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Enzymatic Sulfurylation Mechanisms

Irving Hyman Goldberg

ENZYMATIC SULFURYLATION MECHANISMS

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Irving H. Goldberg, B.S., M.D.

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PREFACE

I should like to express my sincere appreciation and thanks to Dr. Fritz Lipmann for the opportunity to work in his laboratory, and for the encouragement and stimulating discussions provided by him during the course of this work. I am also indebted to all the other people who have been members of the Lipmann laboratory at the Rockefeller Institute over the past three years for their help in equipping me with much of the technical knowledge necessary for carrying out the experiments reported here. In particular, I should like to thank Drs. Phillips W. Robbins, John D. Gregory, Samuel B. Weiss, and Furio D'Abramo. I should also like to express my gratitude to Miss Christian Gillespie who aided considerably in the preparation of this manuscript.

Some of the experiments reported here on sulfurylation in marine organisms were carried out during the summer of 1958 at the Marine Biological Laboratory, Woods Hole, Massachusetts.

ABSTRACT

Sulfate activation and transfer to lipids, polysaccharides, and amino-alkyl phenols have been studied in a variety of biological systems. The transfer of $S^{35}O_4$ from radioactive 3'-phosphoadenosine 5'-phosphosulfate ($PAPS^{35}$) into lipid-soluble material has been investigated in cell-free preparations of rat brain and liver. In brain of 10-20 day old rats activity was found in the particulate fraction of the homogenate, whereas in young adult rat liver activity resided in the supernatant fraction (105,000 x g). In both systems incorporation was stimulated by the addition of a crude mixture of the isomers of N-acetyl sphingosine. Evidence accumulated suggesting that the radioactive sulfolipids produced by these two system were probably different.

Synthesis of a Cerebroside Sulfate-like Compound in Rat Brain

By partial acid hydrolysis of the brain S^{35}-sulfolipid, a radioactive fragment was obtained which appeared to be a sugar sulfate. It is suggested that the compound formed by the brain particles is the N-acetyl analogue of cerebroside sulfate. The synthesis seemed to go by way of the ceramide (N-acyl sphingosine). A partial dependency on added ATP and covalent cation could be demonstrated. Stimulation of incorporation by uridine nucleotides, however, was not consistent.

Direct Sulfurylation of N-acetyl Sphingosine in Rat Liver

The liver system was investigated more extensively. The threo isomer of N-acetyl sphingosine was the active species. Sphingosine, cerebroside, and other ceramides with longer-chained fatty acids were only slightly active, if at all. The relation of structure and solubility properties to the suitability of the lipid as a substrate for the enzyme system is described.

Chemical Evidence - The enzymatically produced sphingolipid has been characterized by partition and ion-exchange chromatography, paper electrophoresis, and chemical degradation. The formation of a compound resembling glycolaldehyde sulfate following alkaline hydrolysis and periodate oxidation indicates that the sulfolipid formed in the liver system is probably, at least in part, the direct primary hydroxyl-sulfate conjugate of N-acetyl sphingosine.

$$\text{N-acetyl sphingosine} + \text{PAPS} \longrightarrow \text{N-acetyl sphingosine} - SO_4$$

Enzymatic Evidence - The direct sulfurylation mechanism in the liver is also suggested by the enzymatic data. Fractionation of the liver supernate has been carried out by acid precipitation, alcohol fractionation, and DEAE-cellulose chromatography. Complementary 0-20% and 20-60% fractions were obtained by the alcohol step. The combined fractions synthesized sulfolipid only from PAPS and not from inorganic sulfate plus ATP. The stimulation of incorporation into sulfolipid provided by the addition of ATP to the combined ethanol fractions, could not be found when the DEAE fractions were tested. This finding supports the thesis of a direct sulfurylation of the ceramide by PAPS. The role of the second alcohol fraction, and the stimulation by ATP before the column procedure have not been clarified.

