

The sulfolipids*

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The ubiquity of sulfate in biology, mainly bound by ester linkage, is attested to by the large number of classes of such compounds which have been described and are being newly discovered. A sulfate chemistry, perhaps as extensive as we now know for phosphate, is within the realm of possibility. The list of such compounds now includes: (a) the sulfated mucopolysaccharides; (b) the steroid sulfates; (c) the phenol sulfates, including tyrosine-O-sulfate found free or in peptide linkage, triiodothyronine sulfate, and triiodothyroacetic acid sulfate; (d) bilirubin sulfate; (e) the arylamine sulfates; (f) choline sulfate in lichens; fungi and marine algae; and (g) the sulfolipids.¹

Although the history of the study of sulfur associated with lipid dates back to at least the 1880's (1, 2, 3), subsequent investigation in this area has been characterized by sporadic periods of activity. Within the past few years, with the development of methods for the separation and isolation of these compounds from related substances, in large part due to the efforts of Lees *et al.* (4, 5) and Radin *et al.* (6, 7, 8), there has been a renewed interest in their biosynthesis and metabolism. The relative importance of the sulfolipids in biology is implied by the finding that in certain forms the sulfur-containing lipids challenge the phosphatides as the major ionic lipid component. With the advance in our understanding of mechanisms concerned in the formation of the complex sphingolipids (9 to 12) and of the enzymatic reactions leading

to the activation and transfer of sulfate to a host of acceptors (13 to 22), the point has been reached where systematic studies on the cell-free biosynthesis of sulfolipids can be attempted.

This review will attempt to summarize what little is known regarding the sulfolipids as concerns their chemistry, procedures used in separation and isolation, and studies on the biosynthesis and metabolism of these compounds *in vivo* and *in vitro*. Since much of this work is of relatively recent origin and remains necessarily incomplete, this review will be more in the nature of a progress report designed to stimulate interest in what appears to be an exciting and promising area.

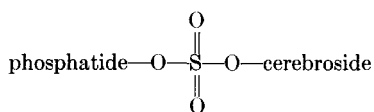
BRAIN SULFATIDE

Chemical Characterization. The sulfur-containing lipid which has been most thoroughly investigated and about which we know most is the so-called sulfatide found in normal mammalian brain. In 1874 Thudichum (1, 2, 3) reported the presence of sulfur in a preparation of cerebroside prepared from brain. Brain protagon, the white mass resulting after exhaustive acetone-ether extraction of brain, was thought by many to represent a single lipid species with both sulfuric and phosphoric acids in the structure of its molecule. Thudichum, however, believed that the sulfolipid was a distinct lipid, having some properties in common with the phosphatides. Unfortunately he failed to separate the two substances. The ratio of sulfur to phosphorus in his purest samples was 3:2. Some years later Koch (23) isolated a sulfate-containing lipid from an ether-insoluble residue which contained 1.91% sulfur, 1.80% phosphorus, and 12.8% sugar. Koch was led to the conclusion that the sulfolipid contained an equimolar proportion of sulfuric and phos-

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¹ Sulfolipid is used here to refer to all lipid-soluble compounds which contain sulfur in covalent linkage. Abbreviations used: UDP-gal = uridine diphosphate galactose; acyl-CoA = acyl-Coenzyme A; ATP = adenosine triphosphate; ADP = adenosine diphosphate; PP = pyrophosphate.

phoric acids. The analytical data led him to formulate the following structure:

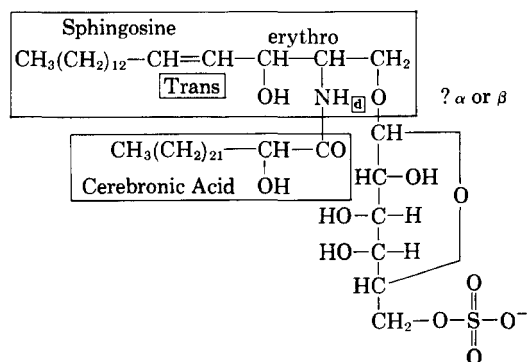


Thus the phosphatide-cerebroside-sulfatide of Koch contained all the elements that were supposed to be parts of protagon. This controversy was finally settled by Levene (24) who, in studies on the lipids of beef brain, isolated a sulfur (2.66%) containing lipid which was entirely free of phosphorus. Later, in work on the characterization of the haptenic substance present in the protagon of beef brain and horse kidney, Landsteiner and Levene (25, 26) isolated an orcinol-positive lipid, having 2.87% sulfur and no phosphorus. So the matter lay until relatively recently when, as will be discussed later, the issue of a phosphate-containing sulfatide has again been raised.

