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LIPIDS AND BIOMEMBRANES OF
EUKARYOTIC MICROORGANISMS

CHAPTER 4

Sulfolipids and Halosulfolipids

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I. Introduction

For one hundred years sulfolipids have been known to be associated with mammalian systems, especially brain (Thudichum, 1874). Their occurrence in eukaryotic microorganisms was first described, however, only ten years ago (Benson *et al.*, 1959a), and since this first report they have been reported in all photosynthetic plants (including algae and plankton),

protozoa, fungi, bacteria, insects, and invertebrates. Like the phospholipids with which they are generally associated, the sulfolipids have a wide variety of structures only one of which includes glycerol in its formulation. Also like the phospholipids, the sulfur atom is found as both a sulfate ester ($RCH_2OSO_3^-$) and a sulfonate ($RCH_2SO_3^-$). Although the former has been found in a variety of chemical forms, the latter has been described only in the glycolipid 6-deoxyhexose-6-sulfonate. In the case of the chloroplast sulfolipid, this hexose is glucose.

All membrane preparations that have been analyzed with respect to their lipid composition have been found to contain phospholipids. In contrast, each sulfolipid has been reported as one component of a particular membrane. This uniqueness has provided a tool for exploring the biosynthesis and metabolism of one membrane of an organism in the presence of the other membranes.

For the most part the current status of sulfolipids in eukaryotic microorganisms is that of establishing the structures and localizing the substances within the organelles of the cell. Very little has been done on intermediary metabolism, largely because the structures themselves are so new. Although the phospholipids are generally present in higher concentrations than sulfolipids in tissues, it is nonetheless surprising that the structures of most of the phosphatides had been well established before any of the sulfolipids (except for brain sulfatide) were even discovered. There are two major reasons for this, and since these reasons aid in our understanding of the sulfolipids, we shall explore them.

The first and most significant reason for the delay in the discovery of new sulfolipids was the absence of a reliable and sensitive colorimetric test for sulfate comparable to the molybdate test for phosphate. Until the introduction of radioisotopes, the only reliable means for the determination of sulfate was the gravimetric barium sulfate method. The appearance of radioisotopes and chromatography, especially thin-layer chromatography, set the stage for the rapid discovery of sulfolipids.

In addition to the difficulties of analysis and isolation, the physical properties of the sulfolipids have retarded their discovery. The sulfolipids are more polar than the phospholipids or glycolipids. Although sulfatides are usually extracted from tissue by chloroform-methanol mixtures, they are usually sufficiently water soluble to remain in the aqueous phase in an ether-water system. When there is an interfacial fluff, it often contains a high proportion of these substances. Furthermore, in the procedure of Folch *et al.* (1957), the sulfolipids generally are completely extracted into the polar phase. Many investigators working on eukaryotic lipids use the Folch procedure and *discard this phase.*

The recent recognition of the significance of membranes and the emergence of polar lipids as key constituents of membranes have sparked considerable interest in the sulfolipids, as they have the other lipids in this volume. All the work on the sulfolipids of the eukaryotes has been motivated by this consideration, coupled with the emergence of a sensitive colorimetric assay for sulfolipids (Kean, 1968). There is every reason to indicate that this trend will continue.

A review by Goldberg (1961) has been preempted by radical changes in our knowledge of the sulfolipids. Even the structure of cerebroside sulfate has since been corrected (Yamakawa *et al.*, 1962; Stoffyn and Stoffyn, 1963). The plant sulfolipid was reviewed by Benson (1963). The chemistry of all the known sulfolipids was reviewed by Haines (1971). A major portion of the latter review is devoted to hydrolysis, analysis (including physical methods), and synthesis of sulfate esters and sulfonic acids.

This chapter will contain an extensive discussion of the two sulfolipid types that have been well described: the plant sulfonolipid (6-sulfo-*O*-α-quinovosyl-(1 $\rightarrow$ 1)-glycerol diglyceride) and the halosulfatides (polyhalo derivatives of 1,14-docosane disulfate and 1,15-tetracosane disulfate). Mention will be made of other eukaryotic sulfolipids that have been discovered but not yet characterized. The two eukaryotic sulfolipids that have been well described have turned out to be radically different from any previously known lipids, and the characterization of the reported but uncharacterized eukaryotic sulfolipids may well turn out to be as interesting.

II. Nomenclature

The term *sulfolipid* was first used from the earliest reports of Thudichum (1874) to describe the sulfur-containing lipid in brain. This substance, cerebroside sulfuric acid, was first characterized by Blix (1933). Because the material is a sulfate ester of galactose, it has also been referred to as sulfatide. Sulfolipids (including the plant sulfolipid) have been discovered that are *not* sulfatides, as the sulfur is in the form of a sulfonic acid (C—S bond) and not a sulfate ester (C—O—S bonds). Daniel *et al.* (1961) have suggested that the term sulfolipid be used to denote a sulfonate lipid. Baer and Stanacev (1964), on the other hand, have suggested that the term *phosphonolipid* be used to denote phospholipids in which the phosphorus occurs as a phosphoric acid. Furthermore, the term sulfolipid has been used for many years in the literature for cerebroside sulfate and is continuing to be so used (Stoffyn, 1966). Nonetheless, some sharpening

of nomenclature is obviously necessary, as sulfolipids of diverse structure are appearing each year.

The following nomenclature has been suggested (Haines, 1971) to clarify the situation:

1. The term *sulfolipid:* to denote any sulfur-containing lipid.
2. The term *sulfatide:* to identify a sulfolipid in which the sulfur occurs as a sulfate ester.
3. The term *sulfonolipid:* (pronounced sŭl-fŏn-ō-lĭ-pĭd) in reference to sulfolipids that contain sulfur in the sulfonic acid form.
4. The term *thiolipid:* to denote sulfolipids with sulfur in the reduced form (Daniel *et al.*, 1961).

Although no lipid that contains the sulfoxide or sulfone states of sulfur has been reported, it is clear that *sulfoxolipid* and *sulfonolipid* can be used in reference to such compounds should they be identified.

This distinguishing nomenclature is based not only upon the obvious differences of chemical structure but upon the more significant differences of biochemistry. The formation of sulfatides is clearly through an entirely different biosynthetic route than that of the sulfonolipids (as well as the thiolipids), and their metabolic behavior should be radically different. These terms will be used throughout this chapter.

III. The Plant Sulfonolipid

The plant sulfonolipid was the first sulfolipids to be reported in eukaryotic microorganisms. Since its discovery over ten years ago by Benson and co-workers (1959a, 1960; Lepage *et al.*, 1961; Daniel *et al.*, 1961; Shibuya and Benson, 1961; Miyano and Benson, 1962a,b), this sulfolipid has been found in all green plants. There is now little doubt that the substance is primarily a chloroplast substituent in green plants, localized in the lamellae. Several lines of evidence suggest that its participation in photosynthesis is not merely as a structural component of chloroplast membrane, and this evidence will be discussed in Section III,E.

The sulfonolipid is not restricted to the higher plants and green algae, as it has been reported in red algae (Benson and Shibuya, 1962; Radunz, 1969), blue-green algae, brown algae, and purple bacteria (Radunz, 1969). Neither its function nor its localization is clear in these organisms. The sulfonolipid constitutes 14 to 18% of the lipids in the red, brown, and blue algae but only 4 to 5% of the lipids of isolated chloroplasts of green plants and algae. This suggests that it may be a component of membranes other than those associated with photosynthesis in the red, brown, and blue-green algae. It should also be noted that its fatty acid composition in these

organisms is radically different from that of green plants (Radunz, 1969), even after considering the seasonal changes to which the fatty acid composition of sulfonolipid is subjected in higher plants (Klopfenstein and Shigley, 1967).

Because the plant sulfonolipid is concentrated in the chloroplast membrane of higher plants and green algae, it presents a unique tool to the researcher on membrane structure and function. Its structure has implications to the structure of the membrane, its presence in the membrane represents a tool for assaying one membrane in the presence of others, and control of its biosynthetic route would offer another handle for manipulating the membrane's biogenesis.

The plant sulfonolipid has not been reviewed since 1963 (Benson, 1963), although its chemistry has been reviewed in some detail by Haines (1971).

A. Isolation

The first structural studies of Benson *et al.* (1959a) did not require the isolation of substantial amounts of material, as the studies were conducted on ^{14}C-labeled material isolated by paper chromatography. Even subsequent stereochemical work (Miyano and Benson, 1962a,b) relied upon small quantities of radioactive material identified by cocrystallization with synthesized enantiomer. Lepage *et al.* (1961) conducted the first isolation of visible amounts by ion exchange chromatography. They note that fresh or dried alfalfa or clover are suitable sources, and most subsequent isolations have used these plants. They are normally poor sources for sulfonolipid. The highest reported concentration is that of a species of alfalfa that yielded 24% of its lipid as sulfonolipid when grown at 30°C (Kuiper, 1970). High concentrations of the sulfonolipid occur in red algae (14.9% of the lipid), brown algae (18.3%), and blue algae (13.9%) (Radunz, 1969). A good procedure suitable for the isolation of a reasonable quantity of rather pure sulfonolipid is that of O'Brien and Benson (1964). These authors use a rather cumbersome and elaborate procedure involving chromatography of the lipid extract of alfalfa or *Chlorella pyrenoidosa* on three successive columns. Nonetheless, their product is pure, and it represents 99% of the ^{35}S-sulfolipid placed on the first column. One gram of alfalfa lipid extract yielded 30 mg of sulfonolipid, whereas the same quantity of *Chlorella* lipid yielded only 19 mg. The authors' description of the procedures for preparing the supports and packing the columns is clear and easy to follow. The columns are Florisil, DEAE-cellulose, and silicic acid essentially that of Rouser *et al.* (1961, 1967)—and these original references should be read before conducting the isolation.

