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A Microbial Sulfolipid. I. Isolation and Physiological Studies*

THOMAS H. HAINES

Department of Chemistry, City College of The City University of New York, New York, N. Y. 10031

SYNOPSIS. A sulfolipid, isolated from the phytoflagellate, *Ochromonas danica*, and from its medium, constitutes over 50% of the sulfur of the cells. It is bound to protein and excreted by the phytoflagellate. When the S^{35}-labeled sulfolipid was placed in the medium it was incorporated by the cells without cleavage of the sulfate group. Thus it passes back and forth between the growth medium and the cells.

The sulfolipid has been found in *O. danica, O. malhamensis, Tetrahymena pyriformis, Chlamydomonas* sp., *Pseudomonas* sp. (sea water bacterium). It was identified in the culture medium of *O. danica, O. malhamensis, Chlorella pyrenoidosa,* and *Streptomyces griseus.* Preliminary evidence was obtained for its presence in the growth medium of white clover and sorghum.

SULFOLIPIDS[1] have always been considered as an appendage to the phospholipids. In 1933 Blix(2) determined the structure of brain sulfatide. Although its structure was later mainly confirmed(22), then corrected(25), it remained the only sulfolipid until discovery of a sulfonolipid by Benson and co-workers (1) in 1958. A 3rd sulfolipid was found shortly thereafter by Middlebrook et al.(16) in tubercle bacilli. A 4th sulfolipid was observed in *Ochromonas danica* (11). Scattered reports in the literature suggest that there are at least 6 other sulfolipids known. Although the structures of 3 of these compounds are now known (25,24,9), their function remains an enigma. It is in this connection that we may see the value of grouping these compounds with the phospholipids.

The recent analyses of tissue sub-fractions impel the conclusion that these polar lipids are primarily (if not entirely) constituents of membranes(13). Considerable evidence has been gathering which suggests that membranes are bi-lipid layers(13,19,23). In this model the non-polar chains of the lipids are back to back and the polar ends face aqueous solution on both sides of the membrane. A major component of the membrane is protein which appears to be on both sides of the bi-lipid layer. In Vandenheuvel's molecular model of myelin(23) sulfatide plays the same role as phosphatide.

The sulfolipid reported in the present paper is bound to protein. It contains well over 50% of the total sulfur in *O. danica*. This substance may well be a membrane constituent of *O. danica*.

MATERIALS AND METHODS

O. danica, O. malhamensis, and *Chlorella pyrenoidosa* were cultured as described(11). Unless otherwise stated *O. danica* was used. The organisms were incubated in the presence of S^{35}-sulfate for 2 hr. The cells were centrifuged from the medium using a Servall refrigerated centrifuge at 9000 × g. The medium was poured off and labeled "Medium." The pellet was washed twice with deionized water at 5°. The washes were *not* combined with the medium. The pellet was frozen at −5° and thawed 3 times, and then extracted with 20 volumes of $CCl_3H:CH_3OH = 2:1$ (v/v).

The techniques of paper chromatography and autoradiography of the extract and medium have been described(11).

An aliquot of the medium was hydrolysed by refluxing in 3 N hydrochloric acid for 16 hr. Another sample of the

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[1] Although it has been suggested by Daniel et al.(3) that "sulfolipid" applies only to lipids containing a sulfonic acid, this is inconsistent with the use of the term long before the discovery of a sulfonic acid in lipid extracts. It is therefore suggested that "sulfolipid" apply to all lipids containing sulfur (8); "sulfatide" where the linkage is esterified sulfate; and "sulfonolipid" for a sulfonic acid.

medium was spotted directly onto the paper and oxidized with H_2O_2 according to Dent(4).