It was not until 1933, however, that this substance was characterized chemically. At this time Blix (27) was able to isolate from normal human brain a sulfatide, as its potassium salt, free of phosphorus, which amounted to 20% to 25% of the total cerebrosides. Its constituents, upon hydrolysis, were cerebronic (hydroxylignoceric) acid, sphingosine, galactose, and sulfate. The composition suggested a compound made up of one part of each of the above substances. The position of the sulfate group was not determined, but Blix suggested that it was probably esterified with the galactose. The galactose was thought to be glycosidically linked to one of the hydroxyl groups of sphingosine and the fatty acid in amido linkage with the sphingosine moiety.

Little, if any, work had been done on the cerebroside sulfuric acid ester from the time of Blix's isolation and characterization until 1951, when Nakayama (28) examined the question of the position of the sulfate group. From his unsuccessful efforts at tritylation of the cerebroside sulfate, he concluded that the primary hydroxyl group of the galactose was esterified by sulfate. Thannhauser *et al.* (29), however, were also unable to tritylate beef brain cerebrosides under the same and more vigorous conditions, so that the negative evidence of Nakayama did not permit any conclusion as to the structure of the cerebroside sulfate. These investigators, working with relatively pure cerebroside sulfate ester (although obtained in poor yield) isolated from beef brain (30), were able to methylate the compound, and by identification of the products of methanolysis, were able to show unequivocally that the sulfuric acid must be esterified to the primary hydroxyl function on carbon-6 of the galactose of the sulfatide.

Based on the work of Blix (27) and Thannhauser *et al.* (29) and in analogy with the known structure of the cerebrosides and sphingomyelin (31 to 39), it is assumed that the structure for cerebroside sulfate may be written as follows:



Cerebroside Sulfate (Sulfatide)

Recently Jatzkewitz (40) was able to separate chromatographically "sulfatides" with cerebronic, lignoceric, or possibly nervonic acid as the fatty acid component. The strongly metachromatic reaction given by sulfatide with toluidine blue suggests the possibility of a polymerized macromolecule (41).

Solubility and Chemical Properties. The solubility of sulfatide in various solvent systems has been exhaustively studied by Lees *et al.* (4, 5) and has been made the basis of extraction and separation procedures. The divalent cation salts of brain sulfatide are more favorably disposed to the chloroform phase for solution than the sodium or potassium salts (7).

The results of partial acid and alkaline hydrolysis of brain sulfatide have been reported only recently (5). Of interest, and contrary to expectation, based on Klenk's experience with the cerebrosides (42), upon hydrolysis with saturated $\text{Ba}(\text{OH})_2$ there was a relatively slow appearance of free amino nitrogen, while the galactosidic linkage of the sulfatide was cleaved relatively easily. Unlike the case with the cerebrosides, such treatment of sulfatide resulted in little or no liberation of psychosine (galactosylsphingosine). It should be noted that the infrared absorption of the sulfuric acid ester grouping has been made the basis for the microdetermination of sulfatide (43).

Separation of Brain Sulfatide. Until recently, the methods used for the isolation and preparation of brain sulfatide have been complicated and tedious, and have resulted in extremely poor yields (27, 30). Within the past few years relatively simple column (Florisil[®] plus ion exchange) (6, 7, 8, 44) and paper (45, 46) chromatographic techniques have been developed for

the separation of sulfatide from closely related contaminating substances. Lees *et al.* (4, 5) have devised a procedure for obtaining brain sulfatide in good yield based on the distribution of brain lipids between the two phases of a series of related solvents.