O'Brien and Benson (1964) also report that ^{35}S-sulfonolipid is distri-

buted after complete equilibration in a chloroform-water system so that 64% of the radioactivity is in the chloroform phase and 36% in the aqueous phase. In a benzene-water system, 98% of the sulfonolipid remains in the aqueous system. Along with phosphatidyl inositol, the sulfonolipid appears to be the most polar lipid on chromatograms (Kates, 1960; Wintermans, 1960; Mumma and Benson, 1961). These data suggest that the Folch *et al.* (1957) extraction procedure might be effective for the preparation of a crude batch of sulfolipid, particularly if a chloroplast preparation rather than whole leaf is used as the source. It is suggested here that repeated washes of the lower phase with the *upper phase* of the Folch procedure would permit isolation of the sulfonolipid in the lower phase.

Several other column procedures have been reported in the literature. Use has been made of Florisil (Russell and Bailey, 1966) DEAE-cellulose (Nichols and James, 1964; Roughan and Batt, 1968; Allen *et al.*, 1966), and ECTEOLA-cellulose (Klopfenstein and Shigley, 1966).

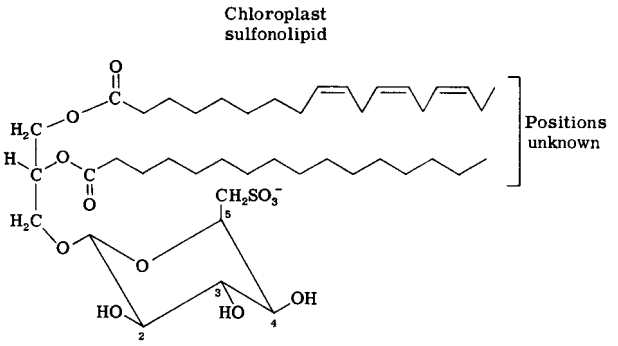
Preparative thin-layer chromatography has proved useful for obtaining small amounts of pure sulfonolipid, which is particularly useful for doing fatty acid analyses of single spots on chromatograms (Pohl *et al.*, 1970; Klopfenstein and Shigley, 1966; O'Brien *et al.*, 1964). The method of Pohl *et al.* (1970) permits a fatty acid analysis of the sulfonolipid after a single thin-layer chromatogram of crude plant lipids. Sodium methoxide is the esterifying reagent.

B. Structural Studies

The structure of the sulfonolipid was evolved by the Benson group, who discovered the substance in green plants (Benson *et al.*, 1960). An elegant combination of isotopes, paper chromatography, and chemistry allowed Benson *et al.* (1959a) to identify the substance as a glycerol lipid containing a hexose-6-sulfonate in glycosidic linkage to the glycerol. At first the sulfonolipid had one fatty acid esterified to the glycerol. It was later found by Yagi and Benson (1962) that this was produced during the isolation by an extremely active lipase from the natural diacyl sulfonolipid.

Using ion exchange resin chromatography, Lepage *et al.* (1961) isolated a sufficient quantity of the deacylated sulfonolipid to allow physical studies on the material. The glyceryl sulfoglycoside exhibited a molecular rotation, $(M)_D^{25}$, of +31,000 degrees, characteristic of alkyl-α-D-glucopyranosides (Daniel *et al.*, 1961). Further evidence for an α-glycoside was obtained from the nuclear magnetic resonance absorption of an anomeric equatorial proton. The rotational shift in Cupra B of −370 degrees indicated three adjacent equatorial hydroxyls, typical of glucosides (Lepage

et al., 1961). The complete structure of the glycerylsulfoquinovoside including the L configuration of the glycerol moiety was confirmed by an X-ray crystallographic analysis of its rubidium salt by Okaya (1964). The structure of the plant sulfonolipid is thus that shown in formula (I).



(I)

Synthesis of the deacylated sulfonolipid with the correct configuration was achieved by Miyano and Benson (1962b). Its infrared spectrum is available (Haines, 1971), as is that of the intact sulfonolipid (Radunz, 1969). Several salts of sulfoquinovose have been prepared by Helferich and Ost (1963) and Lehmann and Benson (1964a,b).

The fatty acids were first identified as palmitic (43%) and α-linolenic (47%) in alfalfa (O'Brien and Benson, 1964). Subsequent work has shown that the condition of the source affects the fatty acid composition radically (Klopfenstein and Shifley, 1967). This will be discussed in more detail in Section III, E.

C. Analysis

The analytical method used in the initial discovery of the sulfonolipid was paper chromatography of ^{35}S-labeled plant tissue. Chromatography on silicic-acid-impregnated paper in chloroform-methanol, 9:1 (R_F = 0.4), was used by O'Brien and Benson (1964). Thin-layer chromatography on silica gel using chloroform-methanol-acetic acid, 65:25:10 (R_F = 0.7) (Klopfenstein and Shigley, 1966), or acetone-benzene-water, 91:30:8 (R_F = 0.4) (Pohl *et al.*, 1970), has been useful. The latter system separates all the plant lipids remarkably well in a single chromatogram. Anion exchange paper has also been used (Mumma and Benson, 1961).

Anthrone has become a unique assay reagent for the sulfonolipid. Wintermans (1960) and subsequently Weenink (1963) have described a

characteristic absorption peak at 592 nm for the sulfonolipid. Galactose and other plant hexoses generally yield adducts with anthrone in sulfonic acid that peak at 625 nm. Application of this method can be used with either the whole lipids or the deacylated lipids. The reaction was used by Isono and Nagai (1966; Nagai and Isono, 1965) in their efforts to characterize the sea urchin sulfonolipid. The blue color of this anthrone reaction was used by Russell (1966; Russell and Bailey, 1966) to routinely assay samples for sulfonolipid. However, Russell's procedure was apparently erratic in the hands of his fellow New Zealanders Roughan and Batt (1968), who used the phenol-sulfuric acid reagent of Dubois *et al.* (1956), which doubles the sensitivity and is quantitative for the sulfonolipid (as well as galacto lipids) while it is still adsorbed to silica. The phenol-sulfuric acid reagent does not distinguish the sulfonolipid from other glycolipids.

A procedure used for the assay of the sulfatides of *Ochromonas danica* by Haines (1965) and elegantly developed for cerebroside sulfate by Kean (1968) has not been applied to the sulfonolipid. The procedure involves mixing the sample with a cationic dye such as azure A and extracting directly in a colorimeter tube with chloroform. It can be conducted on crude samples with only the very slightest contamination from other anionic lipids.

D. Biosynthesis

Little is known about the biosynthesis of the plant sulfonolipid. Most attempts to obtain information on this problem have led to different speculations with a paucity of data to back them up. At least three routes have been put forward in the literature for the biosynthesis of 6-sulfoquinovose.

The first proposal was that of Zill and Cheniae (1962), who suggested in a review that 3′-phosphoadenosine-5′-phosphosulfate (PAPS) transfers a sulfonyl group to a carbon atom of an acceptor molecule. They suggest that the acceptor may be a lipid or a nucleotide-bound precursor.

Benson (1963), in his review of the sulfonolipid, combined this suggestion with one by Kittredge *et al.* (1962) for the phospholipids and proposed that pyruvate was an appropriate carbanion acceptor. He pointed out that the occurrence of sulfolactaldehyde, sulfolactate, and sulfopropanediol in *Chlorella*, albeit in small amounts (Shibuya and Benson, 1961), supports this contention. He suggested that the biosynthesis might occur by a "sulfoglycolytic sequence" from sulfopyruvate. This suggestion was further expanded by Davies *et al.* (1966), who made the proposal in Fig. 1.

A second pathway for the biosynthesis of 6-sulfoquinovose was proposed by Lehmann and Benson (1964a). These authors (Lehmann and Benson,

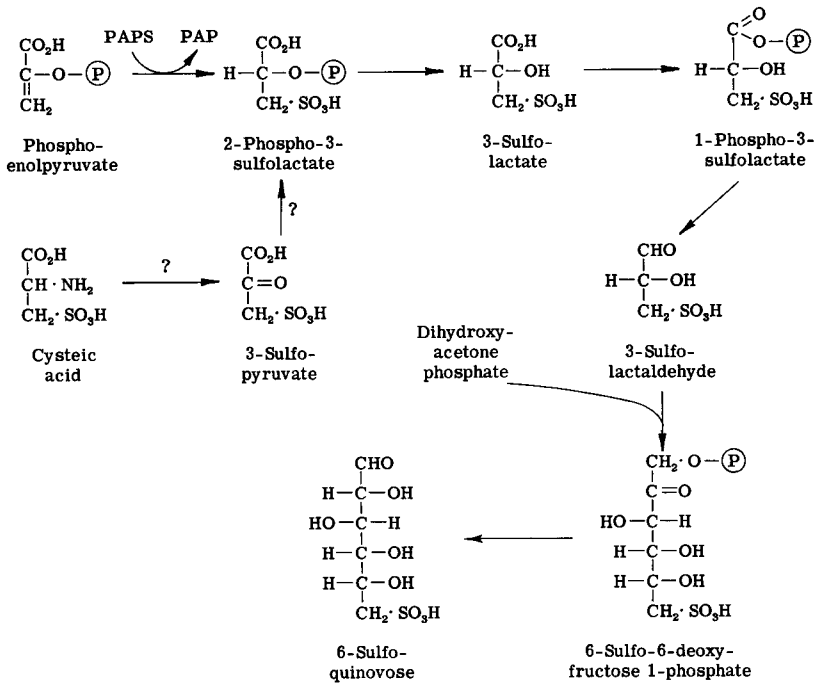


Fig. 1. Pathway for the biosynthesis of 6-sulfoquinovose suggested by Davies *et al.* (1966).

