

May 21, 1969

To the Graduate Council:

I am submitting herewith a thesis written by Susan Tredinnick Bowling entitled "The Utilization of the Sulfur of Sulfolipid by the Albino Rat." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

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THE UTILIZATION OF THE SULFUR OF SULFOLIPID

BY THE ALBINO RAT

A Thesis

Presented to

the Graduate Council of
The University of Tennessee

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by

Susan Tredinnick Bowling

May 1969

ACKNOWLEDGMENT

The author is indebted to many people for their guidance, assistance and moral support during work of this study. An extra thank-you is expressed:

to Dr. John T. Smith, for his guidance in planning this study and for his encouragement, patience, and understanding;

to Dr. Mary Rose Gram and Dr. Ada Marie Campbell, for their help, assistance and helpful suggestions while preparing this manuscript;

to Mrs. Gail W. Disney and Mrs. Jayne T. Morris, for their technical assistance and encouragement;

to the Nutrition Department, for providing financial assistance in the form of a graduate assistantship during the early part of this study;

to her parents, for their much-needed moral support and understanding during the course of this study;

to her husband's family for their helpfulness and encouragement;

and most especially to her husband, Jim, for his patience, understanding, and encouragement during the development and writing of this study.

S. T. B.

ABSTRACT

³⁵S-sulfolipid was isolated from weanling rat brains and incorporated into a diet which was fed to rats for a period of nine days during which time urine and feces were collected daily in the initial study and continuously during the second phase of the study. The ³⁵S-sulfur from the sulfolipid was poorly absorbed, 20 percent of the ingested dose was excreted in the urine and 55 percent in the feces. The amount of radiation excreted in the urine plateaued after the third day with no significant difference occurring until the ninth day when there was a 96 percent increase in urinary excretion and a 63 percent decrease in fecal excretion, suggesting an adaptation in absorption of sulfolipid. Since approximately 75 percent of the ingested dose could be accounted for in excretory products only a small percentage would be available for tissue utilization. It seems unlikely that sulfolipid in a diet could serve as an effective source of exogenous sulfur in view of the poor absorption, the high excretion, and the lack of significant retention by the tissue, including the cartilage mucopolysaccharides. When the uptake of ³⁵S by the cartilage mucopolysaccharides was expressed in counts per minute per mM SO₄ as percent of dose, it was noted that the specific activity was very high and it is thought that some method may exist of selectively incorporating the sulfate of sulfolipid into mucopolysaccharides. Although it seems unlikely that exogenous sulfolipid can serve as a source of sulfur for an animal, it may exert a significant

influence on the sulfation of mucopolysaccharides without contributing to the sulfate pool.

Attempts made to identify the metabolites in the urine, indicate the presence of a galactose - 3 - $^{35}\text{SO}_4$.

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CHAPTER I

INTRODUCTION

In studies (1) done previously in which two week old rats and their mothers were fed diets marginal in protein, low in inorganic sulfur, but supplemented with methionine, it was shown that irregularities in development and utilization of sulfur occurred in the young rats. The results were more pronounced when the diets were low in sulfate and without cod-liver oil. There was a decrease in the width of the epiphyseal plate of the long bones in the leg and an apparent retention of $^{35}\text{SO}_4^{=}$ by the chondrocytes as indicated by autoradiograms. When cod-liver oil was added to diets low in inorganic sulfur, there was a significant increase in the specific activity of the cartilage mucopolysaccharides, in the weight gain and in the feed efficiency. These diets contained vitamins A and D at recommended levels; however, when additional vitamin A and D was added to the level supplied by cod-liver oil, these trends could not be reversed.

It would appear then that cod-liver oil or some component in cod-liver oil plays a role in the development of rats. Analysis of two different brands of cod-liver oil, demonstrated that they contained a small but constant amount of sulfur as sulfate--0.16 percent.¹ When cod-liver oil was fractionated, it was noted that a small fraction was

¹Personal communication from Jayne T. Morris.

chromatographically similar to a sulfolipid isolated from rat brains.² It seemed reasonable to assume that the small amount of sulfur present in cod-liver oil if in a readily available form might contribute sufficient sulfur to the diet to give the observed effects of cod-liver oil in diets low in inorganic sulfur.

Since little information exists with respect to the metabolism of exogenous sulfolipid it is impossible to assess the contribution of the sulfolipid fraction of cod-liver oil to the sulfur pool of an animal. Since the sulfolipid fraction of cod-liver oil and rat brain were chromatographically similar and the rat brain sulfolipid could be isolated as a ³⁵S-derivative, this investigation was carried out to determine the absorption and utilization of ³⁵S-sulfolipid isolated from the brain tissue of weanling rats given ³⁵SO₄⁼.

²Unpublished observations of Dr. John T. Smith.

CHAPTER II

REVIEW OF THE LITERATURE

The study of sulfur containing lipids had its origin in the 1870's when Thudicum as cited by Goldberg (2) reported the presence of sulfur in a cerebroside preparation from brains. Investigations in this area have continued since this time and the importance of sulfolipid and sulfatides have come to the foreground as a result of newer methods of separation and analysis of lipid samples. In this review the term sulfolipid will be used interchangeably with sulfatide; however, the latter term is more indicative of its chemical structure.

I. PLANT SULFOLIPIDS

Sulfur containing lipids have been isolated from plant as well as animal tissues. Benson and coworkers (3,4) assigned the structure of plant sulfolipids, on the basis of radiochromatographic studies with ^{35}S and ^{14}C labeled lipids, as 6 - sulfo - α - D - quinovapyranosyl - (1 $\rightarrow$ 1') - 2', 3' - di - O - acyl - D - glycerol. The fatty acid composition as determined by Obrien and Benson (5) indicated that palmitic and linolenic acids predominated in a sulfolipid isolated from photosynthetic plants and algae.

The relationship of sulfolipid formation and photosynthesis has been studied by Rosenberg and Pecker (6). While studying lipid alterations in Euglena gracilis cells during greening they noted an

accumulation of sulfolipids before measurable amounts of chlorophyll accumulated. They have interpreted these observations as suggesting that sulfolipids may be necessary for the function and orientation of chlorophyll. Trosper and Sauer (7) have recently suggested as a result of their in vitro studies with isolated chloroplasts, that sulfolipids may be involved in pigment organization in an intact plant. Klopfenstein and Shigley (8) have shown that the concentration of sulfolipid palmitic acid increased, while the concentration of sulfolipid linolenic acid decreased during the maturation of alfalfa. When these findings were compared with fatty acid exchanges in phospholipids, the rate of change in fatty-acid composition was greater in the sulfolipids.

II. MICROBIAL SULFOLIPIDS

A microbial sulfolipid structurally identified as 1, 14 - docosyl disulfate has been isolated from the phytoflagellate Ochromonas danica by Mayers and Haines (9). Hopefully this particular sulfolipid will serve as a model and help in solving the problems involved in drug transport, because of its ability to pass back and forth between a medium and the cells without cleavage of the sulfate group (10).

III. ANIMAL SULFOLIPIDS

Isolation and Estimation of Sulfatides

Early isolation and preparative techniques employed in the isolation of brain sulfatides were complicated, tedious and often resulted in extremely poor yields. In recent years, various chromatographic

techniques have been developed which make it possible to isolate sulfatides quantitatively from tissue samples. Because sulfatides are complex polar lipids and tend to enter into a more or less stable association with other substances, which may be essential for their function in the cell, chromatographic separation techniques could be developed (11). Different types of chromatography and sequential chromatography are necessary to assure adequate separation.

Radin and coworkers (12) used a combination of Florisil and mixed ion-exchange resins; Long and Staples (13) used both alumina and silicic acid to separate cerebrosides and sulfatides. Other methods that have been used are ion exchange, DEAE cellulose (14, 15, 16); partition, cellulose (17); and adsorption, charcoal (18). Lees and coworkers (19) have developed a fractionation procedure using weak absorbing supports which enabled them to separate phospholipids and sulfatides. Svennerholm and Thorin(14) first used a mild alkaline hydrolysis and then followed this by silicic acid and DEAE cellulose separation. Rouser and coworkers (20) have developed an analytical procedure which employs column and quantitative thin-layer chromatography to separate the lipids from nonlipids and permits analysis of the lipid classes present.

The procedures available for the determination of lipid sulfate are highly dependent upon the isolation methods previously mentioned. The choice of methods depends upon the nature of the sample to be analyzed. Davison and Gregson (21) have reported analysis of samples using the determination of sulfate turbidometrically as Ba SO_4 . Martensson (22) has described a procedure which involves the reduction

of sulfate to sulfide and then determining the hydrogen sulfide formed. Determination of hydrogen sulfide minimizes the difficulties presented by interfering substances. Stoffyn and Keane (23) have developed a new micro-method for the spectrophotometric determination of sulfur in organic substances in the range of 0.3 to 100 μg . of sulfur in samples weighing up to 3 mg. dry weight. An earlier micro-method proposed by Radin and coworkers (24), was the colorimetric determination of sulfatides based upon the modification of the anthrone sulfuric acid test for sugar content. Svennerholm (25) has proposed a simple procedure based on the estimation of the intensity of the metachromatic staining with cresyl violet of sulfatides on paper chromatograms. A means of determining sulfate in biological materials using the atomic absorption spectrophotometer has been developed by Rowe (26). Kean (27) has recently reported a rapid, sensitive spectrophotometric method for the quantitative determination of sulfatides. It is based upon the formation of a colored complex between the cationic dye azure A and (anionic) sulfolipids and is very sensitive at low concentrations (0.002 μ moles).