Phosphate-containing Sulfatide. The question of the existence of a phosphate-containing sulfatide, which had been considered resolved with Levene's work (24, 25, 26), has been raised again. Radin *et al.* (6) found that about 0.3% of the brain lipid phosphorus (after removal of the phosphatides by Florisil®) was retained by ion exchange resins. In subsequent work these workers (7) reported that when P³²-phosphate was injected into rats and the sulfatides were isolated as the barium salt, about 1.3% of the total lipid activity was found in this fraction. Attempts to separate *in vivo*-labeled P³² and S³⁵ in the sulfatide fraction were unsuccessful. Similarly, Lees (4) found by the method of "linked distributions" that half of the sulfatides of the starting lipid material could be obtained in two main fractions, and that one fraction was phosphorus-free and the other contained both sulfur and phosphorus.

For the first time, by the development of newer methods for the preparation of sulfatides, these two groups of workers were able to isolate this material in respectable yield. It seemed possible, therefore, that previous workers were able to obtain a phosphorus-free sulfatide fraction simply because that which was intimately associated with phosphorus had been discarded by the relatively crude isolation procedures. It is pertinent in this connection that paper chromatography of purified sulfatide by the method of Jatzkewitz leads to the appearance of two "sulfatide" spots, one of which, according to Jatzkewitz (45), contains phosphate, although not in sufficient amount to be part of the sulfatide molecule (40).

The fallibility of arriving at conclusions concerning covalent linkage based on the similarity of physical properties of substances is not new in lipid chemistry. That solvent solubilities and chromatographic characteristics of lipid compounds may be profoundly altered by trace contaminants of other lipid substances is well known. The recent work of Lees *et al.* (15) suggests strongly that the so-called phosphate-containing sulfatide may be a case in point. These investigators have found the phosphorus-free fraction (I) to be probably pure sulfatide, and the second fraction (II) to be a lipid mixture which could be separated into a phosphatide and sulfatide fraction by passage through a Florisil® column. The phosphatide fraction was composed of a mixture, over half of which comprised phosphatidyl serine; the sulfatide fraction separated

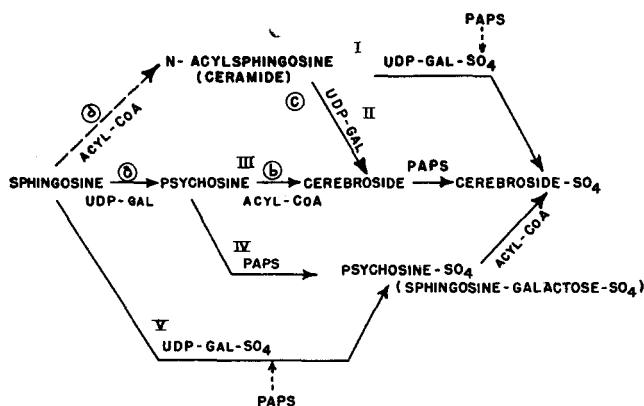
by Florisil® was impure, containing cholesterol and other galactose-containing lipids. The ratio of moles of galactose to atoms of sulfur was 1.4:1. Characterization of this fraction remains incomplete, but so far there is no evidence that the sulfatide present in this fraction (II) differs in composition from that in the other fraction (I). The presence of the phosphatide contaminant was shown, however, to alter the chemical reactivity of the sulfatide. Before removal of the phosphatide contaminant by Florisil®, the sulfatide in this fraction (II) is much more resistant to acid hydrolysis than the pure sulfatide of the other fraction (I). After Florisil® treatment, however, the ease with which sulfate is released becomes the same. A similar resistance to acid could be demonstrated with pure sulfatide when phosphatide was added to the hydrolysis mixture.

Tissue Distribution. In addition to the finding of sulfolipid in horse kidney by Landsteiner and Levene (25, 26), there have been other reports of sulfur-containing lipids in tissues other than that of the nervous system. In 1907, Koch (47) found sulfolipids in liver, testes, submaxillary glands, and muscle. Sammartino (48) has reported finding sulfur-containing lipids in the lung. Blix (27) has suggested that the sulfolipid reported to have been isolated from dog and rabbit liver, beef spleen, horse blood, and muscle by Baldi (49), and from the adrenal of cattle and horses by Manasse (50), is the cerebroside sulfuric acid ester. These sulfolipids had not been further characterized until recently, when Green and Robinson (51) were able to demonstrate what appears to be cerebroside sulfate in rat kidney, liver, and spleen, and mouse mastocytoma. As will be noted later, under pathological circumstances sulfatide may accumulate in large amounts in several tissues outside the nervous system.

