v/v) (R_F 0.15).

Anal. Calcd for $C_{20}H_{42}O_2$ (314): C, 76.43; H, 13.38. Found: C, 76.41, H, 13.40.

The infrared spectrum of II (shown in Figure 2) is consistent with the structure.

Results

Analysis of the Sulfolipid. Calcd for $C_{22}H_{44}S_2O_8K_2 \cdot 2H_2O$ (578): C, 42.94; H, 7.80; S, 10.40. Found: C, 42.56; H, 7.90; S, 10.51.

The infrared spectrum of the purified sulfatide is shown in Figure 1. The spectrum is nearly identical with that of sodium dodecyl sulfate with two important differences. The first is a shoulder at 926–945 cm^{-1} which was reported by Hummel (1962) as characteristic of secondary sulfates. The peak at 830 cm^{-1} is characteristic of primary sulfates and is absent from secondary sulfate esters. A second difference between the sulfatide and dodecyl sulfate infrared spectra is the presence of intense water peaks at 3500 and 1650 cm^{-1}. These peaks justify the inclusion of at least two molecules of water in the calculated elemental analysis. Attempts to remove this water were unsuccessful.

The nmr spectrum in deuterium oxide showed a sharp peak at τ 8.7, typical of aliphatic methylene. A triplet at τ 9.1 was typical of a terminal methyl on a chain. A triplet which appeared at τ 6.5 was identified with the methylene group of the primary sulfate ester. Integration indicated that the triplet contained the proton on the carbon of the secondary sulfate. It was not possible, however, to determine either the splitting pattern or the precise chemical shift of this proton.

Analysis of the Sulfatide Diol. Calcd for $C_{22}H_{46}O_2$ (342): C, 77.19; H, 13.45. Found: C, 76.46; H, 13.59.

The infrared spectrum of the sulfatide diol is shown in Figure 2. The spectrum is clearly that of a long-chain diol. Especially indicative are the peaks at 1060 (primary alcohol CO), 1120 (secondary alcohol CO), and 718 cm^{-1} (long-chain aliphatic CH_2 rocking). The mass spectrum of the diol is presented in Figure 3. The results are tabulated in Table I.

Discussion

The synthesis of eicosane-1,4-diol was based on a novel reaction. The reaction was patterned after the

TABLE I: Mass Spectra of Diols.

1,12-Dihydroxyoctadecane	1,4-Dihydroxyeicosane	Sulfatide Diol	Peak Designation	Ion
				OH | $CH_3(CH_2)_xCH(CH_2)_yCH_2OH^{\cdot+}$
286	314	342	M[a]	
268	296	324	M − H_2O	
250	278	306	M − $2H_2O$	
201	89	229	B[b]	$HOCH_2(CH_2)_yCHOH^+$
215		243	BCH_2	
183	71	211	B − H_2O	
165	53	193	B − $2H_2O$	
				OH | $CH_3(CH_2)_xCH^+$
115	255	143	A[c]	
129	269	157	ACH_2	

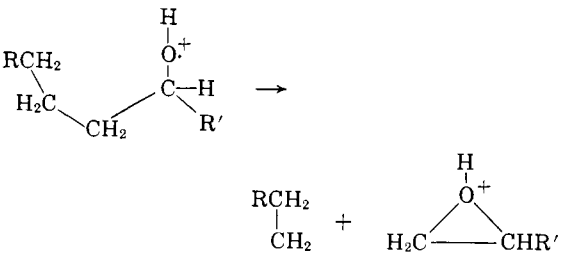
[a] M = molecular ion. The molecular ion contains x methylene groups between the terminal methyl group and the secondary hydroxyl group and y methylene groups between the secondary hydroxyl group and the primary hydroxyl group. x and y are determined from the structure of the diol. [b] B = fragment resulting from cleavage of the CC bond adjacent to the secondary hydroxyl and in the direction of the terminal methyl group. [c] A = fragment resulting from cleavage of the CC bond adjacent to the secondary hydroxyl and in the direction of the primary hydroxyl group.

low-temperature Grignard reaction with anhydrides (Newman and Smith, 1958) which yields keto acids. The success of the reaction appears to be based on the insolubility of the product (magnesium alkoxide) at −65° as reaction of butylmagnesium bromide with ethyl acetate at this temperature produced no detectable methyl ketone. Although Fujimoto (1951) reported a methyl ketone resulting from the reaction of steroid lactone and methylmagnesium iodide the structure of this product was later found to be a tertiary alcohol at the site of reaction (Zwahlen *et al.*, 1957).