1964b) have synthesized 6-sulfoquinovose via the methyl glucoseenide (the double bond between C-5 and C-6 of a glucoside). Their suggestion was based upon the observation that sulfite adds to methyl-α-D-glucoseenide to form the 6-sulfoquinovoside in 5 minutes at room temperature in aqueous solution at pH 6.4 to 7.0. The addition of sulfite to the glucoseenide is a free radical reaction.

A third pathway for the biosynthesis of 6-sulfoquinovose was put forward by Hodgson *et al.* (1971). It is analogous to the displacement of *O*-acetyl or *O*-succinyl groups by thiols or H_2S (Kaplan and Flavin, 1966; Kredich and Tompkins, 1966; Weibers and Garner, 1967; Giovanelli and Mudd, 1968; Kerr and Flavin, 1968). The synthesis of sulfonic acids by displacement with sulfite is, of course, as familiar to the synthetic chemist as the addition of sulfite to double bonds (Haines, 1971).

It is easy to see how many varied proposals are available in the literature. When one looks for hard data on the biosynthesis, however, all such data

are available in only two publications. Nissen and Benson (1964) established that 3-^{14}C-cysteine is not incorporated into the sulfonolipid in *Chlorella*. Davies *et al.* (1966) confirmed this finding for *Euglena gracilis*. They further found that cysteic acid, labeled on either C-3 or the sulfur atom, was incorporated to the same extent into the sulfonolipid. In addition, cysteic acid, but not cysteine, inhibited the incorporation of $^{35}SO_4^{2-}$ into sulfonolipid by *Euglena*, whereas cysteine *but not cysteic acid* inhibited the uptake of $^{35}S_4^{2-}$ by the cells. These data implicate cysteic acid as an intermediate in the biosynthesis of 6-sulfoquinovose.

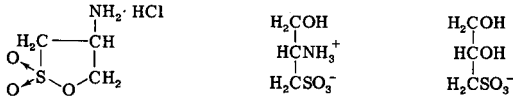
Davies *et al.* (1966) have also found that molybdate (3.0 m*M*) inhibits the incorporation of $^{35}SO_4^{2-}$ into sulfonolipid in *Euglena* but has no effect on its uptake by the cells. These data suggest that PAPS is involved in the biosynthesis of the sulfolipid. It is on the basis of the above that these investigators proposed the pathway described in Fig. 1.

In recent years it has become increasingly clear that a specific and active transport process is involved in the uptake of sulfate by microbes (Pardee, 1966, 1967; Dreyfus, 1964; Ellis, 1964; Kylin, 1964, 1966, 1967; Hodgson *et al.*, 1971). This transport system precedes the formation of PAPS and is independent of it. Thus the uptake of sulfate by *Penicillium chrysogenum* is suppressed by inhibitors of energy metabolism (2,4-dinitrophenol or azide), but sulfate reduction is unaffected (Yamamoto and Segel, 1966), as is that of *Chlorella* (Wedding and Black, 1960) and *Euglena gracilis* (Abraham and Bachhawat, 1965). All the evidence in the literature to date indicates that the first step in the metabolism of sulfate is its activation to PAPS, as described by Wilson and Bandurski (1958). These authors had shown that molybdate inhibited the formation of PAPS by the enzyme ATP sulfurylase (EC 2.7.7.4). Inhibition of sulfate activation by molybdate *in vivo* has been demonstrated in many systems, notably *Escherichia coli* and *Bacillus subtilis* (Pasternak, 1962), rat brain (Pritchard, 1966), and salivary gland (Pritchard, 1967a, b). It is therefore not easy to interpret the molybdate inhibition data of Davies *et al.* (1966). Their findings do not necessarily imply the participation of PAPS directly in the biosynthesis of sulfonolipid.

Recently, Hodgson *et al.* (1971) have isolated *Chlorella* mutants that are unable to reduce sulfate, apparently because of a genetic lesion involving the formation of PAPS. Other sulfur metabolism mutants isolated by these investigators suggest that PAPS is involved in the reduction of sulfate by *Chlorella*. The pathway for the reduction of sulfate by *Salmonella typhimurium* is now becoming clear, and PAPS is definitely involved. In this bacterium PAPS reductase has been identified as the product of the cys H gene (Kredich, 1971). This enzyme reduces PAPS

to sulfite (Noriko *et al.*, 1971). Since PAPS is involved in the formation of sulfite, inhibition of ATP-sulfurylase by molybdate would inhibit the biosynthesis of the sulfonolipid regardless of which of the proposed pathways is correct.

Also consistent with the proposal in Fig. 1 was the finding of sulfolacetaldehyde, and sulfolactate in *Chlorella* by Benson and Shibuya (1961). A report (Wickberg, 1957) that the red alga *Polysiphonia fastigiata* contains cysteinolic sulfolactone (2-L-amino-3-hydroxy-1-propane sulfonic acid lactone) (II) is of some interest in view of the high concentration of the sulfonolipid in red algae (Benson and Shibuya, 1962; Radunz, 1969).



(II) (III) (IV)

Cysteinolic acid (III) has also been identified in brown and green algae (Ito, 1963) and in the freshwater diatom *Navicula pelliculosa* (Busby, 1966), along with sulfopropanediol (IV).

A summary of this discussion on the biosynthesis of 6-sulfoquinovose is illustrated in Fig. 2. The pathway includes the suggestion of a nucleophyllic displacement by sulfite on *O*-acetyl serine as proposed by Hodgson

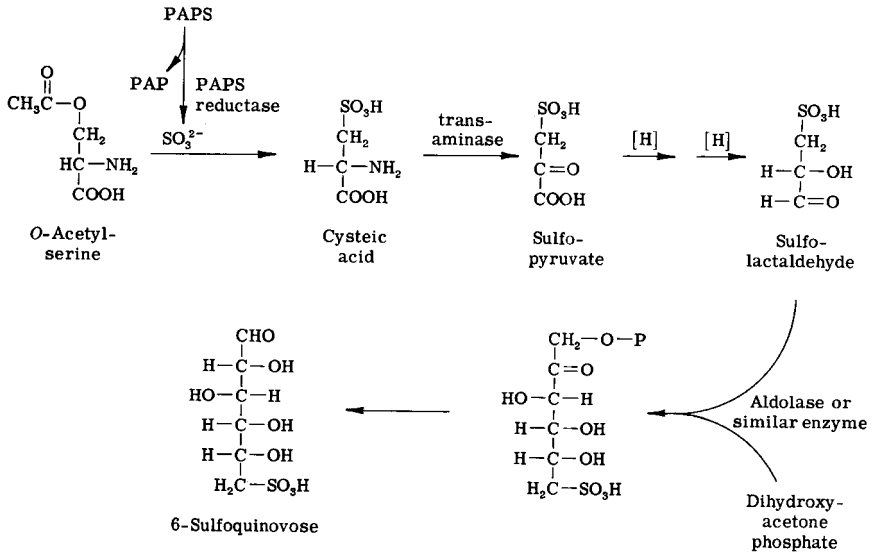


Fig. 2. Proposed biosynthesis of 6-sulfoquinovose.

et al. (1971). The choice of *O*-acetyl serine as the precursor of cysteic acid is based upon its availability as an intermediate in the biosynthesis of cystein (Kredich, 1971). Davies *et al.* (1966) have demonstrated the participation of cysteic acid in the biosynthetic route. A secondary role is proposed for PAPS. The pathway is otherwise similar to that of Fig. 1.

Other studies on the biosynthesis of plant sulfonolipid relate to the participation of nucleoside derivatives of 6-sulfoquinovose as participants in the formation of the glycosidic bond with glycerol. Shibuya *et al.* (1963) identified a nucleoside diphosphosulfoquinovoside among the ^{35}S-labeled components of plant extracts. It was suggested that this compound was the activated intermediate for sulfoquinovosyl glyceride.

A study of the ability of plants to incorporate ^{75}Se-selenate into sulfonolipid was made by Nissen and Benson (1964). Although APSe was identified, no evidence could be found for the formation of either PAPSe or selenolipid.

E. In Chloroplast Membrane

In the very first publications reporting the sulfonolipid in plants it was recognized as an important component of photosynthetic microorganisms and higher plants. Table I shows a list of the microorganisms and plants in which the sulfonolipid has been identified to date. Virtually every higher plant and photosynthetic microorganism that has been investigated has been shown to contain the material. It is therefore tempting to speculate that the sulfolipid plays a role in photosynthesis. Whether or not this is the case, it has now become very clear that the sulfonolipid is a structural component of the chloroplast membrane in higher plants and green algae.