Chemical Structure and Properties

On the basis of a crude lipid extract which contained sulfur and phosphorus, Thudicum as cited by Goldberg (2) proposed that protagon was a sulfolipid, a distinct lipid species, but having properties similar to phosphatides. Later, Koch (28) isolated a sulfate containing lipid and proposed the structure as shown in Figure 1. As a result of his analysis, he proposed that sulfolipid contains equimolar proportions of sulfuric

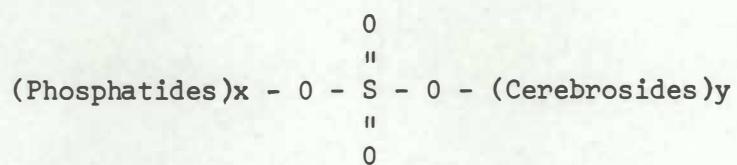


Figure 1. First postulated structure of sulfatide.

and phosphoric acids. Levene (29) using human and beef brain sulfolipid preparations found no phosphorus and less sulfur than Thudicum and less than Koch (28) who also found phosphorus. Levene's substance was dextrorotatory. A similar substance was isolated from horse kidney (30).

Estimation of degradation products. Blix as cited by Thannhauser and coworkers (31) found cerebron sulfuric acid ester to be composed of equimolar amounts of cerebronic acid, sphingosine, galactose, and sulfate. It has been assumed that sulfatides are sulfate esters of cerebro-sides. The position of the sulfate was not determined at this time, but he did propose that it was esterified with the galactose. Lees (19, 30) demonstrated that sulfatides have no free amino groups or reducing sugars, indicating that fatty acids were linked to sphingosine by an amide bond and that galactose was attached by a glycosidic linkage.

Unsuccessful attempts have been made by Lees (19) and coworkers to confirm the linkages by using the same partial hydrolyzing procedures on sulfatides that had been used on cerebro-sides. By desulfation in dilute methanolic HCl, Stoffyn and Stoffyn (32) obtained a product identical to phrenosine and kerasine. The desulfation products identical to phrenosine and kerasine when compared by thin-layer chromatography and infrared spectrophotometry showed a correlation between cerebro-sides and sulfatides. Carter and coworkers (33) were able to convert cerebro-sides to ceramides and sphingosines by periodic acid oxidation, reduction, and weak acid hydrolysis. If alkaline hydrolysis was carried out the desulfated sulfatides gave psychosine (11). The sphingosine

bases present in sulfatides as analyzed by thin-layer and gas-liquid chromatography are identical to those of cerebroside (34).

Position of the sulfate group. The configuration suggested by Blix as cited by Thannhauser and coworkers (31) was supported by Nakayama (35) working with a sulfatide isolated from pig brain. The work by Thannhauser and coworkers (31) also supported the suggestion that sulfation occurred on the C-6 position of galactose. They were able to support this by identifying the degradation products following methylation as 2,3,4 tri-methyl galactose and a small amount of 2,4 di-methyl galactose. Lloyd and Dodgson (36) by means of infrared spectroscopy examination supported the assignment of the sulfate group to the C-6 position. They found an infrared absorption peak at 820 cm^{-1} which has been attributed to the equatorial sulfuric ester of the primary hydroxyl group of the hexopyranose ring. The infrared absorption data were supported by the failure of cerebroside sulfuric ester to react with trityl chloride and the identify of degradation products which were isolated following permethylation.

The suggestion that the sulfate was esterified at the C-3 position rather than the C-6 position of the galactopyranoside moiety as shown in Figure 2 came from two different laboratories (37,38). Yamakawa and coworkers (37) using gas-liquid chromatography methods found that the methylated sugar derivative from permethylated sulfatide obtained from bovine brain agreed closely with the retention time of 2,4,6 tri-methyl galactoside and differed from synthetic methyl 2,3,6 or 2,3,4 tri-methyl galactoside. They also observed that the sulfatide was

Sphingosine

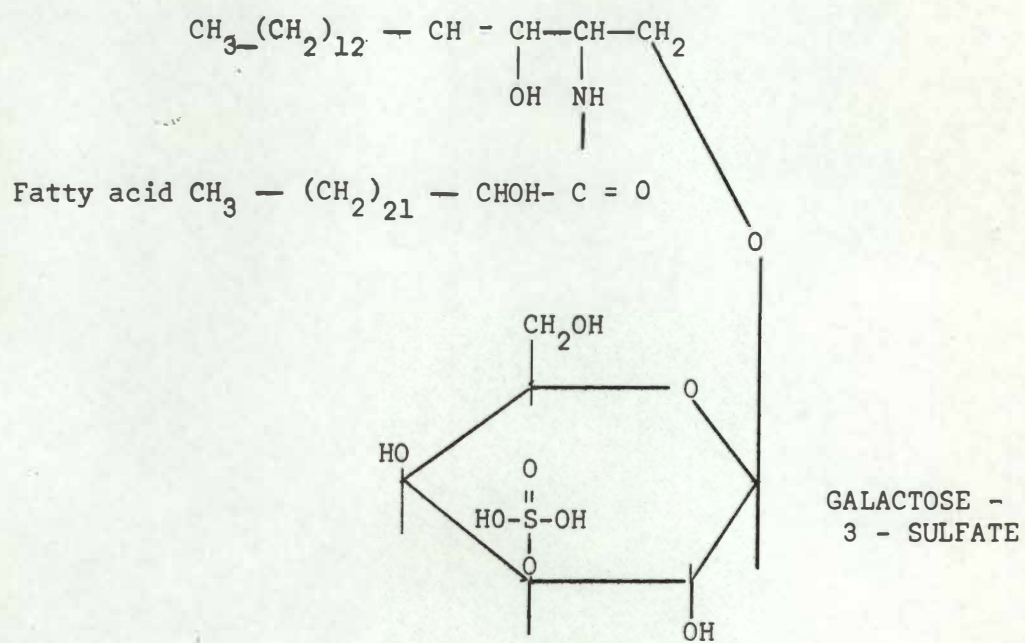


Figure 2. Presently accepted structure of sulfatide.

not affected by periodate oxidation. These data suggest that the 3 hydroxyl of galactose is esterified with sulfate,

In another laboratory, Stoffyn and Stoffyn (38) have observed that the sulfatides are very stable under alkaline conditions. The formation of a 3,6 anhydro derivative was to be expected under these conditions if the sulfate was in the C-6 position (39). Methylation was also performed by Stoffyn and Stoffyn and after methanolysis and hydrolysis of the galactose fraction, a 2,4,6 tri-methyl-D-galactose in high yield was identified by chromatography on paper pregated with di-methyl sulfoxide and by the formation of its crystalline anilide. The main derivative of methylated phrenosine is tetramethyl galactose, and no tetramethyl galactose can be isolated from sulfatides. Again these data point to the presence of the sulfate group esterified at the C-3 position.

Taketomi and Yamakawa (40) reacted a cerebroside from brain dissolved in pyridine, with chlorosulfonic acid. Analysis of the reaction product demonstrated that the sulfate group was esterified to the third carbon instead of the sixth carbon of the galactose moiety of the brain cerebroside. Again the retention time of the methylated sugar of pre-methylated brain cerebroside sulfuric ester agreed with methyl 2,4,6 tri-methyl galactoside but differed from premethylated synthetic cerebroside sulfuric ester which agreed with 2,3,4 tri-methyl galactoside. This was the first time sulfation of natural cerebroside had been achieved.

Recently Peat and coworkers (41) have reported the definitive synthesis of D-galactose 2- and 3- sulfates as well as the 2- sulfate of

D-glucose and 2,3-disulfate of D-galactose. Previously, Peat and coworkers (42) and Turvey and Williams (43) had reported the definitive synthesis of the 4- and 6- isomers of D-galactose monosulfates. They also synthesized the 2- and 3- sulfates of galactose, but their yield was smaller and the procedure was not as accurate as that of Peat and coworkers (41).

Sphingosine moiety. As previously stated the sphingosine bases present in brain sulfatides are similar to those of cerebroside (34). After hydrolysis with 10 percent methanolic sulfuric acid, Jatzkewitz as cited by Moser (44) found only one ninhydrin positive spot corresponding to sphingosine on paper chromatography. However, the possibility does exist that more than one sphingosine base may be present in brain sulfatides. After hydrolysis with Ba(OH)_2 , nitrogen was partitioned equally between chloroform and water and half of the nitrogen in each phase reacted as α amino acid nitrogen (44).

Fatty acid moiety. The fatty acid hydroxylignoceric acid appeared to be the only cerebronic acid found in the sulfatide preparations of Thannhauser and coworkers (31). Jatzkewitz as cited by Moser (44) noted a difference in fatty acid composition of the two sulfatides he had isolated using a combination of chromatographic and countercurrent distribution techniques. One had only hydroxy lignoceric acid, while the other had one-half of its fatty acids as a combination of nervonic, behenic, stearic, and palmitic and the remainder was lignoceric. O'Brien and Rouser (45) determined the fatty acid composition of sulfatides

using gas-liquid chromatography. They noted a large number of both normal and hydroxy fatty acids.

In a study of the human cerebral grey and white matter, Obrien and coworkers (46) using gas-liquid chromatography after hydrolysis revealed that sulfatides contain both normal and hydroxylated long chain fatty acids ranging from 14 to 26 carbons in length. The composition of the sulfatides in gray and white matter was similar. When the infant brains were compared with mature brains, it was noted that there was a smaller proportion of odd-chain and hydroxy fatty acids in the former. In myelin 20 percent of the fatty acid chains are longer than 18-C (47).

Menkes and coworkers (48) studied sulfolipids from both mature and immature brains and observed that as maturation occurs, cerebrosides and sulfatides increased progressively. The amount of 16:0 fatty acids was higher in sulfatides than cerebrosides at each age. They found that the cerebrosides and sulfatides from adult grey and white matter contain predominantly 22 to 26 C fatty acid chains. The major nonhydroxy fatty acids were 24:1 and 24:0.