Mass Spectra of Aliphatic Diols. The mass spectra of secondary alcohols were first studied by Friedel *et al.* (1956). They found that it was possible to determine the position of a secondary hydroxyl on an aliphatic chain by the presence of fragments in the spectrum which were derived from cleavage on either side of the secondary carbon. The fragment which contained the hydroxyl was especially stable. In addition a fragment consistently appeared in these spectra that showed cleavage in the β position in which the hydroxyl was retained. In a study of the mass spectra of hydroxy fatty esters Ryhage and Stenhagen (1960) have obtained similar data. The fragments representing β cleavage were not especially noticeable.

Ryhage *et al.* (1957) also studied the spectrum of eicosane-2,4-diol as a model in determining the structure of phthiocerol. In addition to fragments resulting from cleavage on either side of carbon four they found an unusually intense peak, a C_{15}, implying a β cleavage. Cleavage at other carbons are not distinguishable from cleavage at the carbon bonded to the hydroxyl.

The Mass Spectrum of Eicosane-1,4-diol (II). The mass spectrum of eicosane-1,4-diol (II) is shown in Figure 3. The assignment of the peaks can be seen in Table I. Major peaks at masses 89 and 255 arise through initial cleavage of the molecular ion at the bonds adjacent to the secondary hydroxyl group. As was the case with the other diols, the alkyl ion peaks did not appear in the spectrum. The peak at mass 269 is an example of β cleavage probably stabilized with a bridged oxygen. The molecular ion peak had



low intensity in all three spectra but the peak for M − $2H_2O$ was present in high concentration and together with M − H_2O forms a good basis for the identification of a diol.

The mass spectrum of the sulfatide diol is shown in Figure 3 and summarized in Table I. The peak at mass 342 represents the peak of highest mass and is the molecular ion peak. Peaks at masses 324 and 306 justify the diol assignment and confirm the molecular weight as 342. The major peaks of masses 143 and 229 indicate that the secondary hydroxyl is at C_{14}.

The peaks at masses 211 and 193 show that the fragment of mass 229 contains two hydroxyl groups. The peak at mass 243 is apparently owing to the β cleavage previously described. The absence of peaks (other than those mentioned) which interrupt the normal alkane pattern of fragmentation shows no branching in the chain. The absence of major peaks separated by 14 or 28 mass units indicate that the substance is a single compound rather than a mixture of diols of varying chain length. Thus, the mass spectrum of the diol obtained by acid hydrolysis of the sulfatide shows that the diol is docosane-1,14-diol. The infrared spectrum and elemental analysis support this view. Elemental analysis of the sulfatide indicates that there are two sulfate groups to 22 carbons. The infrared spectrum (Figure 1) of the sulfolipid shows that one is secondary (926–945 cm^{-1}) and that the other is primary (995 cm^{-1}). It is, therefore, concluded that the sulfatide is 1,14-docosyl disulfate. It was not possible to obtain the optical rotation of the sulfatide as the preparation was too colored in solution. This problem is currently under study.

The sulfatide is largely bound to protein in the cell (Haines, 1965). Isolation by solvent extraction as reported herein gave a sulfatide–protein complex that chromatographed as a single spot on tlc. Due to its solubility in organic solvents, it may be referred to as a proteolipid (Folch and Lees, 1951).

In experiments reported elsewhere the sulfolipid has been located in a density gradient band that appears to be that of the cell membrane (T. H. Haines, K. Kahn, and G. L. Mayers, manuscript in preparation). These experiments show that this substance, which represents the bulk of the sulfur of the cell (Haines and Block, 1962), is located almost entirely in a single membrane band!

The bilipid leaflet of Danielli and Davson (1935) is not stable for short chains of eight carbons or less. The sulfatide has two sulfate groups. The secondary is eight carbons from the terminal methyl group and the "loop" formed by the methylenes between the two sulfates would extend less than eight carbons into the bilipid leaflet. This compound could not, therefore, contribute substantially to the stability of a bilipid leaflet. Either the bilipid leaflet is not a satisfactory description of the membrane or the sulfolipid although primarily present in the one membrane is a relatively minor component of the membrane. The latter does not appear to be the case, but is currently under investigation. The former suggests that the lipoprotein matrix of membrane structure first described by Benson and Singer (1965) and later by Green *et al.* (1967) is a more accurate description of this membrane.