The occurrence of sulfonolipid in many of the organisms listed in Table I has been confirmed in several laboratories. An outstanding exception has been the photosynthetic bacteria where conflicting information is available. Thus Wood *et al.* (1965) in a survey of five photosynthetic bacteria found that only *Rhodopseudomonas spheroides* contained sulfonolipid and that it was absent from *R. capsulata, R. palustris, R. gelatinosa,* and *Rhodospirillum rubrum.* On the other hand, Benson *et al.* (1959a), using a sample of ^{35}S-labeled *R. rubrum* provided by John Ormerod, reported sulfonolipid in this bacterium. This discrepancy might be explained by the very small amounts of sulfonolipid found in the photosynthetic bacteria. Park and Berger (1967) estimated that the sulfonolipid was only 0.01% of the dry weight of *Rhodomicrobium vannieli.* This is approximately the value found for *R. spheroides* by Radunz (1969). It would appear that ^{35}S-labeling would have been a more sensitive analytical method than that used by Wood

TABLE I

ORGANISMS IN WHICH SULFONOLIPID HAS BEEN IDENTIFIED

Class	Species	% Total Lipid	Reference
Higher plants	Barley (*Hordeum vulgare*)		Benson *et al.* (1959a)
	New Zealand spinch (*Tetragonia expansa*)		Benson *et al.* (1959b)
	Chive sp.		Benson *et al.* (1959a)
	Coleus sp.		Benson *et al.* (1959a)
	Sweet clover sp.		Benson *et al.* (1959b)
	Tomato sp.		Benson *et al.* (1959b)
	Alfalfa sp.		Lepage *et al.* (1961)
	Runner bean (*Phascolus multiflorus*)		Kates (1960)
	Spinach (*Spinacia oleracea*)		Wintermans (1960)
	Sugarbeet (*Beet vulgaris*)		Wintermans (1960)
	Elder (*Sambucus nigra*)		Wintermans (1960)
	Paul's scarlet rose		Davies *et al.* (1965)
	Maize (corn) (*Zea mays*)		Davies *et al.* (1965)
	Fern (*Dryopteris filix-mas*)	4.0	Radunz (1968)
	(*Antirrhinum majus*)	5.2	Radunz (1969)
	Wheat (*Triticum aestivum*)		Nissen and Benson (1964)
	Sunflower (*Heliantus annus*)		Nissen and Benson (1964)
	Red clover (*Trifoleum pratanse* L.)		Russell and Bailey (1966)
	Moss (*Sphagnum fimbriatum*)		Collier and Kennedy (1963)
	Rye grass sp.		Collier and Kennedy (1963)
	Selaginella Kraussiana A. Br. (a Pteridophyta)		Collier and Kennedy (1963)
	Broad bean (*Vicia faba*) fluffy pericarp		Collier and Kennedy (1963)
	Kalanchoe crenata tissue culture		Thomas and Stobart (1970)
	Sunflower (*Helianthus annus*)		Nissen and Benson (1964)
	Wheat (*Triticum aestivum*)		Nissen and Benson (1964)
Chlorophyceae (green algae)	*Chlorella pyrenoidosa*		Benson *et al.* (1959a)
	Scenedesmus D_3		Benson *et al.* (1959a)
	Chlorella vulgaris		Nichols (1965)
	Chlorella prototecoides		Shibuya and Hase (1965)
	Ulva lactuca var. *latissima* D.C.		Collier and Kennedy (1963)
	Cladophora sp.		Collier and Kennedy (1963)
	Enteromorpha compressa Grev.		Collier and Kennedy (1963)
	Scenedesmus obliquus		Yagi and Benson (1962)
	Chlorella ellipsoidea		Miyachi and Miyachi (1966)

TABLE I (Continued)

ORGANISMS IN WHICH SULFONOLIPID HAS BEEN IDENTIFIED

Class	Species	% Total Lipid	Reference
Phaeophyceae (brown algae)	*Fucus serratus*		Collier and Kennedy (1963)
	Fucus vesiculosus	18.3	Radunz (1969)
Rhodophyceae (red algae)	*Rhodymenia palmata* Grev.		Collier and Kennedy (1963)
	Plumaria elegans Schmitz		Collier and Kennedy (1963)
	Antithamniou plumula Thuret		Collier and Kennedy (1963)
	Gigartina stellata Batt.		Collier and Kennedy (1963)
	Dumontia incrassata Lamour		Collier and Kennedy (1963)
	Ceramium rubrum C. A. Agardh		Collier and Kennedy (1963)
	Batrachospermum moniliforme	14.9	Radunz (1969)
Cyanophyceae (blue-green algae)	*Rivularia atra* Roth.		Collier and Kennedy (1963)
	Oscillatoria chalybea	13.9	Radunz (1969)
Athiorhodacae (photosynthetic bacteria)	*Rhodopseudomonas spheroides*	2.6	Radunz (1969) Wood *et al.* (1965)
	Rhodospirillum rubrum		Benson *et al.* (1959a)
	Rhodomicrobium vannielli	0.01	Park and Berger (1967)
Phytoflagellates	*Euglena gracilis*		Rosenberg (1963)
	Ochromonas danica		Miyachi *et al.* (1966)
	Chlamydomonas reinhardii		Ohad *et al.* (1967)
Hemoflagellate	*Crithidia fasciculata*		

et al. (1965), and it would appear that the other *Rhodopseudomonas* species in their study should be reinvestigated.

In addition to its identification in photosynthetic organisms, the sulfonolipid has also been specifically associated with the photosynthetic process and/or membrane. Thus in gross analyses its concentration is highest in those plant tissues associated with photosynthesis (leaves) and lowest in nonphotosynthetic tissues (root, stem, and seed), although it is generally identified in plant tissue in at least trace amounts.

The first study of the variation of sulfonolipid concentration with chloroplast "concentration" (greening) was that of Rosenberg and Pecker (1964). These authors (see also Rosenberg and Gouax, 1967; Helmy *et al.*, 1967) were able to show a direct correlation between the appearance of chlorophyll and that of the galactosyl glycerides and sulfonolipid in *Euglena gracilis*. A dark-grown culture of the organism was exposed to light, and changes in the lipid composition were noted.

Kennedy and Collier (1963; Collier and Kennedy, 1963) have reported three sulfolipids in green plants, green algae, brown algae, and blue-green algae. In contrast, red algae and the fluffy pericarp of the broad bean *Vicia faba* contained only one sulfolipid. Unfortunately, these investigators did not positively identify the sulfur in each of their spots on the paper chromatograms of extracts but did a sulfur analysis on the mixture of three isolated from brown algae. Furthermore, the analysis was low (found, 3.68%; theoretical, 5.5%) based upon sulfonolipid structure. Their method of detection was based upon staining chromatograms with ionic dyes. In this system, the sulfolipids were characteristically pink, apparently because of their acidity. Many lipids were tested in their system (Kennedy and Collier, 1963) but not phosphatidyl glycerol. According to analyses in many other laboratories, this material should have appeared in their chromatograms. Since this very acidic lipid would likely stain as a sulfolipid in their system, it is probable that one of their spots was phosphatidyl glycerol. Additionally, Shibuya and Benson (1961) have shown that an unusually active lipase in photosynthetic tissue converts the sulfolipid to lysosulfolipid. It would therefore appear that the "three plant sulfolipids" in the chromatograms of Collier and Kennedy (1963) are phosphatidyl glycerol, sulfonolipid, and lysosulfonolipid. One implication of this interpretation of their results is that nonphotosynthetic plant tissue (fluffy pericarp of broad bean) and red algae contain the sulfonolipid but not phosphatidyl glycerol and do not contain an active sulfolipase. In addition, Collier and Kennedy (1963) reported a different sulfolipid that they found in two fungi, which will be discussed in Section V.

A striking observation in the phytoflagellate *Ochromonas danica* was made by Miyachi *et al.* (1966), who were surprised by the absence of sulfonolipid in the early reports of Haines (Haines and Block, 1962; Haines 1965) describing the alkyl disulfates in this microbe. The cells of these investigations contained chloroplasts but not the sulfonolipid. The cells had been grown under constant light in a sucrose medium. Benson's group repeated these analyses and obtained similar results. They then cultured the organism autotrophically with the result that the sulfonolipid appeared on chromatograms of lipids, although its concentration was still low (approximately one-fifteenth that of the alkyl disulfates). These experiments imply that the sulfonolipid plays a very direct role in photosynthesis, since it was the utilization of photosynthesis as the principal food supply that enhanced the sulfonolipid concentration. The organism contained large amounts of chloroplast membrane in both heterotrophic and autotraphic cultures.

A study of alfalfa leaf sulfonolipid composition by Klopfenstein and

Shigley (1967) showed that the sulfonolipid concentration varied seasonally. Higher concentrations are noted in spring, and the level drops gradually throughout the summer. They also noted changes in the sulfonolipid's fatty acid composition throughout the season. During the period of most active photosynthesis, the linolenic acid composition is highest. Palmitic acid increases and linolenic acid decreases with age.

The involvement of the sulfonolipid in photosynthesis might well be explained by its fatty acids. Erwin and Bloch (1963, 1964) had proposed that linolenic acid is involved in photosynthesis on the basis of the analytical data then available. Although subsequent studies have not yet demonstrated a direct connection, it has been shown that of the major chloroplast lipids—monogalactosyl diglyceride, digalactosyl diglyceride, phosphatidyl glycerol, and the sulfonolipid—both galacto lipids contain almost exclusively linolenic acid (Weenink, 1962; Sastry and Kates, 1963; Benson, 1963) and the sulfonolipid is approximately 50# palmitic acid and up to 50% linolenic acid (O'Brien and Benson, 1964; Klopfenstein and Shigley, 1966; Radunz, 1969), although the relative concentrations are variable under different growth conditions. Linolenic acid concentration in *Chorella* has been correlated with photosynthetic oxygen production (Appleman *et al.*, 1966). Recently, Brand *et al.* (1971) were able to show an absolute requirement for polyunsaturated fatty acid glycerides (with linolenic most active) in photosystem I (spinach) that had been extracted with heptane. Thus triglycerides containing polyunsaturated fatty acids but *not* plastoquinones, vitamin K, β-carotene, or α-tocopherolquinone restored photosynthetic activity. Ohad *et al.* (1967) have also reported a lower level of sulfonolipid in dark-grown *Chlamydomonas reinhardii* (which has lost nearly all its chloroplasts). They also found that greening is accompanied by a net synthesis of chloroplast membrane.

An extensive study on the greening of callus (tissue culture) of *Kalanchoe crenata* was recently conducted by Thomas and Stobart (1970). The molar ratios of various lipids to chlorophyll were examined through each of seven generations of cells. It took seven generations for the cultures to achieve the full green. Sulfonolipid was found to appear in lipid extracts in the third generation—*prior to the appearance of chlorophyll.* Although dark-grown cells contained mono- and digalactosyl diglycerides, no sulfolipid could be detected in these cells. Furthermore, although the galacto lipids never reached a constant molar ratio with respect to chlorophyll, the sulfonolipid rapidly achieved a chlorophyll-to-sulfonolipid ratio of 4.4 and remained at that level through greening. The appearance of the sulfonolipid prior to the appearance of chlorophyll was consistent with the earlier data of Rosenberg and Pecker (1964), and the constant molar

ratio of chlorophyll to sulfonolipid was in remarkable agreement with Lichenthaler and Park (1963). These data suggest the sulfonolipid may be involved in orienting the chlorophyll molecules in the membrane—whether or not participation in photosynthesis occurs.