Digestion of cells with enzymes. In a previous paper the isolation of radioactively pure S^{35}-sulfolipid(11, Unknown No. 1) by paper chromatography from extracts of *O. danica* was described. The S^{35}-sulfolipid was fed back to the organism. The cells were then extracted 3 times as described, and the residue from the extraction was digested with Taka amylase as follows:

The dry residue (0.846 g) was ground to a smooth paste and taken up to a final volume of 50 ml with water. The pH was adjusted to 4.5 by the dropwise addition of glacial acetic acid. The suspension was boiled for 10 min. After cooling the suspension was adjusted to pH = 7.0 by the addition of N NaOH. Taka amylase (5 mg) was added together with a few drops of toluene. The mixture was incubated at 37° for 24 hr with occasional shaking. The suspension was centrifuged. The dried residue (0.437 g) was suspended in 50 ml of 5% (w/v) trichloroacetic acid and heated in a boiling water bath for 30 min. The cooled suspension was centrifuged. The residue was rinsed twice with 50 ml ethyl ether and once with 50 ml acetone. The residue now weighed 0.287 g.

The residue was digested with Pronase (Enzyme Development Corp.) as follows:

The powdered residue (287 mg) was suspended in 50 ml water and 5 ml ethanol. Pronase (3 mg, 45,000 P.U.K.) was added and the suspension incubated at 40° for 48 hr, yielding a clear, straw-colored solution.

The supernatants from the amylase and Pronase digestions were chromatographed and autoradiograms were made of the paper chromatograms.

Isolation of the sulfolipid from the cells. The broken cells (60.0 g) were extracted with 1,200 ml CCl_3H:CH_3OH = 2:1 (v/v). The extraction was repeated until the extracts were clear. The solvent was removed *in vacuo* and the residue suspended in 600 ml of 0.2 N KOH in methanol and maintained at 37° for 45 min. The suspension was diluted with 600 ml water and extracted with 1200 ml petroleum ether (b.p. = 30-60°) 3 times. The methanol:water fraction was adjusted to pH = 6.0 with 6 N HCl with vigorous stirring. The suspension was again extracted with 1,200 ml petroleum ether 3 times. All operations up to this point were conducted under N_2 as the fatty acids were used in other experiments(10). The water layer, after the removal of the fatty acids, was washed once with 1,200 ml chloroform. An equal volume (1,000 ml) of 2 M Na_2CO_3 was added to the water and the solution extracted 3 times with 1,000 ml *n*-butanol. The butanol extracts were combined.

The cell residue from the original extraction was digested with Pronase as described above. The digestion mixture was saponified directly by the addition of an equal volume of 0.4 N KOH in methanol and incubated at 37° for 45 min. As the fatty acids were not obtained from this fraction the saponified mixture was neutralized to pH ~6.0 with 6 N HCl and washed twice with $CHCl_3$. After the addition of 2 M Na_2CO_3 the sulfate ester was extracted into *n*-butanol as just described. The *n*-butanol extract was combined with the same fraction obtained from the lipid extract. The *n*-butanol was removed *in vacuo* and the residue dissolved in 50 ml of water and dialysed against deionized water (2 liters) for 2 hr. (The pH of the dialysand should be 7.) The contents of the bag were diluted with 10 volumes of *n*-butanol. The *n*-butanol was removed *in vacuo* and the residue dried with acetone which was also removed *in vacuo*. After this point manipulations were conducted under N_2 as the substance is hygroscopic. The procedure is summarized in Fig. 1. The resulting powder is a crude preparation of the sulfolipid (260 mg).

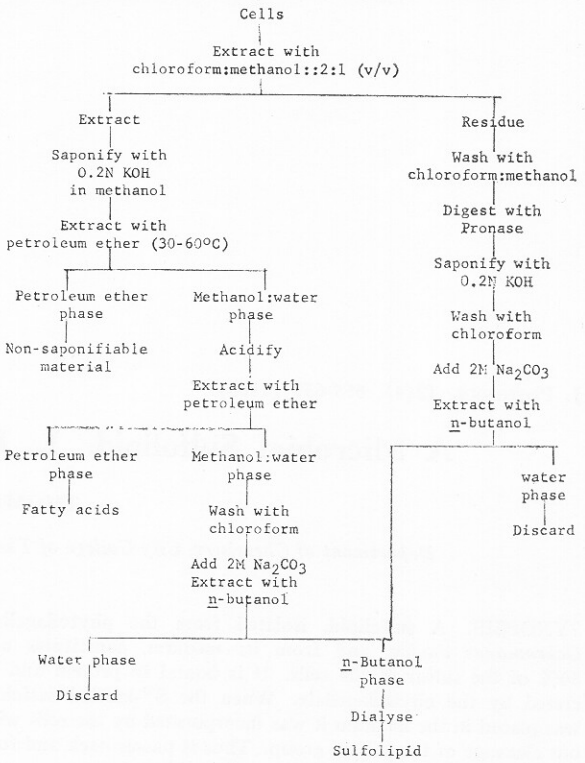


Fig. 1. Procedure for the isolation of sulfolipid from *O. danica* cells.