In Vivo Biosynthesis and Metabolism. One of the earliest studies on the incorporation of radioactive sulfur into the brain was reported in 1945 by Dziewiatkowski (52), who found 0.02% of a dose of orally-administered S³⁵-sodium sulfide to be located in the brain. Bostrom and Odeblad (53) found the uptake of radioactive sulfate to be highest in the gray matter. The incorporation of parenterally-administered radioactive sulfate into isolated sulfolipid of rat brain has been demonstrated by Holmgård (44). By the administration of C¹⁴-galactose and S³⁵-sulfate to rats, Radin *et al.* (7) were able to measure the turnover of chromatographically-isolated brain sulfatide and compare its metabolism with that of cerebroside. It was found that the rate of incorporation of C¹⁴-galactose into sulfatide was slower than that into cerebroside. The rates were compatible with the cerebroside's being the precursor

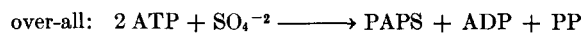
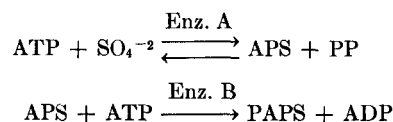
of the sulfatide. No turnover of brain sulfatide was demonstrated. In confirmation of the results of Koch and Koch (54), who had found that brain sulfatides continued to accumulate throughout a rat's life span, these workers showed that an adult rat was still able to incorporate S^{35} -sulfate into brain sulfatide. The interesting possibility of a relation between sulfatide accretion and the aging of nerve cells or the memory processes was pointed out by these authors. The continued accumulation of cerebroside sulfate in the brain of human beings has also been found (55). In addition, sulfatides have been found to make an earlier appearance in development than the cerebroside (55). Similarly, Green and Robinson (56) have compared the rates of turnover of injected $S^{35}O_4$ that was incorporated into brain mucopolysaccharide and brain sulfolipid. The turnover of the sulfate moiety of cerebroside sulfate was extremely slow compared with that of the mucopolysaccharide. By contrast in rat liver and spleen, and mouse mastocytoma labeling of sulfatide and release of radioactivity was much more rapid than in brain (51). It remains to be shown, however, that this turnover of sulfate is representative of the whole sulfatide molecule and not due simply to sulfate transfer or exchange.

Possible Schemes of Cerebroside Sulfate Biosynthesis. Based on available information regarding mechanisms of synthesis of complex sphingolipids and what has been firmly established as concerns the activation and transfer of sulfate, several possible schemes for the biosynthesis of sulfatide can be formulated:



In all schemes the immediate donor of activated sulfate is taken to be 3'-phosphoadenosine, 5'-phosphosulfate (PAPS) (14, 15, 22). From the work of Bandurski and his collaborators (16, 17, 19) and Robbins and Lipmann (18, 20, 21), it has been established that PAPS is formed enzymatically from inorganic SO_4 and ATP by a two-step reaction. The first reaction catalyzes the displacement of inorganic pyrophosphate

from ATP with the formation of adenosine-5'-phosphosulfate (APS). The second reaction is the phosphorylation by ATP of the 3'-hydroxyl group of APS to form PAPS:



That PAPS is the common metabolic donor of activated sulfate has been repeatedly confirmed in a variety of systems with a wide variety of acceptor compounds. The enzyme systems concerned with the transfer of sulfate from PAPS to the acceptor compound have been termed sulfokinases (22).