Shibuya and Hase (1965) have also studied the destruction of the chloroplast membrane by bleaching *Chlorella protothecoides*. In addition to a decrease in sulfonolipid concentration during bleaching, they noted a large increase in 6-sulfoquinovosyl glycerol, implying that a sulfolipase is involved in the destruction of the chloroplast.

In a recent study of the lipid composition of chloroplast grana and stroma lamellae Allen and Park (1971) have found that these two membranes have very similar composition. These data are consistent with the model chloroplast membrane proposed by Weier and Benson (1967). This latter summary of the status of the chloroplast membrane problem places the sulfonolipid and the galacto lipids in the same role in the membrane.

IV. The Sulfolipids and Halosulfolipids of *Ochromonas*

In 1962, Haines and Block identified some ^{35}S-labeled lipids in extracts of the phytoflagellate *Ochromonas danica*. These substances, which have turned out to be strange membrane components, dominated the ^{35}S-labeled compounds in the cell. The substances were soon identified as sulfate esters that were present in amounts greater than most phospholipids or glycolipids in the cells. Elovson and Vagelos (1969) found them to constitute 3% of the dry weight of the cell. Although early reports indicated that these sulfatides are widespread in microbes and algae (Haines, 1965), some doubt is raised by recent attempts to label the halosulfatides in a wide variety of microbes with ^{36}Cl (Emanuel *et al.*, 1972). Of 12 microbes screened, only in *O. danica* and *O. malhamensis* were halosulfatides identified in the organisms. There is considerable evidence in the literature, however, that microbes excrete lipoid sulfate esters (Haines, 1965; Mumma and Gahagan, 1964; Roberts *et al.*, 1957). The nature of these sulfatides has not been described, but several were found to cochromatograph with the sulfatides herein described. Mumma and Gahagan (1964) had originally reported the ^{35}S-labeling of sulfatides excreted by higher plants. These substances were later found to be absent from axenic culture of the plants and probably produced by contaminating *algae* (Mumma, 1967).

That these halosulfatides are present in membrane is implied by their

large concentration as polar lipids. Evidence obtained from fragmented cells is consistent with this notion.

A. Isolation

The early studies of Haines and Block (1962) and later of Haines (1965) were conducted on labeled material and did not require the isolation of any significant amounts of material. Sufficient information was obtained, however, to permit a large-scale isolation of material by Mayers and Haines (1967). At first, cells were extracted with chloroform-methanol, 2:1, and the extract was saponified with 0.2*N* KOH at 37°C for 45 minutes. The latter procedure, which saponifies the phospholipids and glycolipids completely, does not attack the sulfatides. After removal of the non-saponifiable fraction by ether extraction followed by acidification and further ether extraction to obtain the fatty acids, the neutralized aqueous solution is extracted with n-butanol. Butanol has several advantages in this procedure: (1) It is the most polar alcohol that is not miscible with water, (2) It inhibits foaming during flash evaporation, and (3) it forms an azeotrope with water so that upon evaporation the sample is dry (several triturations with hexane is generally used to remove the last traces of butanol).

Initial attempts at isolating a pure sample of sulfatide for structural work were thwarted by the tenacious appearance of protein in the preparation. This was overcome by the digestion of the crude mixture with the

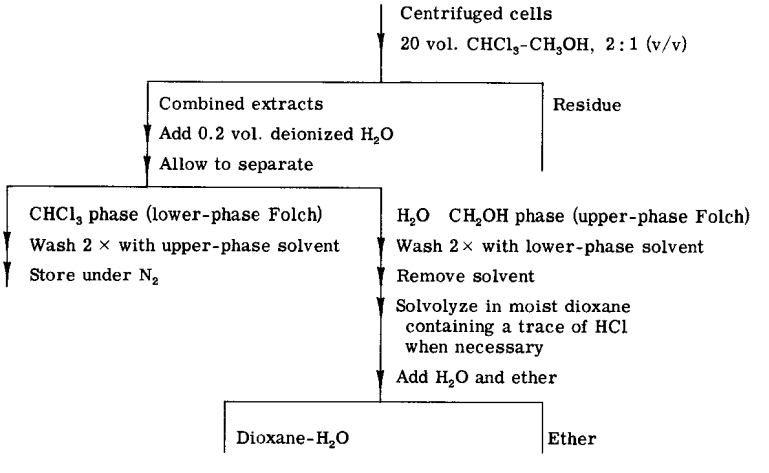


Fig. 3. Folch extraction of halodiols.

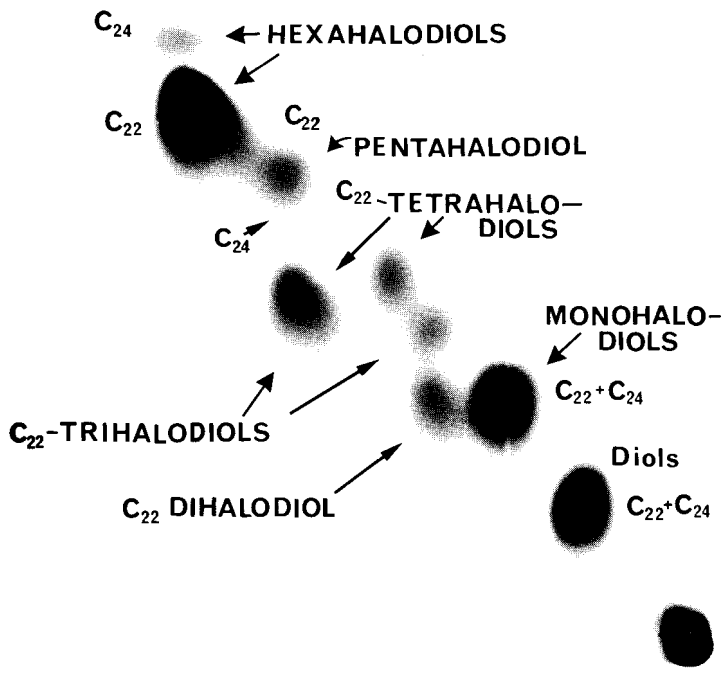


Fig. 4. Autoradiogram of a two-dimensional thin-layer chromatogram of halodiols after 1-^{14}C-laurate was incubated with *Ochromonas danica*.

proteolytic enzyme pronase (Haines, 1965; Mayers and Haines, 1967). Once the sulfatides were identified as disulfates, the requirements for pure sulfatides diminished, and crude preparations were hydrolyzed or solvolyzed so that structural work could be conducted on the diols that were obtained (Mayers *et al.*, 1969; Elovson and Vagelos, 1969; Haines *et al.*, 1969).

A simpler extraction procedure is that of Folch *et al.* (1957; Lees *et al.*, 1959); see Fig. 3. Although this procedure gives a crude product, it is quick and the *upper phase* contains approximately 90% of the sulfatides that are extracted from the cells by the chloroform-methanol. Unfortunately less than 60% of the sulfatide is extracted by the solvents; the remainder may be released from the fat-extracted residue by digestion with pronase (Haines, 1965).

The sulfatides are only poorly separated into their constituent components by thin-layer or column chromatography as sulfate esters. In addition to the streaking usually associated with acids of this type, the

chromatogram is further complicated by the fact that different salts of the sulfatides chromatograph to different positions. Since the mixture consists of halogenated alkyl diol disulfates, hydrolysis or solvolysis produces a quantitative yield of the halogenated alkyl diols, which may then be separated on thin-layer, column, or gas-liquid chromatography. The mixture of diols is quite complicated, however, and two-dimensional thin-layer chromatography (Mooney *et al.*, 1972) has proved quite useful as a method for separating the diols into their components (Fig. 4).

In the early identification work, Mayers and Haines (1967) had separated the nonhalogenated diol by repeated crystallization of the mixture from hexane. Of the entire mixture of diols shown in Fig. 4, only the nonhalogenated diols are insoluble in hexane; these may readily be separated from the halogenated diols by recrystallization.

B. Structural Studies

The structures of all the sulfatides in the mixture have not yet evolved, but the three principal components have been identified, as have several of the minor ones, and the pattern is rather clear. The first in the mixture was identified by Mayers and Haines (1967) as 1,14-docosanediol-1,14-disulfate. This was demonstrated by analysis and by the identification of primary and secondary sulfate in the infrared spectrum. Removal of the two sulfate groups was effected by hydrolysis or by solvolysis in dioxane, which was found to leave the orientation of a secondary C—O bond undisturbed (Mayers *et al.*, 1969). The resulting diol was identified by mass spectrometry as 1,14-docosanediol. This was confirmed by synthesis of 1,14-docosanediol and comparison of the infrared spectra of the two substances. Rotation of the diol established the configuration of the secondary hydroxyl as *S*, and since solvolysis of the secondary sulfate did not disturb the C—O bond, the original sulfatide was 1-(*S*)-14-docosanediol-1-(*S*)-14-disulfate. The diol resulting from solvolysis of this disulfate is the major diol shown in Fig. 4 and labeled "diols."

Elovson and Vagelos (1969) confirmed the above data and, using a gas-liquid chromatograph-mass spectrometer hookup, were able to identify a second diol, which Mayers and Haines (1967) could identify only as a tetracosane diol from its retention time on gas-liquid chromatography. The substance turned out to be 1,15-tetracosanediol. The ratio of the C_{22} to the C_{24} diols is about 8:1, as shown by a gas-liquid chromatogram (Haines, 1971). These diols are shown in Fig. 4 near the origin of the chromatogram.