Basic hydrolysis of the sulfatide. A preparation of the sulfatide was saponified with 0.5 N KOH in ethanol:water = 1:1 (v/v) by refluxing for 5 hr. The ethanol was diluted with an equal volume of water and extracted with petroleum ether. The aqueous layer was neutralized with 9 N H_2SO_4 and again extracted with petroleum ether. The petroleum ether fractions were each dried over $CaSO_4$ and the solvent removed *in vacuo*. The product was chromatographed by gas-liquid chromatography(7).

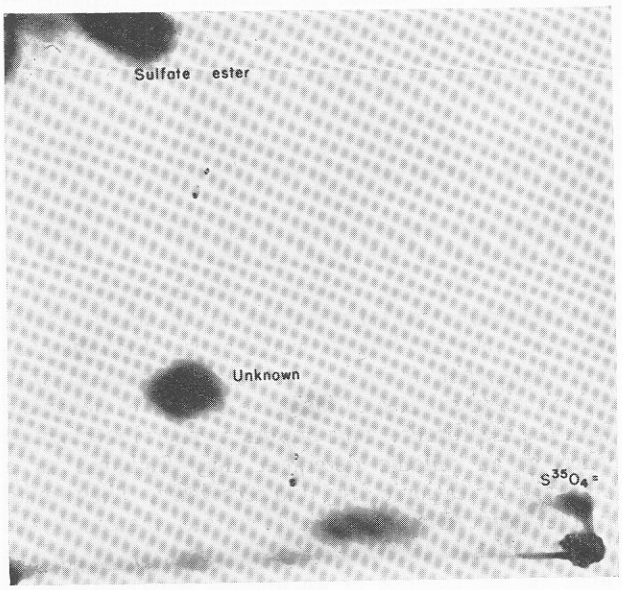
Quantitative estimation of the sulfatide. Spectrophotometric estimation of the sulfatide was conducted by the following modification of the method of Jones(14):

To 1 ml of a solution of sulfatide was added 1 ml of methylene blue reagent and 5 ml of $CHCl_3$. The test tube was stoppered, shaken vigorously, and permitted to stand. After 10 min the $CHCl_3$ layer was read in a Klett colorimeter with filter #66 (red). A standard curve of molar concentrations is obtained with Na dodecyl sulfate. The methylene blue reagent consists of 50 g of Na_2SO_4, 10 ml of conc. H_2SO_4 and 100 mg of methylene blue HCl dissolved in a liter of deionized water. The test is clearly not specific for the sulfatide as it may be used to identify lipid soluble sulfates and sulfonates (14) including steroid sulfate(20). It is likely that the test could be used to estimate the quantity of cerebroside sulfate in brain and other tissue lipids.

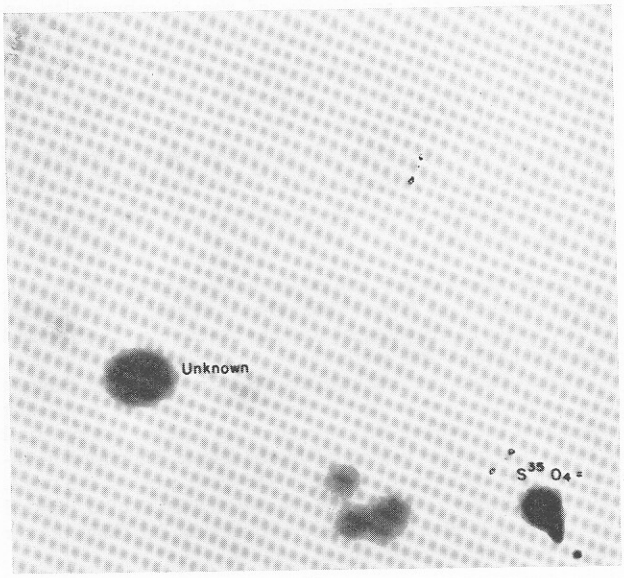
RESULTS

The excretion of sulfolipid into the medium. An autoradiogram of a paper chromatogram of the medium of *O. danica* after incubation with S^{35}-sulfate is shown in Fig. 2. The media of the other two organ-