Svennerholm and Stallberg-Stenhagen (49) studied the changes in fatty acid composition of human nervous tissue with age and found a ratio of normal fatty acids to hydroxy fatty acids in the sulfatides of 0.6 to 0.8. In adult nervous tissue, the dominant fatty acids were C₁₄ 2-hydroxy saturated acids. When fatty acid changes in sulfatides with age were compared with those of fatty acids in cerebrosides similar results were obtained; however, the changes in sulfatides occurred later in life. The adult fatty acid composition pattern with regard to

degree of unsaturation and total percentage of C_{22} to C_{26} in cerebroside and sulfatides was reached as early as two years, but the percentage of odd-numbered (C_{23} and C_{25}) fatty acids continue to increase up to age ten-fifteen years.

Kishimoto and coworkers (50) and Kishimoto and Radin (51) investigated the metabolism of fatty acid moieties. They found a steady rise in activity of 24:0 and 18:0 sulfatide acids and a decline in 18:0 cerebroside. From this they postulated a rapid conversion between 18:0 cerebroside to 18:0 sulfatide.

Anomeric configuration. A β -configuration is generally attributed to the glycosidic linkage in cerebroside. In sulfatides, except for the presence of a peak at 11.22μ in the infrared spectrum, there has been no proof of a β -configuration of galactosidic linkage. A method has been recently developed for determining the configuration of anomeric center of cerebroside. When this was applied to cerebroside obtained by desulfation of sulfatides (32) similar results occurred demonstrating the β -configuration in sulfatides (11).

Occurrence of Sulfatides in Nonneural Tissue

In a review of sulfolipids, Goldberg (2) reported that several investigators have found sulfatides in the liver, testes, submaxillary glands, muscle, and lungs. They have also been isolated in various organs of the dog, rabbit, and horse. However, these earlier preparations may have been contaminated and were not adequately characterized.

Green and Robinson (52) demonstrated by fractionation that sulfatides differed in brain, kidney, and liver. Partition studies with rat brain, kidney, and liver have shown brain sulfatides in the mitochondria; kidney sulfatides in the microsomes; and liver sulfatides in the insoluble particulate fraction of the liver. No cerebroside could be extracted from blood cells or plasma.

Levene in 1917 (53) isolated a cerebroside containing galactose, sphingosine, and fatty acid from the kidney. Later, Svennerholm (54) using the best methods available found the percentage of sulfolipid in the kidney and spleen from a two month old infant to be 0.13 and 0.20 percent of the dry weight of the kidney and spleen respectively.

Makita (55) and Makita and Yamakawa (56) while studying the biochemistry of organ glycolipids reported the isolation of four glycolipids from the kidney. Of main interest is the presence of a sulfatide similar to that in the brain which they call galactocerebroside-3'-sulfate. Further analysis demonstrated that it contained a large amount of hydroxy fatty acids. The other glycolipids were thought to be cerebroside, a ceramide dihexoside, and ceramide trihexoside.

Mårtensson (57,58,59) has isolated two different sulfatides from the human kidney. Based on infrared absorption data, one has a chemical composition similar to that from brain in that it has an absorption maximum at 1240 cm^{-1} , while the other exhibited an absorption maximum of $1120\text{--}30\text{ cm}^{-1}$ which is indicative of a high hexose content. On the basis of hydrolysis and periodate studies the latter compound has been tentatively identified as galactosyl-glucosyl ceramide, esterified with sulfate

at the three-position of galactose. The two sulfatide fractions and all the neutral kidney glycolipids have fatty acid patterns with common characteristic features which may reflect the close relationship between the substances.

Recently studies (60,61) have been reported concerning the presence of phytosphingosines in human kidney glycolipids. Their presence in kidney sulfatides has not been fully elucidated.

Metabolism of Sulfatides

Biosynthesis. In a survey of the literature, several different but related schemes for the formation of sulfatides have been reported. The similarity of cerebrosides and sulfatides has led many investigators to postulate a relationship both in synthesis and turnover. Based on studies done by Radin and coworkers (62), it was concluded that sulfatides were formed from cerebrosides. They observed that cerebrosides undergo a slow turnover but appear to be in a dynamic state. Sulfatides, however, appear not to break down in the normal brain and may continue to be formed over the life span of the rat. Hauser (63) postulated that it would not be possible for sulfatides to be the precursors of cerebrosides. He noted that the specific activity of the cerebrosides was higher than seven times that of sulfatides. He was unable to decide at that time whether or not the sulfate was attached at an earlier stage in the synthesis process.

As a result of extensive studies (64,65), it has been established that 3'-phosphoadenosine-5'-phosphosulfate (PAPS) is the active sulfate

in biological reactions. Several specific sulfokinases have been identified as catalyzing the transfer of the sulfate from PAPS to the acceptor. Hauser (63) questioned whether the sulfate acceptor was a cerebroside or an earlier precursor.

Using a cell-free system Goldberg and Delbrück, and Goldberg (66, 67) have found that the homogenates of young rat brain and liver catalyze the transfer of $^{35}\text{SO}_4$ from PAPS to lipid fractions. Incorporation occurred in the particulate fraction of the brain and in the 100,000 X g supernatant. The compound formed in the brain was thought to be a sulfatide. An acid hydrolysis test was used to support this idea. The scheme as proposed in Figure 3 is supported by the fact that only ceramide N-acetylsphingosine stimulated the incorporation of labeled sulfate into the homogenate, while cerebroside, psychosine, and cerebrohyl ceramide were inactive.

Just as Goldberg and Delbrück, and Goldberg (66, 67) were unable to demonstrate the ability of natural cerebroside and psychosine to stimulate formation of sulfatides, several other investigators have been unable to produce sulfatides using labeled cerebroside (68, 69). These labeled cerebroside were injected intracerebrally or intraperitoneally in 10 day old rats. The low activity of the sulfatides indicated that there was little or no direct sulfation of cerasin. They suggest cerebroside are metabolized in some other manner (68). In a recent study reported by Cumar and coworkers (70) involving the sulfation of glycosphingolipids and related carbohydrates by enzyme preparations the findings of the previous study (68) are questioned. They wonder whether

1. UDP - galactose + PAPS $\longrightarrow$
UDP - galactose sulfate + PAP
2. UDP - galactose sulfate + Ceramide $\longrightarrow$ SULFATIDE

Figure 3. Possible scheme for the synthesis of sulfatides.

the injection of substances as complex as cerebroside could reach the sites in the cell where synthesis occurred.

The incorporation of labeled sulfate into sulfatides was used by McKhann and coworkers (71,72) as index of in vivo and in vitro synthesis of sulfatides. They suggest that the transfer of PAPS into galactocerebroside to form a sulfatide involves a galactocerebroside sulfo-transferase. A galactocerebroside sulfotransferase can be solubilized from the microsomal fraction of rat brain and is specific for the formation of sulfatide this way. They call this sulfate transfer enzyme--"galactocerebroside sulfokinase" and it is involved in the transfer of sulfate from one lipid to another (72).

Balasubramaniam and Bachkawat (73,74,75) have reported extensive studies concerning the enzymic synthesis of sulfatides from PAPS. The enzyme system from rat brains with which they worked had an optimum pH of 7.0 and required divalent metal ions for maximum activity (73). Sheep brain extracts were also capable of catalyzing the formation of sulfatides from ³⁵S-PAPS (74). Their findings indicate that there was only a transfer of sulfate from PAPS which they believe means that the acceptor is in the enzyme preparation itself. The presence of ATP, UTP, UDPG and N-acyl-sphingosine did not stimulate the formation of sulfatides. They concluded that the galactose containing acceptor was present in a protein bound form for the following reasons: (1) after prolonged dialysis the enzyme retained its sulfate transferring activity and (2) after passage through a sephadex column the activity was retained. This protein bound acceptor could be a cerebroside and

sulfation could only occur when it is bound. This may be another reason why previous attempts (68,69) at sulfation of injected cerebroside failed. Balasubramaniam and Bachkawat (75) extended their studies to include the effect of age of rat brain enzyme and tissue location of the enzyme. By the addition of galactose oxidase to the enzyme preparation they proved that the reaction studied was the transfer of sulfate to the acceptor and not just sulfate exchange reaction with any sulfatide present. No enzyme protein was present in rat brain to catalyze the synthesis of sulfatides at age of one and seven days when the natural acceptor was provided. Very little sulfatide synthesis occurred until the rats were nine days of age pointing out that very little acceptor may be present. A study of tissue distribution of the enzyme in the rat showed that the kidney had three times higher sulfatide synthesizing activity compared to the brain and the liver whereas the activity in the spleen was negligible. They were unable to state with certainty whether or not the protein which is bound to cerebroside is actually required for the sulfation or whether it aids in the solubilization of cerebroside. On the basis of the previous discussions the scheme presented in Figure 4 is suggested for the synthesis of sulfatides. A deficiency of cerebroside sulfotransferase has recently been reported in a myelin disorder (76). It appears that a metabolic block occurs between cerebroside and cerebroside sulfate involving the enzyme which transfers the sulfate. This leads to an insufficiency of myelin sulfatides which may result in defective myelination.

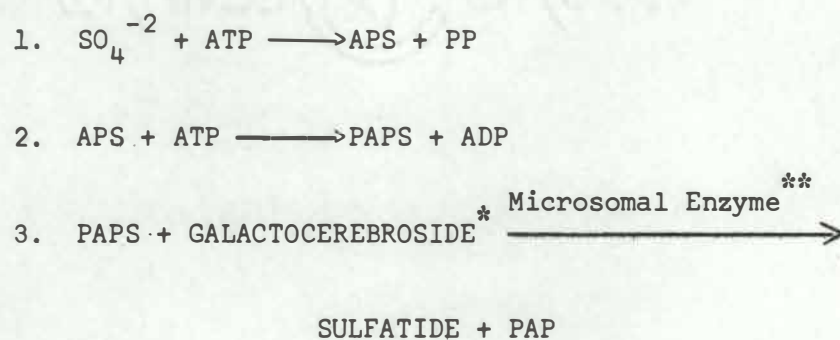


Figure 4. Synthetic incorporation of ^{35}S -sulfate to form Sulfatide.

*Protein-bound.