The spot above these substances has been characterized by Haines *et al.* (1969) as *threo*-(*R*)-13-chloro-1-(*R*)-14-docosanediol derived from

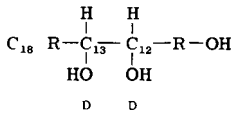
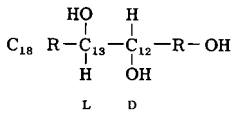
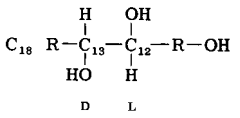
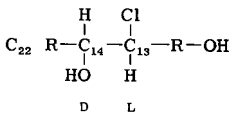
Compound	Configuration	Rotation (deg)
	*erythro*	−1.7
	*threo*	−23.8
	*threo*	+23.8
	*threo*	+14.7

Fig. 5. Rotations of some dihydroxy and chlorohydroxy long-chain alcohols. The glycol data are those of Morris and Wharry (1966). The chlorohydrin data were obtained by Haines *et al.* (1969). Other chlorohydrins in the literature have rotations similar to glycols of analagous configuration.

the corresponding disulfate. The diol was identified by its mass spectrum, infrared spectrum, rotation, analysis, and nuclear magnetic resonance spectrum. The configuration of the chlorohydrin was determined by rotation with comparison to those of Morris and Wharry (1966); see Fig. 5. The configuration of the chlorohydrin was confirmed by conversion to the *cis*-epoxide and comparison in thin-layer chromatography to authentic *cis*- and *trans*-epoxides and *threo*- and *erythro*-chlorohydrins. The identification of the 13-chloro was also confirmed by a mass spectrum of the silyl derivative obtained by Elovson and Vagelos (1969). These investigators also identified a small amount of 14-chloro-1,15-tetracosane-diol by the gas-liquid chromatography-mass spectrometry combination.

Two dichloroalkane diols have been identified in the mixture to date: 11,15-dichloro-1,14-docosanediol by Elovson and Vagelos (1969) and 2,2-dichloro-1,14-docosanediol by Pousada *et al.* (1972b). They have been identified by mass spectral evidence only, but include derivatization in each case.

The trichloro and tetrachloro derivatives have not yet been identified, although one can tell from the mass spectra how many chloro groups are

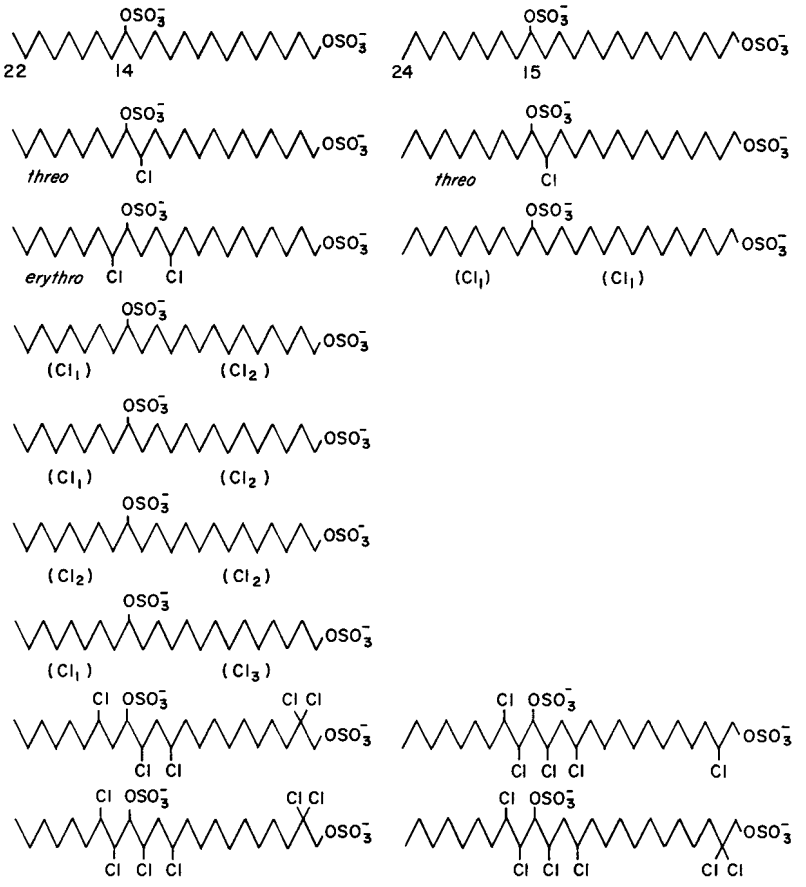


Fig. 6. Structures of chlorodiol disulfates characterized to date.

distal or proximal to the secondary hydroxyl. These are summarized in Fig. 6.

The pentachloro docosanediol was identified by Pousada *et al.*, (1972b) as 2,2,11,13,15-pentachloro-1,14-docosanediol by mass spectral evidence coupled with a nuclear magnetic resonance spectrum. In a like manner these investigators identified 2,12,14,16,17-pentachloro-1,15-tetracosanediol and 2,2,12,14,16,17-hexachloro-1,15-tetracosanediol.

In an elegant combination of degradative chemistry, ^{36}Cl radiocounting, and mass spectrometry, Elovson and Vagelos (1970) identified the major hexachlorodocosanediol as 2,2,11,13,15,16-hexachloro-1,14-docosanediol.

It appears that the two latter hexachloro compounds are the end products of a series of chlorinating enzymes. The structures of all the known compounds are shown in Fig. 6.

In addition to the chlorosulfatides, Pousada *et al.* (1972a) have isolated a series of bromosulfatides of similar structure. In this series only the *threo*-(*R*)-13-bromo-1-(*R*)-14-docosanediol-1-(*R*)-14-disulfate has been characterized. It was identified by its mass spectrum, nuclear magnetic resonance spectrum, rotation, and analysis. The 2,2,11,13,15,16-hexabromo-1,14, docosanediol was also obtained from these cultures, as were several of the tri- and tetrabromo intermediates. These were identified by their relative positions on a two-dimensional thin-layer chromatogram comparable to that in Fig. 4 and by their mass spectra. The bromine was also confirmed by the incorporation of ^{89}Br into the respective bromodiols.

C. Analysis

Probably the surest and quickest method of analysis of halosulfolipids is ^{36}Cl labeling followed by thin-layer chromatography of the upper phase of a Folch extract of the tissue. This method, which will obviously not identify the nonhalogenated 1,14-docosanedilo-1,14-disulfates, is not as simple as it would appear. Since ^{36}Cl-chloride ions chromatograph up the plates, Emanuel *et al.* (1972) found it necessary to cospot silver nitrate on the thin-layer plate in order to prevent ^{36}Cl-chloride from moving from the origin. This modification of the above procedure permitted the rapid screening of organisms for chlorosulfatides.

The use of ^{35}S-sulfate, which was the original method of identification (Haines and Block, 1962), is also available. Since unknown sulfolipids may cochromatograph with the halosulfatides in a given solvent system, it would be advisable to solvolyze the sulfatides and chromatrograph the resulting diols for a positive identification. 1,12-Octadecanediol is commercially available as a standard for thin-layer chromatography of 1,14-docosanediol. It should be noted that the halogenated diols run ahead of the unsubstituted diols on thin-layer chromatograms. The solvolysis procedure (Mayers and Haines, 1967) is especially desirable for the identification of sulfate esters because of its high specificity for this functional group.

The methods available for the analysis of sulfatides have recently been reviewed (Haines, 1971). They include oxidative, reductive, colorimetric, turbidimetric, flame photometric, infrared spectrophotometry, activation analysis, and radiometric methods. Of special note is the colorimetric method used by Haines (1965) for these compounds. This method has been

improved by Kean (1968) and used by him as a very selective nondestructive method for the identification and quantitation of sulfatides in a complex mixture of crude lipids.

D. Biosynthesis and Metabolism

The biosynthesis of the halosulfatides is not yet understood. The structures of the various compounds provide some clues to their biosynthetic route, and perhaps these should be discussed first. There are only two series of aliphatic disulfates in the mixture—1,14-docosanediol-1,14-disulfate and 1,15-tetracosanediol-1,15-disulfate. The difference between these series is that the chain of the tetracosane series is longer by one methylene group *both proximal and distal* to that of the docosane series.

Mooney *et al.* (1972) have shown that ^{14}C-acetate and ^{14}C-octanoate are efficiently incorporated into the sulfatides (Fig. 7). This suggests that the chain is biosynthesized by the usual fatty-acid-synthesizing enzymes. It is presumed that the sulfate is derived from PAPS, as this intermediate has been reported to be the intermediate in the biosynthesis of all the sulfate esters where the biosynthesis has been investigated (Roy and Trudinger, 1970). As PAPS sulfates hydroxyl groups, it remains to determine how the hydroxyl is incorporated into the chain.

Three routes are possible for the synthesis of hydroxy fatty acids. The first is that of synthesis during the chain-lengthening process and would presumably be anaerobic. A second conceivable route is direct hydroxylation by a hydroxylase using molecular oxygen such as the microsomal P-450 hydroxylase of the mammalian liver. A third possibility is the hy-

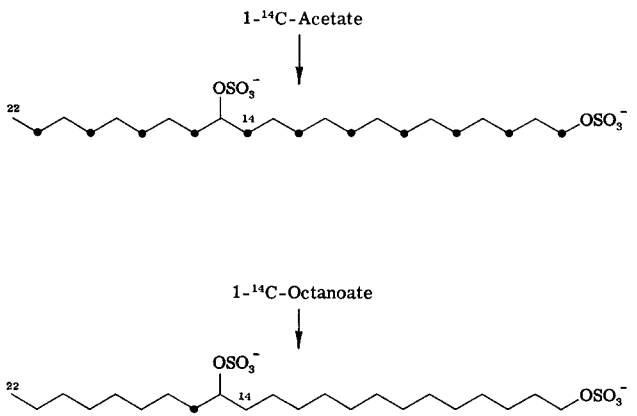


Fig. 7. Incorporation of acetate and octanoate into 1,14-docosanediol-1,14-disulfate.