isms gave identical results. The sulfolipid is clearly excreted into the medium by all 3 organisms. The medium was hydrolyzed as described, and an autoradiogram of the paper chromatogram of the hydrolyzed medium is also shown in Fig. 2. Disappearance of the sulfolipid was accompanied by an appropriate increase in the radioactivity of the S^{35}-sulfate.



A.



B.

Fig. 2. Autoradiograms of 2-dimensional chromatograms of the culture medium supporting *O. danica* after growth in the presence of $Na_2S^{35}O_4$. The chromatograms were developed in the vertical direction in *t*-butanol: 88% (w/v) formic acid: water = 14:3:3 (v/v/v). In the horizontal direction phenol: water = 5:1 (v/v) was used. A. Growth medium. B. Portion of the same medium after hydrolysis by refluxing in 3 N HCl for 16 hr.

The "unknown" in these two figures was not found in the cells. It was resistant, as seen in Fig. 2, to hydrolysis.

The sulfolipid is bound to protein. It was previously reported(11) that when *O. danica* was incubated in the presence of the S^{35}-sulfolipid, the cells incorporated the compound. Furthermore, only 60% of the substance in these cells was extractable with $CHCl_3$: CH_3OH. The nature of the binding of this material to the cell was investigated by digestion of the residue with enzymes. Amylase released *no* sulfatide, but protease released the remainder of the substance. It was therefore concluded that the sulfolipid is attached to protein.

Identification of the sulfatide in tissue extracts. In a cooperative effort(10) to identify the fatty acids of *O. danica* it was noticed that a mild saponification (0.2 N KOH, 37°, 45 min) yielded a fatty acid composition virtually identical with that yielded by vigorous saponification (4 N KOH, refluxing, 4 hr) with one outstanding difference. A peak appeared after vigorous saponification in gas-liquid chromatograms which had a retention time of a fatty acid methyl ester of "12.5" carbons(7). A purified sample of the sulfatide was saponified with the vigorous procedure and gave a single peak with the expected retention time. This peak was thus found useful for the identification of sulfatide in tissue extracts.

A 2nd method used for identification was paper chromatography of extracts of S^{35}-labeled cells developed in the solvents used in Figs. 2 and 3.

A 3rd method was the methylene blue method of Jones which is not specific for the substance but may be indicative. This method was used on the media of white clover and sorghum after these plants were grown in hydroponics. The medium was concentrated *in vacuo* to small volume and dialyzed as described in the methods. The dialysate was tested by the methylene blue method. It should be noted that microbes, although minimized, were not vigorously excluded from the medium. These results must be considered tentative.

The results on identification of the sulfatide are summarized in Table 1.

Where more than one technique was used the results were the same. The substance constituted a major component (5-10% of the total fatty acids as estimated by retention volumes) of *O. danica, O. malhamensis, T. pyriformis,* and *Pseudomonas* sp. Only a trace was present in *Gingko* leaves. It constituted, however, 70% of the total fatty acids of Pronase. The approximate concentration of the white clover and sorghum media was 0.2 mM.