**Enzyme-galactocerebroside sulfotransferase.

Turnover of sulfatides and myelination. The relationship of sulfatides to the structure and composition of myelin has emerged as a result of indirect evidence obtained from studies of grey and white matter during maturation. Jacobson (77) has reported in detail the sequence of myelinization in the brain of the albino rat with emphasis on the cerebral cortex, thalamus and related structures. Recently, Vandenheuvel (78,79) has presented models of the lipid of the myelin sheath.

The major lipid components of myelin are cerebrosides, sulfatides, proteolipids, sphingomyelin, inositol phosphatide, cholesterol, lecithin, and ethanolamine phosphatide (80). Cerebroside concentration in the white matter is greater than in the grey and sulfatides are more abundant in white than in grey matter (81). In addition to the sulfatides in the myelin, some are found in the mitochondrional and microsomal fraction and are thought to be associated with the grey matter (21,52). The sulfatides that have been found in nonmyelin areas of the brain may be characteristic of lipoprotein membranes of white matter other than myelin (82).

Turnover has been demonstrated regardless of the age of the animal in kidney, liver, and spleen (52). In the nervous system, the synthesis of sulfatides proceeds slowly and varies with the age of the animal; degradation occurs even more slowly (52). Just as early investigators (2) pointed out that sulfatides of the brain increased as myelinization occurred more recent investigations support these findings. These have led to the postulation that sulfatides play an important role

in the structure of the brain. As has already been stated, during myelinization the fatty acid composition changes which indicates a conversion of cerebrosides to sulfatides (48,51). Also during this time of increased activity of myelination, the enzyme system necessary for sulfatide synthesis also increases in activity (88). When $\text{Na}_2^{35}\text{SO}_4$ was injected intraperitoneally into rats, it was taken up into the total lipid fraction of the whole brains at a maximum rate in 15-20 day old rats which correlated with maximum myelin synthesis (83). Characterization of the sulfolipid by column and thin-layer chromatography indicated that 90 percent of the radioactivity recovered was in the areas corresponding to sulfatides. The in vitro study carried on simultaneously showed a sharp increase in the transfer of $^{35}\text{SO}_4$ from PAPS to galactocerebroside at day 20.

The relationship of myelination and structural role of sulfatides has been supported by studies concerning the rate of incorporation of sulfate into various tissues and the formation of sulfatides. Green and Robinson (52) found that injected ^{35}S -sulfate was incorporated slowly into sulfatides which may indicate a slow turnover of sulfatides. Heald and Robinson (84) finding slow incorporation suggested that this represents slow synthesis of new sulfatides and that the apparent stability is necessary for the role of a structural component. Davison and Gregson (21) found an increase in sulfatides up to 100 days after birth and little if any increase beyond this time in rats. In the human brain, a rapid increase in sulfatide occurs up to three or four years of age,

followed by a very gradual increase in the adult level, and remaining constant after thirty years (44).

It has been demonstrated in both the rat and mouse, that when injected with labeled glucose, the most rapid incorporation of labeled glucose occurs between 10 and 20 days after birth (85). Similar results were obtained with labeled galactose (86) and sulfate (21). After an intraperitoneal injection of ^{35}S -sulfate about 0.5 percent of the dose was incorporated into the brain of a 15 day old rat, whereas 0.08 percent was incorporated into the adult rat brain (21).

Radin and coworkers (62) observed in the sulfatide fraction no change in radioactivity after an injection of ^{14}C labeled galactose when observed for a period of ten days. Davison and Gregson (21) injected 15 day old rats and measured radioactivity for a period of 233 days with a mean half-life of 210 days. Smith (87) using adult female rats found that sulfatides were inactive after initial incorporation of labeled glucose during the next 60 days.

Recently there have been reports in the literature concerning the changes that occur in the developing brain (88,89,91,92) which point out that while myelin is not entirely a metabolic unit, some components of myelin may be more actively metabolized than others. Dittmer (89) while studying the post natal changes in the concentration of the lipids of the developing rat brain noted that lipids such as cerebrosides, sphingomyelin, and galactosyl-diglyceride rapidly increased throughout the period of active myelination. Sulfatides were similar to these except they began to increase before myelination began. Galli and Cocconi (90)

also noted an increase of sulfatides in rat brains up until 50 days though they were not present at birth. Cuzner and coworkers (92) however were able to isolate sulfatides from rats at 10 days (0.01 moles/whole brain).

Subcellular and cellular sulfatides have received attention. Cerebrosides and sulfatides are often considered as a single unit. This creates a problem because the synthesis of brain cerebroside and sulfatide continue in the adult, with degradation of sulfatide occurring very slowly; yet, sulfatides do not increase in amount. Davison and Gregson (21) have investigated this situation. They studied subcellular fractions of rats that had been injected eight months earlier. No radioactivity was found in microsomal fractions, but the radioactivity was still high in the sulfatides of myelin and mitochondria. This indicates microsomal sulfatides turn over more rapidly. Smith (87) also found a high concentration of sulfatides in myelin and mitochondria. In a later study by Davison and Gregson (93) on the metabolism of subcellular membrane sulfatides in rats of all ages, the fast turnover of microsomal sulfatide during development was related to the process of myelination. Radioactivity was found to be more extensively incorporated after intracerebral injection, especially in adult animals. They noted a small but rapidly exchanging pool of myelin sulfatide; however, the pool is small and myelin sulfatides can still be considered inert.

Pritchard (94) noted that in rats injected intraperitoneally with ³⁵S-sulfate prior to the onset of active myelination both myelin and nonmyelin sulfatides were labeled, thus indicating that some myelination

occurs while animals are still young. The brain stem was the most active center of sulfatide synthesis. He, also, found a high concentration of sulfatides in the mitochondria.

As has already been stated, the enzyme system necessary for the formation of sulfatides is located in the microsomes (72). The rate of incorporation of sulfate to form sulfatide may be limited by the inavailability of galactocerebroside; also, the sulfotransferase enzyme appears to be more active around the time of active myelination (85). The results of a study done by Herschkowitz and coworkers (95) suggest that the sulfatide synthesized in the microsomes is transported to the myelin as a water soluble sulfatide containing lipoprotein.

In the previous study (95), it was noted that the microsomal fraction had two very different compartments--a rapidly turning over fraction and a fraction which is relatively stable. The observation of two different compartments in the mitochondria is similar to the postulate of Hajra and Radin (96). They assumed that the brain had groups of lipids having separate roles. One of these was a rapidly metabolizing fraction which may be "functional" in that it is involved in brain metabolism. The second more slowly metabolizing fraction may be "structural" and is found in older rats. The "functional" fraction (96) and the rapid turnover fraction (95) are similar to the rapidly metabolizing sulfatide in the microsomal and supernatant fractions which Davison and Gregson (93) suggest are involved in the synthesis of nonmyelin sulfatides.



Undernutrition and Myelination

The effect of undernutrition on myelination of the central nervous system has received considerable attention in recent years. The term "undernutrition" as used in this sense refers to an inadequate diet or in some instances underfeeding. Dobbing (97) concluded as a result of extensive study that undernutrition can affect myelination depending on the timing of the stress, its severity, and its duration. The period during which active myelination occurs has been shown to be most vulnerable with respect to the influence of undernutrition on brain development (98). Benton and coworkers (99) showed that myelination was significantly retarded when rats were deprived of adequate nutrition from birth until weaning at 21 days. They also found that if the rats were given an adequate diet for the next three weeks, the formation of myelin lipids was accelerated to the extent that they could not be distinguished from the controls. Of the lipids analyzed in this study, brain cerebroside were the most greatly affected--5 percent less than controls.

In a later study carried out by Chase and coworkers (100), the in vivo and in vitro synthesis of sulfatides during myelination was decreased in the brains of nutritionally deprived rats. In contrast to the previous study, they did not find indications of a catch-up period. The peak and onset of myelination was the same in the control and nutritionally deprived rats, and the peak activity of the latter was less than one-half that of the former. In in vitro studies when the level of PAPS and galactocerebroside was kept constant, a significant

difference in activity of the cerebroside sulfokinase was noted in the 19 and 20 day old rats. The level of the galactocerebroside was decreased in the nutritionally deprived rat. At this time, however, they are not certain as to whether undernutrition during the neonatal period is selective or general in its effect on the metabolic processes occurring during myelination.

Metachromatic Leucodystrophy

Metachromatic Leucodystrophy (MLD) or Sulfatide Lipidosis which affects the white matter of the human brain, is a progressive, hereditary disorder in which demyelination affects the nervous system. In 1959, Austin (101) reported above normal amounts of sulfatide concentration in the central nervous system. A similar increase of sulfatides has also been reported in the gall bladder, liver, kidney, urine, and peripheral nervous system (102,54). Austin first pointed to an enzymetic defect in MLD by demonstrating a deficiency of arylsulfatase A (103). Mehl and Jatzkewitz (104) have isolated an enzyme from normal human kidney which was responsible for desulfation of sulfatide, but absent in patients with MLD (104). They postulate that the cerebroside sulfatase activity depends upon two components of high molecular weight--one "heat-stable," the other "heat-labile." In MLD, there is a deficiency of the latter and the activity of this component can be assayed conveniently by measuring arylsulfatase A activity.

A failure to elongate fatty acids beyond 18 carbons of the brain cerebroside and sphingomyelin was thought to be the basic defect in

MLD (105). Malone and Stoffyn (106) confirm the fact that cerebrosides have a fatty acid composition of shorter chain fatty acids--C-16 and C-18. An absence of C-14 fatty acids in sphingomyelin has been noted (107).

Malone and coworkers (108) have studied the sulfatides in MLD. They concluded as a result of permethylation tests that the galactose moiety in MLD was identical to normal brain. The sulfatide fatty acid distribution was normal, but cerebrosides and sphingomyelin showed a deficiency as previously mentioned. In MLD, the sulfatides in kidney increased 10 to 20 times their normal value. They are not an "overflow" from some other site, because their fatty acid compositions are similar (106).