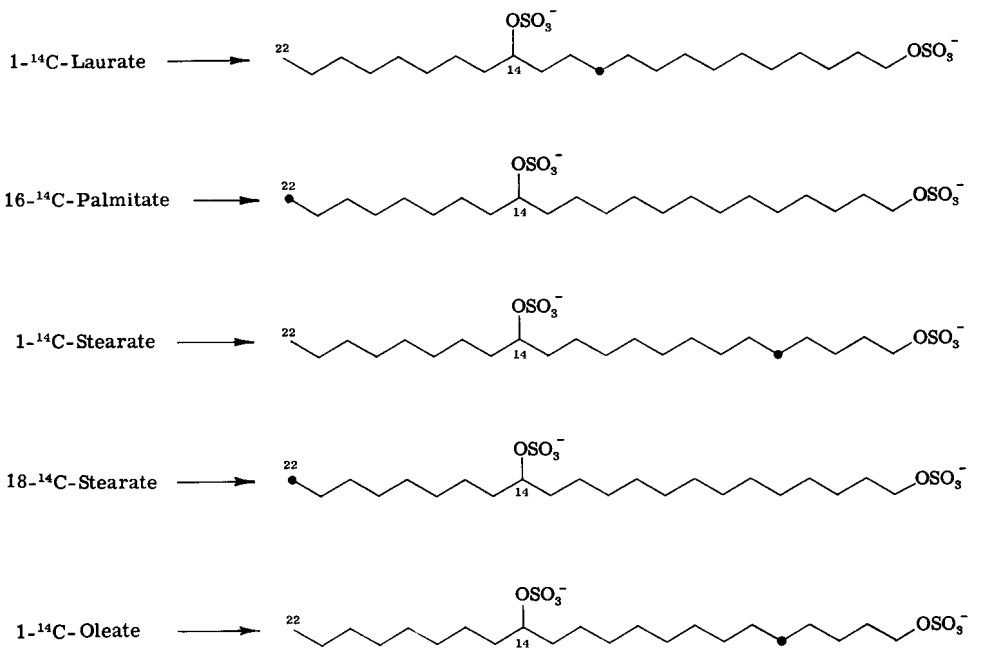


Fig. 8. Incorporation of fatty acids into 1,14-docosanediol-1,14-dusulfate.

dration of a double bond that could itself be introduced either during the chain-lengthening process (anaerobically) or into the saturated chain. An example of the latter is the biosynthesis of 10-hydroxystearic acid from oleic acid by a pseudomonad, NRRL-B-2994, (Niehaus *et al.*, 1970). Mooney *et al.* (1972) have reported the rapid incorporation of laurate, palmitate, stearate, and oleate into the chain of the halosulfatides. These data (Fig. 8) show that the hydroxyl function is introduced onto the unsaturated chain and not during the chain-lengthening process.

Since the hydroxyl is on the 14 position in the docosane series and on the 15 position in the tetracosane series, the double bond of oleic acid could be hydrated after chain lengthening to C_{22} or C_{24}. In the first case the hydroxyl would be placed on C-10 of oleic acid and in the second case it would be situated on C-9. This biosynthetic route could explain the one-carbon difference in position. Mooney *et al.* then fed ^{14}C-oleic acid to the organism. The oleic acid was incorporated into the sulfatides as indicated in Fig. 8. It therefore appears that a double-bond intermediate is likely for the introduction of the hydroxyl.

Two important aspects of the metabolism of these substances in cells have been established. The first was noted in the very first paper by Haines

and Block (1962). It was found that *Ochromonas* is unable to cleave the sulfate groups from the chain. Thus ^{35}S-sulfate is used efficiently for the biosynthesis of cystine and methionine in proteins, whereas cultures that were incubated with the ^{35}S-labeled sulfatides did not label the sulfur amino acids. In these experiments the ^{35}S-sulfatides were incorporated into the cells to the same extent as sulfatide was when the label originated as sulfate. The compounds are thus remarkably inert metabolically.

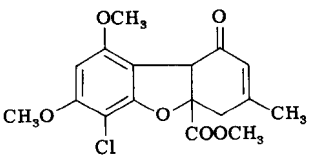
A second aspect of their metabolism was noted first by Elovson and Vagelos (1969), who observed a dramatic increase in the amount of hexachlorosulfatide in the presence of a media rich in chloride. This was confirmed by Pousada *et al.* (1972c), who also found that the organism survived in a chloride-free culture medium but that its morphology was changed somewhat and that it produced the nonchlorinated diol exclusively.

The sulfatases in *Pseudomonas* $C_{12}B$ described by Payne and Painter (1971) were shown to cleave 1,12-octadecanediol-1,12-disulfate and, judging from the similarity of structure, are very likely to hydrolyze the sulfate esters in these compounds.

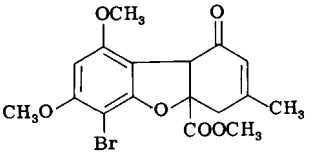
An autoradiogram of a two-dimensional chromatogram of the diols obtained from ^{14}C-laurate-labeled sulfatides is shown in Fig. 4. The pattern is identical to that obtained from the charred diols in extracts of the cells. It is also identical to autoradiograms obtained from ^{14}C-palmitate- and ^{14}C-stearate-labeled diols. Of special importance is the fact that the C_{24} and the C_{22} halogenated diols are labeled to the same extent. This suggests that they are derived from the same intermediate and that the chloro groups are introduced *onto the saturated chain.* This implies that the ^{14}C-fatty acids are *first* incorporated into the nonhalogenated diol disulfate and that this molecule is then the substrate for the chlorinating enzyme(s). This is consistent with the location of the chloro groups around the sulfate groups on the molecule. It is also consistent with the shift of one carbon for each of the chloro groups around the 15-sulfate in the tetracosane series.

A large number of halogenated compounds are reported in the literature. Most of the chlorinated compounds have been reported in fungi or actinomycetes. Several of these are shown in Fig. 9 along with the bromo derivatives that have been obtained in each case by excluding chloride from the medium and adding bromide. It was by this approach that Pousada *et al.* (1972a) were able to obtain bromosulfolipids. It might be noted that to date no natural chloro compounds have been characterized or reported in marine organisms, although at least ten bromo compounds have been described. The only halogenated compound whose biosynthesis has been investigated is that of Caldariomycin in the fungus *Caldariomyces*

Griseofulvin
(penicillium)

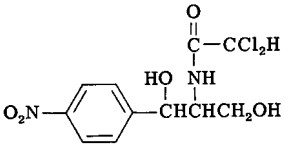


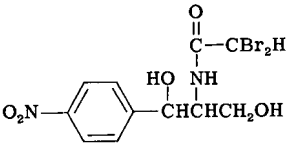
Oxford, Raistrick, and
Simonart, 1939



MacMillan, 1954

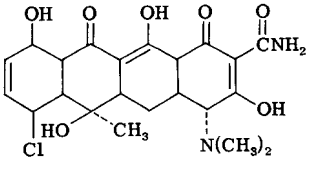
Chloramphenicol
(streptomyces)

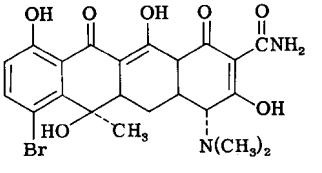




Smith, 1958

Tetracycline
(streptomyces)





Sensi, de Ferrari, Gallo,
and Rolland, 1955

Fig. 9. Some natural chloro compounds that have also been isolated as bromo compounds. In each case the organism was grown in the absence of chloride and in the presence of bromide.

fumago by Hager *et al.*, (1970). Hager's group has isolated and characterized the chloroperoxidase that inserts the chloro group on the antibacterial product (Brown and Hager, 1967; Morris and Hager, 1966). The purified enzyme does not have a high specificity for the organic substrate and is capable of bromination as well as chlorination, although the rate of bromination is much slower than that of chlorination. This is consistent with the lower amounts of bromolipids produced and the considerably slower growth rates observed in bromide media for *Ockromonas danicd.*

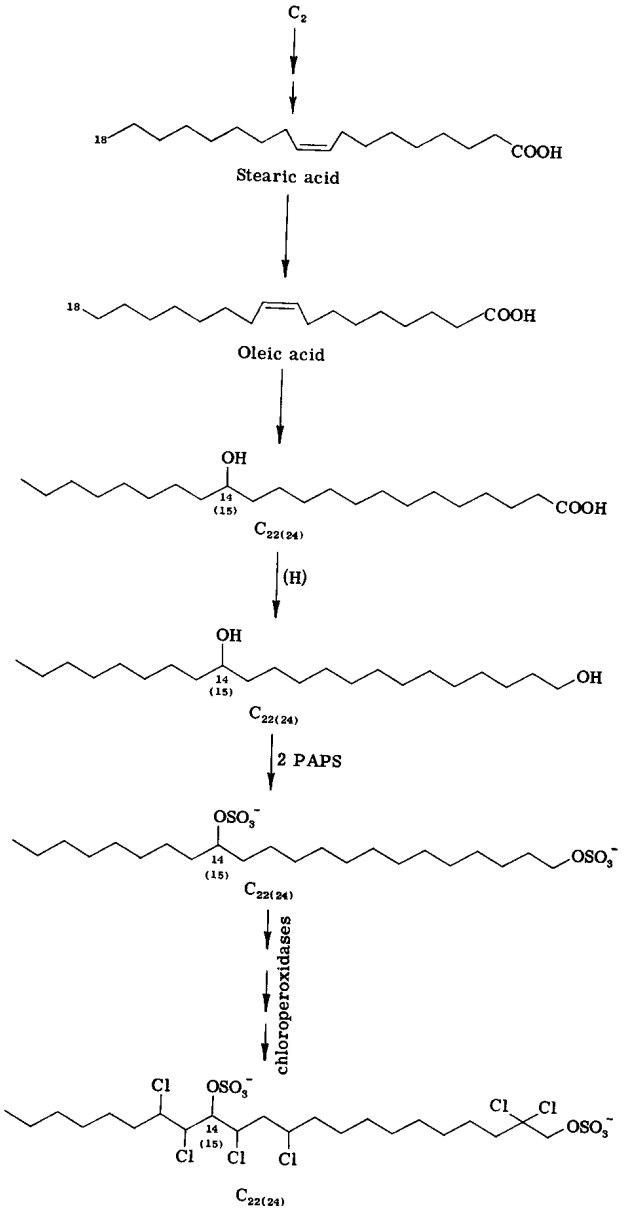


Fig. 10. Proposed biosynthesis of the halosulfatides.