A long-chain alkyl sulfate has not previously been reported in nature. Such compounds have been synthesized commercially for use as synthetic detergents for many years. Their use has increased as a result of recent problems introduced by the nonbiodegradability of the branched alkylbenzenesulfonates. The natural sulfatide, judging from its behavior in the laboratory, is a potent detergent. It is assumed that a compound produced by nature is degraded by nature. It has been found (Haines, 1965) that the substance is synthesized by *Ochromonas malhamensis*, *Chlorella pyrenoidosa* (Van Neil), *T. pyriformis*, *Chlamydomonas*, *Pseudomonas* (sea water bacterium), and *S. griseus*. Thus it appears that new sulfolipid is ubiquitous in microorganisms.

Acknowledgment

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Effects of Experimental Galactosemia on the Measured Serotonin Receptor Activity of Rat Brain*

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ABSTRACT: Serotonin receptor activity was measured by a pharmacological assay which uses a rat stomach strip. Rats were made galactosemic by incorporating galactose as 40% of the total diet, from 19 to 80 days of age. Fractions isolated from the brains by chromatography were measured for serotonin receptor activity, and the galactosemic brains were found to contain less than 10% as much activity as controls. The activity was found in the galactolipid fraction. Chemical comparison of the fractionated galactolipids showed no significant differences in composition or quantity between the two groups of rats, except in the purified fractions which showed receptor activity. Purification of the receptor activity from normal brains yielded an active preparation from the ganglioside fraction which was not observable in the brains of galactosemic rats. Effects of galactosemia on the carbohydrate content of some of the minor galactolipids from the crude receptor fraction were also observed. It was concluded that galactosemia prevented the synthesis of normal serotonin receptor.

The possible relationship of this effect to the mental retardation of untreated human galactosemics was discussed.

The metabolic defect in galactosemia has been known for some time to be a genetic lack of the enzyme, galactose 1-phosphate uridyl transferase, such that galactose and galactose 1-phosphate accumulate in the tissues (Kalckar and Maxwell, 1958). The chemical explanation of the idiocy which results from the high levels of galactose and its derivatives in the tissues has not been found, aside from the demonstration that it is these excesses which provoke the mental failure. Several years ago, Woolley (1962) suggested that a deficiency of serotonin receptors might be the cause. It was later shown (Woolley and Gommi, 1964) that rats, made galactosemic by long-continued feeding of excessive galactose from 19 days to 11 weeks of age, had a specific deficiency of serotonin receptors in their stomachs. The specificity of the deficiency was indicated by the finding that the responsiveness to acetylcholine was normal. Responses to both acetylcholine and serotonin by uterus were unaffected by the galactosemia.

This work was followed by the development of a bioassay for serotonin receptor through the use of normal rat stomachs in which the receptor had been specifically damaged by neuraminidase and EDTA (Woolley and Gommi, 1966). Once it was possible to compare substances for receptor activity, it was shown that the most active materials were obtained from ganglioside preparations (Woolley and Gommi, 1965). Because gangliosides are galactolipids, they might be affected by derangements of galactose metabolism.

The present work was undertaken to learn whether the serotonin receptor of brain was demonstrably affected by galactosemia, and whether the amounts or composition of other galactolipids in brain were influenced by the disease. It was found that the major galactolipids were not significantly affected by the disease, but that a ganglioside fraction which contained receptor activity in the controls was affected. The lipid with the highest activity in the receptor assay was absent from galactosemic brains. Some other, minor lipids from this fraction also were found to differ in amount and composition between the two groups.

Experimental Section

Treatment of Rats. Female, 19-day-old albino rats from Carworth Farms were used. Groups of 15 rats were given either the highly purified diet of Sterling and Day (1951) (controls) or the same diet in which D-galactose replaced sufficient glucose to account for 40% of the total diet (galactosemics). Rats were individually caged on screen floors and fed the experimental diets *ad libitum* for at least 8 weeks. The con-

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