Metachromatic leucodystrophy can be diagnosed clinically by the demonstration of excessive sulfatide excretion in the urine (109). Austin and coworkers (110,111) have developed a colorimetric test to measure the activity of arylsulfatase in the urine.

The accumulation of sulfatides as a result of diminished sulfatase activity is the reason for the demyelination that occurs in the central nervous system. Malone and Stoffyn (112) conclude that the demyelination occurred as a result of abnormal myelin formation. Clinically, the disease is characterized by progressive paralysis and dementia which becomes apparent during the second year of life and is usually fatal. There are also adult forms. The pattern of the disease as a result of study indicates that it is autosomal recessive and at this time no ethnic differences have been noted (113).

The literature concerning sulfolipids provides very little information concerning the metabolism of exogenous sulfolipid. Therefore a study to determine the absorption and utilization of sulfolipid may indicate the contribution of the sulfolipid fraction of cod-liver oil to the sulfur pool of an animal.



CHAPTER III

EXPERIMENTAL

I. GENERAL PLAN

As was previously stated, the purpose of this investigation was to study the absorption and utilization of sulfolipid extracted from rat brain by the albino rat. The sulfolipid extracted from rat brain is similar to the sulfolipid fraction of cod-liver oil. In the second phase of this investigation an attempt was made to identify the ^{35}S -metabolites in the urine.

The sulfolipid material was extracted from the brains of 40-50 weanling rats that had been injected with 25 μC of $^{35}\text{SO}_4^{=}$, forty-eight hours prior to death by a modification of the method of Svennerholm and Thorin (14). The sulfolipid extract was checked for radioactive purity by thin-layer chromatography and then incorporated as part of the fat content in the diet presented in Table 1.

Five adult male albino rats of the Wistar strain from the Nutrition Department colony were used. These rats were littermates. They were placed in individual metabolism cages for 9 days and fed the diet and distilled water ad libitum. Urine and feces were collected daily. One ml. of 0.1 N HCl was added to each urine vial to retard spoilage. The animals were then killed by decapitation and the brain, liver, lungs, testes, heart, kidneys, spleen, and ribs were excised and stored at

Table 1
Composition of the diet

Component	Quantity per 100 grams g
Casein	18.00
Cornstarch	30.00
Sucrose	30.00
Wesson oil	2.00
Crisco	6.00
Sulfolipid	2.00
Salt mixture ¹	3.00
Vitamin mixture ¹	2.00
Nonnutritive bulk ¹	7.00

¹Nutritional Biochemicals Corporation, Cleveland, Ohio.

-20 degrees C. until they could be analyzed for ^{35}S activity. The samples were counted by the method of Katz and Golden (114) using a Nuclear-Chicago, windowless, automatic, gas-flow chamber.

In the second phase of this investigation two male albino rats were placed in individual metabolism cages and fed the diet and distilled water ad libitum for nine days. The urine was collected continuously during this period. The animals were killed by decapitation and the organs were treated as previously described. The urine samples were analyzed using column chromatography, thin-layer chromatography, and electrophoresis in an attempt to identify the metabolite in the urine.

II. METHODS

Extraction of Sulfolipid from Rat Brains

A modification of the method of Svennerholm and Thorin (14) was used to extract the sulfolipid from the brains of weanling rats. The brains were homogenized in a blender with 10 ml. of chloroform-methanol (1:2 v/v) per gram of tissue. The homogenate was then transferred to glass beakers and brought to boil for ten minutes in a 60° C. hot water bath. The homogenate was separated by suction filtration using a Büchner funnel and Whatman No. 2 paper. The filter cake was reextracted with 5 ml. of chloroform-methanol (1:1 v/v) per gram of original tissue. The filtrates were combined and evaporated to near dryness with a flash evaporator. The concentrated filtrate was transferred to a large beaker and 4 ml. of distilled water per gram of original tissue was added and allowed to stand overnight. When necessary the pH of the extract was

adjusted to 4-5 with 4 N HCl. At this point 500 ml. of chloroform was added and the mixture was shaken thoroughly and allowed to stand for 24 hours. The two immiscible layers that formed were separated using a suction flask attached to a pipette. The bottom³ layer was saved and dialyzed against running water for 24 hours. The contents of the dialysis tube were centrifuged at 713.1g and the bottom layer removed by suction and saved. Fifty ml. were removed to be used for purity check and the remainder was combined with Crisco and Wesson oil and evaporated to near dryness in a 60° convection oven in preparation for addition to the diet.

Determination of Radioactive Purity of Sulfolipid Extracted from Rat Brain

Radioactive purity of the isolated sulfolipid was determined by thin-layer chromatography on Silica Gel G. Fifty ml. of the lipid extract was concentrated to approximately 5 ml. About 300 λ was stripped on a Silica Gel G plate. The plate was developed in a tank saturated with a chloroform, methanol and water (120:70:16 v/v/v) solvent. The solvent was prepared fresh and the tank and solvent were allowed to equilibrate for at least one hour. The bands were visualized under ultraviolet light after the plates had been sprayed with a Rhodamine reagent (0.5 g. Rhodamine/100 ml. ethanol). The bands were scraped into centrifuge tubes and washed free of color with methanol by centrifugation. The supernatants were collected and evaporated on a hot plate; 50 percent HNO_3 was added, followed by combustion mixture composed of

600 ml. concentrated HNO_3 , 300 ml. 70 percent perchloric acid, and 9.0 g. cupric nitrate in the method of Katz and Golden (114). The ^{35}S -sulfate was precipitated as $\text{Ba}^{35}\text{SO}_4$ on a weighed glass-fiber filter paper disc. During the collection process the 24 mm. filter paper disc was supported by a small piece of Whatman No. 1 filter paper and secured between a perforated rubber disc and a glass funnel cut from tubing. The side arms of the funnel were attached to a vacuum flask with spring. The precipitate was washed onto the paper with several volumes of distilled water, 0.5 N HCl , and 95 percent ethanol followed by acetone. A dry cake of barium sulfate of constant area was formed. The filter paper was weighed again, attached to center of aluminum planchet with rubber cement, put into drying oven for approximately 3 minutes, allowed to cool, and counted with a Nuclear-Chicago, windowless, atomic, gas-flow chamber. The counts per minute of each band were calculated.

Determination of the Specific Activity of the Diet and Residue From Brain Extract

The specific activity of the diet was determined by combusting approximately 1.0 g. samples in a Parr, series 1900, oxygen bomb sulfur apparatus containing 10 ml. of distilled water. Oxygen was added to a pressure of 35 atmospheres to charge the bomb. It was then ignited and allowed to cool in a water bath for 15 minutes or more. The contents of the bomb were transferred to a 25 ml. Erlenmeyer flask, collected, and counted as previously described. The counts per minute per gram of diet were calculated.

The residue after extraction of the brain was combusted along with the filter paper. One ml. of carrier sulfate was added to each sample, they were then precipitated, collected, and counted as previously described. The counts per minute were calculated and added to the counts calculated for the extracted brains.

Determination of the ^{35}S -radioactivity of Urine

The daily urine samples collected from each of the rats were filtered using Whatman No. 2 paper and diluted to 25 ml. in a volumetric flask with distilled water. Duplicate 5 ml. portions from each sample were combusted in 25 ml. Erlenmeyer flasks with 10 ml. of combustion fluid and 0.5 ml. of carrier sulfate (114). After combusting, the samples were precipitated as barium sulfate, collected, and counted as previously described. The results were expressed both as total counts per minute and counts per minute as a percentage of the ingested radioactivity.

Determination of ^{35}S -radioactivity of Feces

The daily fecal samples were dried in a 60° C. convection oven for several hours and weighed. A mortar and pestle were used to grind the feces to a fine powder. Duplicate 0.3 g. samples were combusted with 8 ml. of combustion mixture, precipitated, collected, and counted as described previously. The results were expressed both as total counts per minute and as counts per minute as a percentage of the ingested radioactivity.

Determination of the ^{35}S -radioactivity of Sulfolipid Extracted from Brains of Rats Fed Diets Containing Sulfolipid

Each brain was weighed and extracted separately with chloroform-methanol as previously described. The liquid containing the lipid was decanted into 25 ml. or 50 ml. volumetric flasks and diluted to volume with additional chloroform-methanol (2:1 v/v). Duplicate 5 or 10 ml. samples were combusted with 10 ml. of combustion fluid, precipitated as barium sulfate, collected, and counted as described previously. The results were expressed both as total counts per minute and as counts per minute as a percentage of the ingested radioactivity. Most of the chloroform-methanol was allowed to evaporate prior to the addition of combustion fluid.

Determination of the Specific Activity of Costal Cartilage Sulfomucopolysaccharides

A modification of the method proposed by Bostrom (115) was used to prepare a sulfomucopolysaccharide from costal rib cartilage. The cartilage samples were prepared by boiling the excised ribs for approximately five minutes in distilled water. The cartilage was freed of fat and tissue, removed from the rib, and placed into a small preweighed bottle. The weight of cartilage from each rib was determined. The cartilage was then sonified with approximately 5 ml. of acetone to dry using a Bronwell, Biosonik probe. The acetone was discarded and the samples were extracted with 4 ml. of 0.5 N NaOH for three minutes by sonification. The liquid portion was then decanted into a 15 ml. centrifuge tube. The cartilage

was sonified a third time with 2 ml. of 0.5 N NaOH for two minutes. The liquid was decanted into the 15 ml. tube containing the product of the first extraction. The pH of the decanted liquids was adjusted to 6 using 10 percent acetic acid and centrifuged in an International Model SBV centrifuge for ten minutes at 2000 r.p.m. The liquid was decanted into a 50 ml. centrifuge tube, and 3 or 4 drops of 20 percent sodium acetate was added. The solution of mucopolysaccharides was precipitated with three volumes of 95 percent ethanol overnight at -20° C. The following day the mixture was centrifuged at 713.1 g for ten minutes, and the supernatant fluid discarded. The precipitate was dissolved by stirring with 3 ml. of 0.5 N NaOH and centrifuged at 713.1 g for ten minutes. The supernatant was transferred to 15 ml. centrifuge tubes and the pH was adjusted to 6 with 10 percent acetic acid. Twenty percent sodium acetate was added and the mucopolysaccharide precipitated as described above. The next day, the solution was centrifuged at 713.1 g for ten minutes, the supernatant fluid discarded, and the precipitate hydrolyzed with 6.0 N HCl for three hours at 100° C. according to the method of Dodgson and Rice (116) for hydrolyzing ester sulfate linkages. If a black precipitate formed during this process, the sample was filtered through Whatman No. 2 filter paper and washed with about 5 ml. of water. One ml. carrier sulfate solution was added. The samples were precipitated, collected, and counted as previously described. The counts per minute per millimole of sulfate were calculated.