DISCUSSION

Fig. 2 shows that the sulfatide and an unknown

TABLE 1. *Distribution of sulfatide among investigated organisms. Presence (+) or absence (—) of the sulfatide in the tissue or medium of a given organism is so indicated. A blank space indicates that the method was not used with the source indicated.*

Source		Method* 1	2	3
Ochromonas danica	cells	+†	+(11)	
Ochromonas danica	medium	+†	+(11)	+
O. malhamensis	cells	+†	+(11)	
O. malhamensis	medium		+(11)	+
Chlorella pyrenoidosa	cells	—†	—(11)	
Chlorella pyrenoidosa (Van Niel)	medium		+(11)	
Tetrahymena pyriformis	cells	+†	+‡	
Chlamydomonas (sp.)	cells		+‡	
Euglena gracilis	cells	—†		
Pseudomonas sp. (sea water bacterium)	cells	+†		
Streptomyces griseus§	medium	+†		
Ginko	leaves	+†		
Sorghum	medium			+
White clover	medium			+
Rat	liver	—†		

* *Method 1:* Gas-liquid chromatography of the saponification product. *Method 2:* Paper chromatography of extracts of S^{35}-labeled cells. *Method 3:* Methylene blue method of identification. See text for details. Figures in parentheses are references.

† The gas chromatograms were the generous contribution of J. L. Gellerman and H. Schlenk, University of Minnesota, Hormel Institute.

‡ Reggio, R. Boyce Thompson Institute for Plant Research, Inc. Personal Communication.

§ This determination was done on the enzyme preparation Pronase (Enzyme Development Corp.) obtained from the growth medium of *Streptomyces griseus*(18).

compound were excreted into the medium. As this "unknown" was resistant to hydrolysis (Fig. 2) it cannot be a sulfate or sulfite ester, glycoside, etc. Oxidation by Dent's procedure(4) did not alter its chromatographic properties. It is thus not a thiol, sulfide or sulfoxide. It appears therefore that the material is a sulfone or a sulfonic acid. It does not cochromatograph with taurine, cysteic acid, 6-sulfonoquinovose or methionine sulfone. It does not react with ninhydrin.

When the S^{35}-labeled sulfolipid was incubated with cells in the logarithmic phase of growth it was incorporated by the organism but not *metabolized*(11). When incorporated it was firmly bound to protein. Thus the substance may be part of a membrane. This is consistent with the quantity of the substance in *O. danica*. Experimental data has been obtained to support this contention(12).

If the Na salt of the sulfatide is placed in a CCl_3H: H_2O system the substance remains in the water. If Ca^{++} or Mg^{++} is added, these metals could be isolated from the $CHCl_3$ layer along with sulfatide. It is well known that alkyl sulfates or sulfonates may be precipitated from water by divalent metals. These salts are generally more soluble in organic solvents than in water.

The presence of divalent metals in the medium of a microbe into which the sulfatide is excreted would probably yield such a precipitate. The precipitate would then come into contact with a lipid membrane.

Mumma & Gahagan(17) found sulfatides which may or may not include the sulfatide here reported—in roots, but not in leaves—of a large variety of higher plants.

Presumably a sulfatide excreted by microbes and possibly by higher plants would be found in soil. Freney(5,6) and Lowe & Delong(15) have reported that ⅓ to ½ the sulfur in soil is present as esterified sulfate.

Sokolski et al.(21) studied the effect of surface-active agents on the permeability of *O. danica* to neomycin. Although nonionic, cationic, and amphoteric detergents had no effect, the anionic alkyl sulfates increased permeability. It would appear that the cationic neomycin formed a non-polar complex with anionic alkyl sulfates to facilitate contact of neomycin with the membrane. This implies a general mechanism whereby an anionic detergent may find use as a synergist for cationic drug and vice versa. They found further that Na heptadecyl sulfate increased the toxicity of salt to *O. danica*. Whereas 1.5% KCl is toxic in the absence of an anionic detergent; 0.5% is toxic in its presence. They infer that anionic detergents increase the sensitivity of the membrane to osmotic pressure through "a wetting of the membrane." Sokolski et al.(21) were unaware of the presence of sulfatide in the medium. Possibly the increased toxicity to salt of *O. danica* might be due to the interference of the synthetic alkyl sulfate with the natural sulfatide's carrier mechanism. Thus the K_2SO_4 added to the medium effectively converts the sulfate esters to their K salts.

I regret that it is too late to thank the late Dr. Richard J. Block, formerly of Boyce Thompson Institute; I am grateful to Dr. James A. Johnston of Rutgers University. Their critical guidance enabled this work to be accomplished.

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