In schemes I and II, the ceramide is considered to be the precursor of the more complex sphingolipid, whereas in schemes III, IV, and V, the sphingosine base is the precursor. Cleland and Kennedy (10) have demonstrated reaction *a* and probably *b* in microsomes of rat and guinea pig brain, although some suggestive evidence for reaction *c* could be obtained with the whole homogenates of such tissues. These workers were careful to point out that "it is possible that psychosine in an intermediate in cerebroside synthesis, and ceramides the intermediates in the syntheses of more complex glycolipids." Evidence for reaction *d* has been provided by Zabin (11).

The question of the point in time at which the sulfate is transferred to the hydroxyl function of the sugar moiety has its analogy in mucopolysaccharide biosynthesis. The mechanism of sulfurylation in the formation of sulfated polysaccharides has been the subject of considerable speculation and experimentation (57 to 63). It is possible to conceive of the sulfate's being esterified to the galactose, in the form of a sulfurylated uridine nucleotide (UDP-gal-SO₄), before polymerization occurs in mucopolysaccharide formation or before the sugar is attached to the lipid moiety in the case of the biosynthesis of the sulfatide (schemes I and V). Precedence for such a nucleotide intermediate has been provided by the finding of Strominger (64) of uridine-diphospho-N-acetylgalactosamine-4-sulfate in hen oviduct. Although this compound would appear to be an obvious intermediate in chondroitin sulfate synthesis, the evidence to date does not suggest such a role (62). In fact, the direct sulfurylation of the already completed polysaccharide has been shown to take place (61, 62, 63).

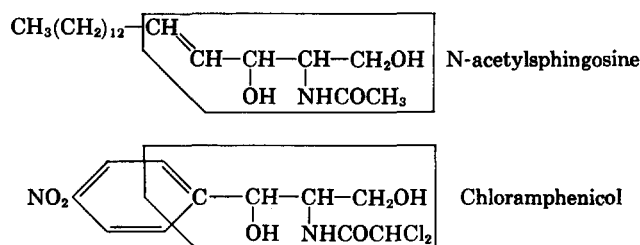
In Vitro Biosynthesis and Metabolism. The available data are as yet too incomplete to allow one to select intelligently one scheme over another as the

probable route of sulfatide biosynthesis. Experiments at the cell-free level designed to determine the mechanism of formation of the sulfatide and sulfur-containing lipids in general have been described only recently (65, 66, 67). Evidence has been obtained that extracts of rat kidney and liver as well as brain were able to catalyze the incorporation of radioactive sulfate, $S^{35}O_4$, from PAPS³⁵ into a lipid which may be the Blix compound (based on preliminary identification of the brain substance). In fact, kidney and liver preparations were found to be more active in this regard than brain. Despite repeated efforts, no evidence for a compound such as UDP-gal-SO₄ could be obtained, making schemes I and V less likely as pathways of significance in sulfatide synthesis. Of course this possibility is not ruled out. The enzymatic activity resided in a particulate, probably mitochondrial, fraction in young rat brain, whereas, in the liver a high speed (105,000 × *g*) supernatant fraction of the homogenate was active. Interestingly, a similar subcellular distribution of sulfatide in rat brain and liver has been recently reported by Green and Robinson (51). The enzymatic experiments mentioned above are of a preliminary nature and make no distinction between net synthesis and exchange of sulfate in pre-existing sulfatide.

Scheme IV is attractive in that all intermediates between the starting sphingosine and final cerebroside sulfate possess considerable water solubility. Efforts to show sulfate-accepting ability for either psychosine or natural cerebroside, however, have so far not been successful (65). Such experiments, especially in the case of the latter substances, demand cautious interpretation because of unresolved problems of substrate solubility and, hence, availability to the active site of the enzyme. A possible role for the ceramide was indicated by the stimulation of incorporation of $S^{35}O_4$ into sulfolipid by addition of N-acetylsphingosine to the crude brain particulate system.