Determination of the ^{35}S -radioactivity of the Heart, Lungs, Spleen, Kidneys, and Testes

Each organ was minced and placed in an Erlenmeyer flask and digested with 50 percent HNO_3 and then combusted with 10 ml. of combustion mixture. The sample was precipitated, collected, and counted using the Geiger tube counter as they failed to count on the gas-flow counter. The counts per minute and the percent isotope retention were calculated.

Determination of the ^{35}S -radioactivity of the Liver

Each liver was homogenized with approximately 3.0 ml. of cold water per gram of liver using a Potter-Elvehjem glass homogenizer with a motor driven teflon pestle. Duplicate 5 ml. samples of each homogenate were digested with 10 ml. of 50 percent HNO_3 and then with .0 ml. of combustion mixture. The samples were precipitated, collected, and counted as previously described. The counts per minute and the percent isotope retention were calculated.

Preparation of Galactose-Sulfate Standard

The method of Peat and coworkers (42) was used to prepare the galactose sulfate standards which were compared with similar urine fractions. Five grams of galactose dissolved in 100 ml. of dry pyridine, was treated with 3 moles (13.26 g.) of pyridine-sulfuric anhydride reagent. The mixture was stirred under anhydrous conditions on a 55°C . water bath for seven hours. When the solution had cooled, 100 ml. of distilled water was added and stirred for one more hour. The pH was then adjusted to 9 with saturated aqueous barium hydroxide (6 g./100 ml.).

The precipitated barium sulfate was removed by centrifuging for 15 minutes at 713.1 g. The solution was evaporated at 35° C. on the rotating evaporator with water being added periodically to maintain the volume until all the pyridine had been removed. The excess barium was precipitated with carbon dioxide and the filtrate was evaporated to dryness. At this point, if a colloidal precipitation had retained barium carbonate, the residue would have been dispersed in a small amount of water, filtered, and poured into ethanol.

The first attempt to separate the sulfated galactose was on a cellulose column; the second, ion-exchange chromatography using Dowex-50 and Dowex-1. The methods used will be given below.

Separation of galactose-sulfate on cellulose column. The mixture obtained by sulfating galactose at 55° C. for seven hours was dissolved in 50 ml. of distilled water and the solution was passed through a column (2 X 22 cm.) of Dowex 50 X 8, 200-400 mesh and washed with 200 ml. of distilled water.

The combined eluates and washings were applied to a cellulose column (70 X 4 cm.) which was eluted in a stepwise manner with 85 percent aqueous ethanol (3 liters) containing 0.5 percent (v/v) formic acid; 75 percent ethanol (3 liters) also containing formic acid (43). The fractions were collected in 500 ml. beakers and pooled. Fractions 9 - 12 which hopefully contained galactose - 3 - sulfate were concentrated by evaporation. The identity of the eluted fractions was determined by various chromatographic techniques which will be described later.

Separation of galactose-sulfate fractions by ion-exchange. The mixture obtained by sulfating galactose at 55° C. for seven hours was dissolved in 50 ml. of distilled water and the solution was passed through a Dowex 50 X 8, 200-400 mesh column. The combined eluates and washings were then passed through a Dowex 1 X 8, 200-400 mesh column and washed with several volumes of water. The column was then eluted in a stepwise manner with the following solutions (42): 4.25 liters, 0.005 M Na_2SO_4 ; 0.25 liters, 0.005 M Na_2SO_4 ; 2.5 liters, 0.01 M Na_2SO_4 ; 1 liter, 0.1 M Na_2SO_4 ; 0.5 liter, 2 N NH_3 ; and 0.5 liter, 0.4 M Na_2CO_3 . The fractions were combined in beakers to represent the respective peaks and the beakers placed in a 60° convection oven to concentrate to dryness. The identity of the eluted fractions was determined by various chromatographic techniques which will be described later.

Determination of Galactose-Sulfate Moiety in Rat Urine

The urine samples collected from the two rats in the second phase of this investigation were pooled and chromatographed on a Dowex 1 X 8, 200-400 mesh, column and eluted as mentioned previously (42). The fractions were collected and pooled as indicated by the volume and composition of the eluent. The fractions were placed in a 60° C. convection oven to evaporate. As each fraction was dried a portion was saved for chromatographic purposes. After the fractions had concentrated, the precipitate was dissolved in a small amount of methanol. Due to the formation of a precipitate in the methanol, the samples were

then transferred to 100 ml. volumetric flasks and diluted to volume with distilled water.

Duplicate 1 ml. samples from each flask were pipetted into counting vials and 10 ml. of scintillation fluid¹ was added and thoroughly mixed, separation occurred in some of the vials due to the presence of water. The samples were then placed in the Picker Nuclear Liquimat 220 to determine radioactivity. The corrected counts per minute of each sample were calculated.

Chromatographic Techniques Used to Identify Metabolite in the Urine

Many different methods were used in the attempt to compare the metabolite in the urine with synthetic sugar sulfates. After the sulfated sugar had been chromatographed on the cellulose column and fractionated, various methods were used to identify the fraction. A chromatogram was prepared using Whatman No. 1 paper and spotted with 5 λ to 25 λ of the sugar sulfate fractions. It was placed in a tank that had been saturated for an hour with water saturated methyl-ethyl ketone (100 ml.) and cetyl pyridinium chloride (3 g.) solvent (117). The chromatogram was visualized by the development of dark brown or black spots as a result of the following procedure (118): The dried, developed chromatogram was first washed in chloroform to remove the cetyl pyridinium chloride. After it had dried, it was passed rapidly through a silver nitrate solution,

¹Picker Nuclear packaged scintillator [98 percent 2,5-diphenyl-oxazole and 2 percent p-bis (O-methylstyryl benzene)] mixed in 1 liter of toluene.

prepared by diluting 0.1 ml. saturated aqueous silver nitrate to 20 ml. with acetone and adding dropwise distilled water with shaking until the silver nitrate which separates on addition of acetone has redissolved. The paper was sprayed when dry with an 0.5 N NaOH solution in aqueous ethanol, made by diluting 3 ml. of 50 percent NaOH diluted to 90 ml. with 95 percent ethanol. Brown AgO_2 was produced which facilitates even spraying. When the reaction was complete, the excess AgO_2 was dissolved by immersing the paper for a few minutes in 6 N ammonium hydroxide, after which the paper was washed for at least an hour under running tap water and then dried in an oven. Black or dark brown spots were obtained. A sample from each fraction was spotted on Silica Gel G plates that had been channeled so each sample would have own solvent front and had been activated for 30 minutes. The chromatogram was developed using n-propanol-ethyl acetate- H_2O (70/20/10) (119). The plate was visualized by spraying with a silver nitrate in acetone solution (1 ml./20 ml.). The plate was placed in an ammonia saturated tank and kept in the dark for 15 minutes and then heated until dark spots could be located.

After the urine and sulfated sugar had been fractionated and pooled various chromatographic methods were attempted in order to identify the metabolite. Fifty λ of each fraction was spotted on a chromatogram using Whatman No. 1 paper, developed, and visualized by the method of Rees (117) and Trevelyan and coworkers (118). It was then decided to do electrophoresis as this had been successful in the past (42,43).

A thirty λ sample of 5 percent galactose solution, and the first urine fraction were spotted on a Whatman 3M strip 7 X 19 cm., 6 cm.,

from one edge. The samples were subjected to electrophoresis in 0.067 M PO_4 buffer pH 6.8 at 10 v/cm. for four hours in a Gelman Electrophoresis chamber. The strip was visualized as previously mentioned (118). The separation of the sugar achieved with the phosphate buffer was not satisfactory; therefore, the separation was repeated using a 0.1 M acetic acid:pyridine buffer of pH 6.5 which according to the literature afforded better separation and visualization since it was more sensitive to spray reagents (44). Various electrophoretic chromatograms were prepared in order to determine the amount of the sample to apply.

Using the same procedure 50 λ samples were stripped and subjected to electrophoresis. The bands were visualized and cut apart. They were then placed directly in the counting vials and 20 ml. of scintillation fluid were added. The vials were counted and the counts per minute calculated.

Using the thin-layer chromatography method according to Stahl (119), 10 λ was spotted of fractions from the urine and corresponding sugar fractions and developed. Bowker and Turvey proposed a method for detecting sugar sulfates (120). Silica Gel G plates were spotted with 10 λ of fractions after the plate had been activated for one hour. The plate was developed with methyl ethyl ketone saturated with water containing 1 percent (w/v) cetyl pyridinium chloride for 90 minutes. The spots are detected with diphenyl analine reagent which was made fresh using 4 g. diphenylamine, 4 ml. analine, and 20 ml. of syrupy 80 percent orthophosphoric acid in 200 ml. acetone. It was necessary to heat

the plates longer than four minutes to develop them. It was also noted that the plates change color with time.

Statistics

The method of paired comparisons was used to determine the statistical significance of the data. Mean and standard error of the mean were calculated where applicable. These calculations were according to the method of Steel and Torrie (121).