An important difference between these halosulfatides and the natural and synthetic substrates for the chloroperoxidase of Hager, *et al.*, (1970) should be noted. The Hager enzyme could use Cl^+ as a chlorinating intermediate, as it chlorinates phenyl rings or benzyllic carbons, whereas this enzyme (these enzymes) places chloro groups on a saturated hydrocarbon chain. This suggests a more energetic free radical intermediate, possibly a hydroperoxide.

These proposals are summarized in Fig. 10, which suggests a complete biosynthetic scheme for the compounds. It is assumed that there are two sulfating enzymes.

E. In Membrane Vesicles

The occurrence of these compounds in a membrane is of significance because of their unique structure as polar lipids. Recent evidence obtained from freeze-etch electron micrographs (Pinto da Silva and Branton, 1970) and from X-ray electron density profiles (Wilkins, 1972) indicates that there is a "cleavage plane" or region of very low electron density down the center of a variety of natural membranes. These data are consistent with the earlier proposals of a bilipid leaflet model for membrane structure (Gorter and Grendel, 1925; Danielli and Davson, 1935), which was subsequently demonstrated to be the structure of myelin by Schmitt and Bear (1939). The application of the freeze-etch and X-ray techniques to artificial bilayers of fatty acids or phospholipid micelles lends further support to this model of membrane structure. The picture does not appear to be as simple as the model would suggest, however, for there is much evidence that proteins are deep within the hydrophobic region of many membranes and furthermore that much of the hydrophobic region of membranes is occupied by proteins (Green and Perdue, 1966; Weier and Benson, 1967). Additionally, judging from their inability to cleave with the freeze fracture technique, some membranes such as mitochondrial membranes do not appear to contain such a cleavage plan. This is especially significant in view of the fact that the mitochondrial membrane has a trilamellar structure as viewed in thin-section electron microscopy, as do other membranes.

The bilipid leaflet as a model for membrane structure is based upon the stability of a monolayer of aliphatic hydrocarbon chains, which terminate as a methyl group at one end and a polar hydrophylic group at the other end. This is the structure of all polar lipids known to date. The halosulfolipids present an interesting exception to this pattern, as they all contain a sulfate group at one end of the chain and a second sulfate near the other end of the chain. These groups are negative at all aqueous pHs. Thus the lipid is not capable of forming a monolayer and likewise, presumably, a

bilayer. It is thus of some value to determine whether or not these compounds are present in a membrane of the organism. Several attempts have been made to establish that this is the case, with limited success.

It was observed early that these compounds represented well over 50% of the sulfur in the cell (Haines, 1965). Elovson and Vagelos (1969) reported that they constitute over 3% of the dry weight of the cell. Aaronson and Baker (1961) and also Haines (1965) had found that lipids represent about 10% of the dry weight of the cells. This indicates that these compounds represent about one third of the lipid. In our experience the value varies from about 10% to about 40%, depending on the culture conditions. The low value would be the case for a chloride-free culture medium and the high value for a high bromide medium. It should be noted that the halogen itself may contribute substantially to the weight of the sulfatide mixture.

Early experiments by K. Kahn and T. H. Haines, which were summarized by Haines (1966), demonstrated that the sulfatide appeared in a major peak in a Ficoll density gradient that was designed for the examination of membranes by Kamat and Wallach (1965). The peak of ^{35}S-activity corresponded to a band in the density gradient that had the physical appearance of a membrane band. Numerous attempts to repeat these experiments were not successful, although Poncz and Haines (1972) have found a sharp band on a density gradient that has a very high ^{35}S-sulfatide-protein ratio.

It has been found (Haines and Block, 1962) that the sulfatides are excreted into the medium in some quantity. Subsequently, Gellerman and Schlenk (1964) reported that a pellet obtained by high-speed centrifugation of the medium (after removal of the cells) contained a large amount of a substance identified with the sulfatides (Haines, 1965). It was therefore of considerable interest to find that the organism grown under the same culture conditions excretes membrane vesicles (Orner *et al.*, 1972). These vesicles were examined as thin-section electron micrographs and have the trilamellar appearance of other biological membranes. Studies are currently under way to determine the precise lipid composition of these vesicles that are rich in halosulfatides.

V. Miscellaneous Sulfolipids of Eukaryotic Microorganisms

Sulfolipids have been reported in a variety of living systems, which include nearly the entire biosphere. Mammalian sulfatides include cerebroside sulfate, sulfo-lac ceramide, and ganglioside sulfate (Haines, 1971).

A sulfonolipid similar to that of the plant sulfolipid has been described in the sea urchin (Isono and Nagai, 1966).

There are several reports of sulfolipids in bacteria. Kates *et al.* (1967, 1968) have described a sulfate ester of 1-*O*-(glucosylmannosylgaloctosyl-2,3-di-*O*-phytanyl-L-glycerol in the extremely halophylic bacterium *Halobacterium cutirubrum*. Hancock and Kates (1972) have recently reported the 2,3-di-phytanyl-L-glycerol-1-sulfate in the same organism. Marshall and Brown (1968) have also found the glycoside in the extreme halophile *Halobacterium halobium*. Apparently it is not present in moderately halophilic or nonhalophilic bacteria but occurs in all extreme halophiles thus far examined (Kates *et al.*, 1967). Another prokaryote, *Mycobacterium tuberculosis*, contains a sulfatide mixture (Goren, 1971) that contains the 2,3,6,6′-tetraester of 2′-trehalose sulfate. The esters are of unique branched fatty acids, which is not unusual for *Tubercle bacillus*. The sulfatide's concentration in the cell of a variety of strains is apparently proportional to the virulence of the strain (Gangadharam *et al.*, 1963). In their classic study of the metabolism of *Escherichia coli* with radioisotopes, Roberts *et al.* (1957) reported a sulfolipid excreted by the bacterium. The substance constituted 20% of the sulfur in the organism. It was not present in the cells; the substance was identified only as a spot on a paper chromatogram.

A sulfolipid has been found in diatoms that does not correspond to the plant sulfonolipid (Kates and Tornabene, 1972). The substance appears to be a sulfate ester.

The conidia of the fungus *Glomerella cingulata* contain a sulfolipid (Jack, 1964). The substance was identified by thin-layer and paper chromatography of lipid extracts after incubating the organism in the presence of ^{35}S-sulfate. The compound was slightly more polar on thin-layer chromatograms than the most polar phosphatides and was not positive to ninhydrin.

Another investigation of fungal lipids was conducted by Collier and Kennedy (1963). They reported a sulfolipid in the fungus *Coprinus atramentarius* Fr. that did not cochromatograph with the sulfolipid they found in the photosynthetic microorganisms [presumably the sulfoquinovose compound of Benson *et al.* (1959a)]. A sulfolipid that cochromatographed with this new material was also reported in the fungi *Psalliota campestris* Quél and in the fruit bodies of *Clitocybe aurantiaca* Fr. It should be pointed out that the identification of these substances as sulfolipids is based solely upon their staining properties as very acidic lipids. The weakness here has already been discussed (Section III, E).

A variety of sulfolipids has been reported in insects, chicken eggs, etc., and has been reviewed by Haines (1971).

These scattered reports indicate that in our quest for sulfolipids only the surface has been scratched. Although there is a greater multiplicity of phospholipids and although they constitute a larger portion of the lipids in membranes, it appears that sulfatides are ubiquitous and that they are generally present in selected membranes.

VI. Sulfolipids and Membranes

The eukaryotic microorganisms are characterized by the presence of more than a single membrane. It is precisely this quality that makes a study of sulfolipids in eukaryotic microorganisms especially interesting. Where they have been studied, there has been but one sulfolipid in each membrane that contains them, and frequently this is the only sulfolipid in the microorganism. This substance then represents a tool for studying the membrane in question—its biogenesis, its metabolism, and its structure. For example, should the biosynthesis of the sulfolipid be inhibited selectively, then the biogenesis of that particular membrane would probably be blocked as well or its structure distorted. Studies of the structure and function of the membrane may be conducted by a replacement of a natural sulfolipid with analogs while the biosynthesis of the natural sulfolipid is blocked genetically or with an inhibitor. These approaches have been used in the study of chloroplast membranes by several investigators, as discussed earlier, but the full use of the approach has not yet been realized in this area. This has also been the case with the halosulfatides. In neither case is there a detailed understanding of the route of biosynthesis, nor have any mutants been reported to be missing appropriate enzymes. The whole area of lipid genetics is only just emerging in the prokaryotic microorganisms.

A final point should be made about a possible special role of the sulfolipid in membranes. The sulfate ester or sulfonic acid is unique in biological systems because of the extremely low pK value of the anion. It is therefore highly probable that a counterion will be present under nearly all circumstances in biological systems. It may very well be that their principal role is that of transporting the cation. Furthermore, those systems such as the stomach in which an extremely low pH is *maintained* are likely to contain a sulfate ester or sulfonic acid as the proton carrier, since few other organic groups (if any) can maintain such a low pH.

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