Sulfurylation of N-acetylsphingosine and Chloramphenicol. A more detailed investigation of the enzymatic systems for sulfate incorporation into lipid from PAPS in the soluble fraction of rat liver has led to the study of an interesting reaction which may be of some importance in sphingolipid metabolism in general. The reactions described above presumably depend on the presence of endogenous lipid substrate precursor as sulfate acceptor. In addition, it was found that the rat liver supernatant fraction was markedly stimulated in its ability to transfer $S^{35}O_4$ from PAPS into lipid when N-acetylsphingosine (the threo isomer was found to be more active than the erythro—whether this is related only to the greater ease of emulsification of the

former has not been settled) was added to the incubation mixture. Evidence was obtained with purified protein fractions suggesting the direct transfer of sulfate from PAPS to the primary hydroxyl group of the ceramide to form N-acetylsphingosine-O-sulfate, a compound not previously known. An interesting consequence of these studies was the finding that these same partially purified enzyme fractions were also able to sulfurylate chloramphenicol (the D-erythro isomer is most active), a compound which bears striking structural similarities to the ceramide.



Both possess a 2-amino-1,3-propanediol backbone with two asymmetric centers; chloramphenicol has a benzylic hydroxyl and a dichloroacetamido group, whereas in N-acetylsphingosine there is an allylic hydroxyl and an acetamido group. That the same enzyme system was probably involved in the sulfurylation of both compounds was indicated by the ability of the chloramphenicol to compete with the ceramide for sulfurylation in the presence of excess PAPS.

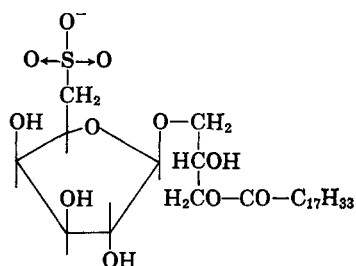
The physiological significance of this reaction is not presently known, but it is of some interest that rat liver, which lacks the ability to sulfurylate choline, is able to sulfurylate the ceramide, whereas *Neurospora*, which can sulfurylate choline, cannot sulfurylate the ceramide. Both systems appear to be absent in rat brain and marine snail extracts (65). Whether the ceramide sulfate is yet another example of a liver detoxification product or has a biological role of its own remains to be determined. The ability of the chloramphenicols to compete with N-acetylsphingosine for sulfurylation warrants a similar examination of other reactions in which the ceramides take part, i.e., sphingomyelin synthesis. Likewise the chloramphenicol free base deserves study as a competitor for the sphingosine base in psychosine formation. Possible implications in sphingolipid disease states are obvious.

Sulfatides in Diffuse Metachromatic Sclerosis. Of considerable interest have been the investigations of Austin (68 to 71) on the nature of the metachromatic straining lipid granules in the urine of children with the so-called metachromatic form of diffuse cerebral sclerosis, a familial progressive demyelinating disease of the nervous system. Pathologically this same meta-

chromatic material is distributed in large excess in many tissues outside the nervous system. This material has been identified by Austin (41, 70, 71), Jatzkewitz (45, 72), and Hagberg *et al.* (46) as sulfatide. This disease appears to constitute a sulfatide lipidosis (71).

SULFOLIPIDS IN PLANTS AND TUBERCLE BACILLI

Recently reports have appeared on the finding of two new sulfur-containing lipids in plants and virulent tubercle bacilli. Benson and his co-workers (73, 74, 75) have described a sulfolipid present in photosynthetic microorganisms and higher plants. Its concentration in *Chlorella* ($4 \times 10^{-3}M$) exceeds that of the phosphatides. This compound has not been fully characterized, but its properties upon acid hydrolysis suggested to the authors that it might contain a sulfonic acid attached to a galactosyl residue. The proposed structure is as follows:



1-O-(β-6'-deoxy-aldohexopyranosyl 6'-sulfonic acid)-3-O-oleoylglycerol

The other report comes from the laboratory of Middlebrook (76), who has provided evidence that the material responsible for the fixation of neutral red in pathogenic human and bovine varieties of *M. tuberculosis* is a new type of sulfolipid. Characterization is in a preliminary form. There appears to be about 1 mole of neutral red fixed in salt form per atom of sulfur, and an acid equivalent weight of about 3,000. The fraction constitutes 0.1% to 0.2% dry weight of a fully pathogenic human strain. Some evidence has been obtained for there being a group of closely related sulfolipids with slight differences in polarity. The only data on the nature of the sulfur in the lipid come from infrared spectrophotometry, which indicates sulfur-oxygen vibrations.

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