CHAPTER IV

RESULTS AND DISCUSSION

As was previously mentioned, the initial purpose of this investigation was to study the absorption and the utilization of the sulfur of sulfolipid by the rat. Preliminary work had shown that cod-liver oil contains some sulfolipid and because it was possible to prepare ^{35}S -sulfolipid from rat brain rather than from cod-liver oil, rat brain sulfolipid was used. The results of the initial investigation indicated that it would be advisable if possible to identify the ^{35}S -metabolite in the urine.

The radioactive sulfolipid was extracted from the brains of weanling rats, checked for radioactive purity and then incorporated into the diet. The data in Table 2 show the amount of feed consumed and the amount of isotope consumed during the nine day period on the initial study. The five rats lost weight, number 5 had difficulty in finding its feed when first placed in the metabolism cages. The rats numbered 6 and 7 who were in the second phase gained weight; however, this gain is questionable. It is thought that they may have been weighed incorrectly prior to being placed on the diet. The first two rats were not weighed prior to death. The total amount of diet consumed by the last two rats was 116 g. and 99 g. It was necessary to give them a diet similar to the sulfolipid the last day. In Table 3, the amount of radiation recovered indicates that most was found in the urine and feces

Table 2

Weight of rats when placed on diet and when killed, feed consumption
and amount of isotope consumed

Rat	Initial Weight	Final Weight	Weight Change	Feed Consumed ¹	Isotope Consumed
	g	g	g	g	cpm x 10 ⁻⁴
1	275	---	---	135	7.6
2	286	---	---	135	7.6
3	280	270	-10	135	7.6
4	280	270	-10	135	7.6
5	272	260	-12	121	6.8
6	168	210	42	96	7.2
7	180	220	40	84	6.3
				<u>120</u> +8.1 ²	<u>7.2</u> +0.2 ²

¹Amount of sulfolipid diet consumed.

²Data are mean $\pm$ standard error of the mean of seven animals unless otherwise indicated.

Table 3

³⁵S-radioactivity recovered from urine, feces, and various tissues
of rats fed sulfolipid diet

	Rat						
	1	2	3	4	5	6	7
	total cpm						
Urine	16,213	19,022	14,167	14,749	12,753	8,900	13,053
Feces	37,687	30,042	40,351	44,645	39,764	51,474	33,366
Liver	1,816	1,412	436	509	1,209	128	99
Brain	156	111	63	156	112	28	28
Ribs	10	46	2	38	56	41	122
Other tissues ¹	100	51	97	150	113	117 ²	236
Total recovered	$5.6 \times 10^{+4}$	$5.1 \times 10^{+4}$	$5.5 \times 10^{+4}$	$6.0 \times 10^{+4}$	$5.4 \times 10^{+4}$	$6.1 \times 10^{+4}$	$4.9 \times 10^{+4}$
Percent isotope recovered	73	66	72	79	79	84	74

¹Includes heart, lung, spleen, kidney, and testes.

²Sample of testes lost during analysis.

and the least in individual tissues. An average of 75 percent of the ingested dose was recovered. As can be seen in Table 3 there was great variation in the amount of radiation recovered in the urine, feces, and tissues of the rats used in this study.

The data presented in Table 4 show the total isotope consumed and excreted in the urine and feces as percent of the ingested dose. These data are compared with similar data for ^{35}S fed as inorganic sulfur and as cysteine. Although only one-twentieth as much radioactivity was fed as sulfolipid the comparative absorption and excretion are noteworthy. It is obvious that the sulfur from the sulfolipid is very poorly absorbed--only 45 percent absorbed compared to roughly 90 percent for both sulfate and cysteine. Of the sulfur absorbed from the organic sources approximately 42 percent is excreted in the urine which is much higher than that excreted when $^{35}\text{SO}_4^{2-}$ is fed. As can be seen in Table 5 the amount of radioactivity excreted in the urine appears to plateau after the third day with no significant difference in excretion until the ninth day ($0.01 > P > 0.001$). The amount of radiation excreted in the feces did not increase significantly after the second day ($P > 0.001$) when compared to the first until a significant decrease was noted on the ninth day ($P < 0.001$). On the ninth day there was a 96 percent increase in the urinary excretion of radioactivity and a 63 percent decrease in fecal excretion. The percentages therefore may suggest a mobilization of sulfur. However, since only 20 percent of the ingested dose is excreted in the urine and 55 percent in the feces as indicated previously in Table 4, 96 percent of the urinary excretion is slightly less than

Table 4

Comparison of the form of total isotope consumed and excreted in the urine and feces as percent of dose¹

Form of isotope fed	Isotope consumed cpm X 10 ⁵	Percent excretion	
		Feces %	Urine %
³⁵ SO ₄ ²	19.4 ± 4.2	8.2 ± 1.7	2.2 ± 0.24
³⁵ S-cysteine ³	15.2 ± 0.38	12.8 ± 2.6	36.1 ± 5.6
³⁵ S-sulfolipid	0.7 ± 0.02	54.7 ± 3.7	19.4 ± 1.4

¹Data are the mean ± the standard error of the mean.

²Michels and Smith, (122).

³Fulton, S. F. Unpublished observations.

Table 5
Daily ^{35}S -radioactivity excreted in the urine and feces
of rats fed sulfolipid diet¹

Day	Urine	Feces
	Total cpm X 10^{-3}	
1	0.1 ± 0.04^2	0.4 ± 0.2^5
2	$1.3 \pm 0.30^{2,3}$	5.4 ± 0.4^5
3	1.9 ± 0.40^3	5.9 ± 0.9^6
4	1.9 ± 0.20	$5.1 \pm 0.7^{6,7}$
5	1.8 ± 0.40	6.9 ± 0.8^7
6	1.8 ± 0.20	5.0 ± 0.7
7	1.9 ± 0.20	4.1 ± 0.4
8	1.6 ± 0.20^4	3.8 ± 0.2^8
9	3.1 ± 0.30^4	1.4 ± 0.2^8

¹Data are the mean and standard error of the mean of five animals.

² $0.01 > P > 0.001$ comparing days 1 and 2.

³ $0.1 > P > 0.05$ comparing days 2 and 3.

⁴ $0.01 > P > 0.001$ comparing days 8 and 9.

⁵ $P < 0.001$ comparing days 1 and 2.

⁶ $0.2 > P > 0.1$ comparing days 3 and 4.

⁷ $0.02 > P > 0.01$ comparing days 4 and 5.

⁸ $P < 0.001$ comparing days 8 and 9.

63 percent of the fecal excretion. These data suggest an adaptation for the absorption of sulfolipid through the induction of specialized enzymes or the growth of specialized microorganisms.

Since approximately 75 percent of the ingested radioactivity could be accounted for in excretory products only a small amount of the isotope would be available for use by the tissues as can be seen in Table 6. It is obvious from the table that the only tissue to show an appreciable accumulation of the isotope was the liver. Although the poor absorption and the high urinary excretion make it seem unlikely that the sulfolipid is being significantly metabolized, it may be assumed that a high level found in the kidney as well as the liver is a reflection of their suggested role in the metabolism of endogenous sulfolipid. The rapid uptake of radioactivity of sulfolipid by the liver had been thought to represent an exchange (52). The high percent retention of the dose of isotope and the high number of counts per minute may be taken as an indication of this rapid rate of exchange.

On the basis of the percent dose and counts per minute, it appears that there was some uptake of the ^{35}S from sulfolipid which resulted in the formation of sulfolipids in the brain. Investigators (52,93) have reported the ready incorporation of labeled sulfate into the brain after injection. It may be possible to assume that the radioactive sulfate that was fed in the diet was incorporated into the brain during the synthesis of new tissue which after the period of maximum incorporation proceeds very slowly which would also influence the percent retention of the isotope by the tissue.

Table 6

Isotope retention in selected tissues of rats fed sulfolipid diet¹

Tissue	Isotope retention	
	% of dose X 10 ⁻²	Total cpm
Heart	3.4 $\pm$ 0.8	23 $\pm$ 5.9
Lungs	3.1 $\pm$ 0.5 ²	23 $\pm$ 4.1 ²
Spleen	1.6 $\pm$ 0.4	11 $\pm$ 2.7
Kidneys	4.7 $\pm$ 1.1	33 $\pm$ 7.2
Testes	3.6 $\pm$ 1.2 ²	25 $\pm$ 7.1 ²
Liver	103.1 $\pm$ 33.2	800 $\pm$ 225.0
Brain	12.5 $\pm$ 2.6	93 $\pm$ 20.6
Cartilage muco- polysaccharides	4.4 $\pm$ 1.0 ²	32 $\pm$ 8.72

¹Data are the mean $\pm$ standard error of the mean of seven animals unless otherwise indicated.

²Six animals.

Therefore, in view of the poor absorption, high excretion, and lack of significant retention by any tissue including cartilage mucopolysaccharides it seems unlikely that sulfolipid could serve as a source of dietary sulfur. However, when the data for the mucopolysaccharides were expressed as counts per mMSO_4 as percent of dose to compare with the data obtained feeding ^{35}S as sulfate and as cysteine, an interesting effect was observed as seen in Table 7. It may be observed that when expressed in this manner, although the standard error is high, the specific activity of the cartilage mucopolysaccharides is approximately 10 times that obtained when feeding either ^{35}S -cysteine or sulfate. This is the highest value to be obtained. Disney (123) obtained similar data for the specific activity of cartilage mucopolysaccharides when rats were stressed by feeding malathion and the isotope $^{35}\text{SO}_4$. In this instance the cartilage was labeled before malathion was administered and it was assumed that the high value was obtained because malathion blocked resynthesis of the mucopolysaccharides with nonradioactive sulfate. In this investigation, the radioactivity came from the sulfolipid; therefore, it seems that some method of selectively incorporating the sulfate of sulfolipids into mucopolysaccharides may exist. It would be attractive to assume that the galactose-sulfate moiety of the sulfolipid was incorporated directly into the mucopolysaccharides as sulfated - N - acetyl - galactosamine or as sulfated glucuronic acid. The second postulate is tenable only if the specificity of the enzymes involved is sufficiently broad to accept the sulfated substrate. It seems unlikely that exogenous sulfolipid can serve as a source of sulfur

Table 7

Comparison of radioactivity of cartilage mucopolysaccharides

Form of isotope fed ¹	Percent of ² total cpm
³⁵ S-cysteine (5) ³	0.75 $\pm$ 0.04
³⁵ SO ₄ (5) ³	0.56 $\pm$ 0.04
³⁵ S-sulfolipid (7)	6.00 $\pm$ 1.36
³⁵ SO ₄ malathion (5) ³	4.48 $\pm$ 0.54

¹Numbers in parenthesis are the number of animals per treatment.

²Data are expressed as counts per min. per mM. SO₄ as a percentage of the total ingested radioactivity $\pm$ the standard error of the mean.

³Disney, (123).

for an animal, although it may influence the sulfation of mucopolysaccharides, without contributing significantly to the sulfate pool.

In view of the observed adaptation to absorption, the concomitant increase in urinary excretion and the low retention of the isotope by the kidney, it is reasonable to assume that the isotope is being absorbed as galactose-SO₄ rather than cerebroside-SO₄. Mehl and Jatzkewitz (124) have shown that the arylsulfatase A is 10 times more active toward cerebroside sulfate than galactose-sulfate. It is therefore highly possible that galactose - 3 - sulfate is among the metabolites to be excreted in the urine. Therefore, an attempt was made to isolate and identify the metabolite or metabolites in the urine. Two rats were placed in metabolism cages and fed the same diet as mentioned previously. The urine was collected continuously for nine days. The total counts per minute for each rat (no. 6 and no. 7) are included in Table 3, page 48, and the amount of isotope consumed is noted in Table 2, page 47. The urine was frozen until analysis could be completed. The results of analyses of the various tissues were included in Table 3, page 48, and Table 6, page 53.

Several attempts were made to synthesize galactose-sulfate by the method of Peat and coworkers (42). The first attempt to separate the various sugar sulfates using a 70 X 4 cm. cellulose column was not successful (43). The solvent used to elute the column passed through very slowly and after many attempts to prepare chromatograms, it was decided that the fractions were not being eluted. A second attempt to

separate the various sugar fractions was more successful. This time Dowex-50 and Dowex-1 columns were used and somewhat different fractions of sugar were separated and attempts were made to identify them by electrophoresis and paper chromatography. The urine samples were eluted the same as the sugar samples had been and various attempts were made to identify these by paper chromatography, electrophoresis and also by counting each fraction individually on the scintillation counter. The counts are presented in Table 8. As can be seen there is very little difference in the amount of radioactivity in each fraction. Later, 50% of each fraction was stripped on Whatman 3M paper and electrophoresis was carried out. These chromatograms were developed and the bands cut apart and placed in vials and counted on the scintillation counter. The greatest amount of radiation was found in an upper region of the chromatogram of fraction 4.

Figure 5 illustrates the similarity of the various fractions. It would have been desirable to have had a urine sample from a rat that had been fed a diet that did not contain sulfolipid to compare with these fractions since the radioactivity was lost and the sample from each fraction was very small. Fractions seven and eight developed a heavy precipitate, which made it difficult to chromatograph them. They were thought to contain a mono and a disulfate galactose in a mixture. In an earlier analysis¹ of the urine sample, ninhydrin positive material

¹Personal communication from Jayne T. Morris.

Table 8

³⁵S-radioactivity in the various fractions from the
urine of rats fed sulfolipid diet

Fraction ¹	Counts per minute
1	44.0
2	42.0
3	41.4
4	42.8
5	39.3
6	41.4
7	42.3
8	39.3

¹As separated by ion-exchange. Fraction 1 eluted with eluates from previous Dowex column; fraction 2, water; fraction 3, 0.005 M Na₂SO₄; fraction 4, 0.005 M Na₂SO₄; fraction 5, 0.01 M Na₂SO₄; fraction 6, 0.1 M Na₂SO₄; fraction 7, 2 N NH₃; and fraction 8, 0.4 M Na₂CO₃.

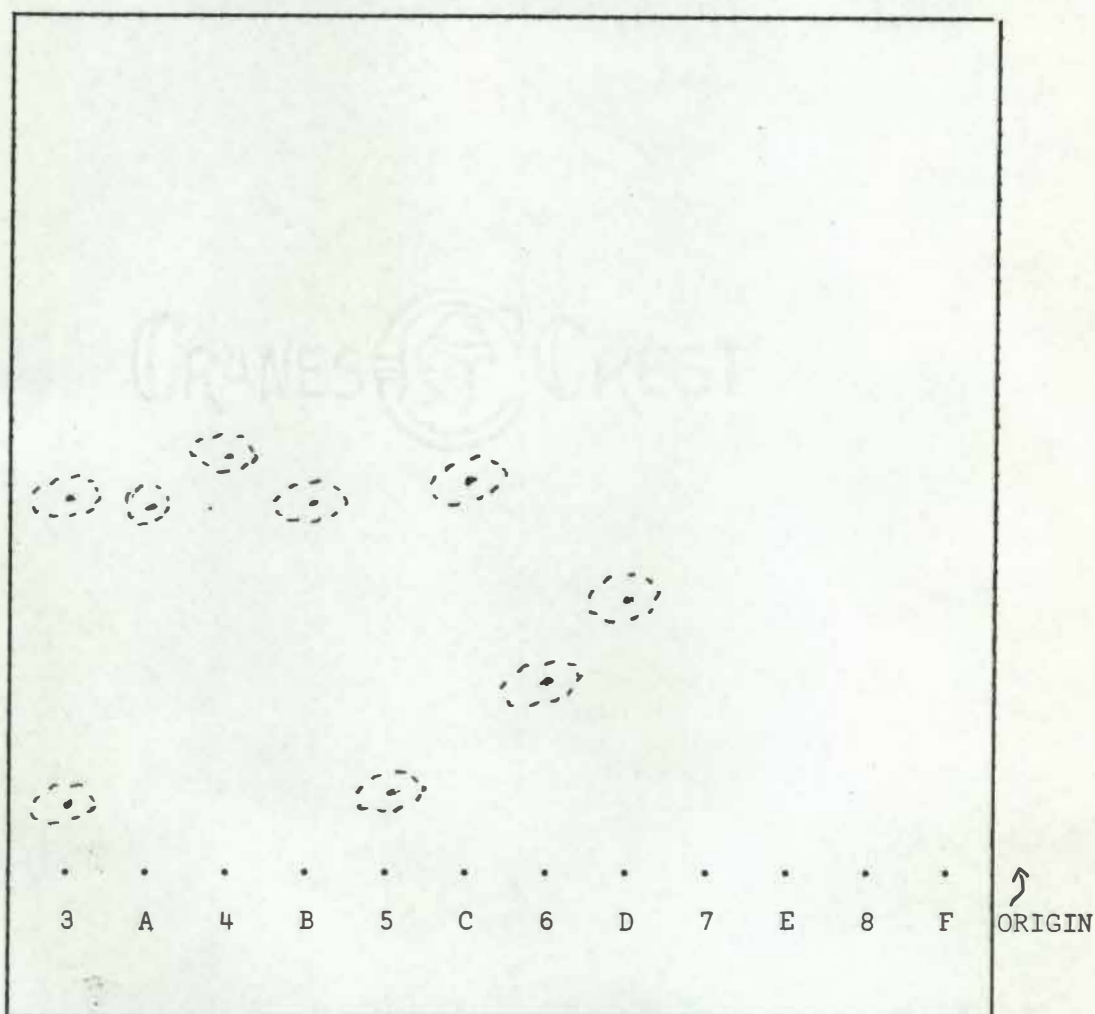


Figure 5. Reproduction of electrophoresis on Whatman 3M paper.

Fractions 3,4,5,6,7,8 urine and fractions A,B,C,D,E,F sulfated sugar. Carried out in 0.1 M acetic acid buffer adjusted to pH 6.5 with pyridine. Visualized with silver nitrate and sodium hydroxide (118).

could be detected. On several different thin-layer plates, more than one spot was noted in fractions where only one metabolite should have been.

CHAPTER V

SUMMARY

^{35}S -sulfolipid was isolated from weanling rat brains and incorporated into a diet which was fed to rats for a period of nine days during which time urine and feces were collected daily in the initial study and continuously during the second phase of study. The ^{35}S -sulfur from the sulfolipid was poorly absorbed, 20 percent of the ingested dose was excreted in the urine and 55 percent in the feces. The amount of radiation excreted in the urine plateaued after the third day with no significant difference occurring until the ninth day when there was a 96 percent increase in urinary excretion and a 63 percent decrease in fecal excretion, suggesting an adaptation in absorption of sulfolipid. Since approximately 75 percent of the ingested dose could be accounted for in excretory products only a small percentage would be available for tissue utilization. The liver was the only tissue to show appreciable accumulation which may reflect its role in metabolism of endogenous sulfolipid. It seems unlikely that sulfolipid in a diet could serve as an effective source of exogenous sulfur in view of the poor absorption, the high excretion, and the lack of significant retention by the tissue, including the cartilage mucopolysaccharides. When the uptake of ^{35}S by the cartilage mucopolysaccharides was expressed in counts per minute per mMSO_4 as percent of dose, it was noted that the specific activity was

very high when compared with results of other studies, it is thought that some method may exist of selectively incorporating the sulfate of sulfolipid into mucopolysaccharides. Therefore, although it seems unlikely that exogenous sulfolipid can serve as a source of sulfur for an animal, it may exert a significant influence on the sulfation of mucopolysaccharides without contributing to the sulfate pool.

Attempts made to identify the metabolites in the urine, indicate the presence of a galactose - 3 - $^{35}\text{SO}_4^-$.



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LITERATURE CITED

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