Parallels with Chloramphenicol-sulfurylation - Additional evidence favoring direct sulfurylation in the liver system is provided by experiments on the sulfate conjugation of the chloramphenicol isomers. It was reasoned that if the direct sulfurylation of the ceramide was taking place in the enzymatic reaction, the same system might also sulfurylate chloramphenicol (D-threo) which bears striking structural similarities to the ceramide. Such proved to be the case. All four isomers of chloramphenicol could be sulfurylated with PAPS by the same partially purified enzyme fractions as above; the D-erythro isomer was about 20-times as active as the natural D-threo. The enzymatic product is the same as chemically synthesized chloramphenicol monosulfate. The sulfate was shown to be attached to the primary hydroxyl function of the chloramphenicol. That the sulfokinase for chloramphenicol and N-acetyl sphingosine are probably the same, was also indicated by the inhibition of sulfurylation of the latter by the former in the presence of excess $PAPS^{35}$. This system was shown to be distinct from the sulfokinases of the phenols and dehydroepiandrosterone. The structural, steric, and electronic requirements for sulfurylation of derivatives and compounds related to chloramphenicol have been studied.

Sulfurylation in Marine Organisms

Extracts of marine organisms were examined for their ability to activate and transfer sulfate. PAPS synthesis, and transfer of sulfate to polysaccharide were demonstrated in cell-free extracts of the mucous gland of the marine snail, Busycon canaliculatum, and in extracts of various marine algae.

The *in vivo* assimilation of $S^{35}O_4$ by photosynthesizing *Porphyra umbilicalis* was also studied. Incorporation of $S^{35}O_4$ into several alcohol-soluble compounds and into polysaccharide was found. The sulfurylation from $PAPS^{35}$ of aminoalkyl phenols such as tyramine, noradrenaline, serotonin, 5-hydroxyl-tryptophan, tyrosine, or their metabolites, was observed in extracts of the snail gland and of rat liver.

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INTRODUCTION

Sulfuric Acid Esters of Biological Interest

The ubiquitous nature 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 and as important in nature as we now know for phosphate, may not be an unreasonable prediction. The list of such compounds now includes: 1. The mucopolysaccharides - the chondroitin sulfates A, B, and C; heparin; heparitin sulfate; keratosulfate; mucoitin sulfates; the polyglucose sulfates; and the homo- and hetero-polysaccharide sulfates of the marine algae. 2. The steroid sulfates. 3. The phenolic sulfates, including tyrosine-o-sulfate, triiodothyronine sulfate, and triiodothyroacetic acid sulfate. 4. Choline sulfate. 5. The sulfolipids, including the cerebroside sulfates. 6. Bilirubin sulfate. 7. The arylamine sulfates. Studies on the formation of these compounds at the enzymatic level have been in progress for a relatively short period of time. Although many of the details remain to be filled in, a major advance in our understanding of these reactions has been provided by the elucidation of the mechanism of "sulfate activation" and the characterization and preparation of the activated compound. The stage has thus been set for the systematic investigation of the transfer reactions.

The purpose of this review is to summarize our knowledge of the mechanism of biological sulfurylation in the formation of these compounds, with special emphasis, where possible, on the use of cell-free systems. Some discussion of recently discovered sulfolipids will also be included. This review is not intended to be complete; only those aspects of the subject that are directly related to the researches undertaken in this paper will be covered.

Conjugation of Sulfate with Phenol: The Development of the Concept of Active Sulfate

Much of our understanding of the mechanism of biological sulfurylation in general, and of sulfate activation in particular, stems from the studies conducted on the conjugation of phenol with sulfate. From the historical point of view, then, it is appropriate that this discussion should start with this aspect of the problem.

In 1876, Baumann (1, 2, 3) established that the formation of arylsulfates takes place in the animal body. He was able to isolate the potassium salt of phenyl sulfate from human urine, and showed that there was a marked increase in the excretion of these compounds following the administration of various phenols to dogs. Using organ perfusion, Embden and Glässner (4, 5) demonstrated that phenol conjugation occurred in the dog liver, to a small extent in the kidney and lung, and not at all in muscle.

The mechanism of formation of sulfate conjugates has been a source of much controversy. Baumann (1, 2) and Hele (6) believed that they arose by combination of inorganic sulfate and phenols. On the other hand, Sherwin (7) suggested that mercapturic acids were intermediates in the formation of the ethereal sulfates. Recent experiments by Marsden and Young (8) with S^{35}-labelled mercapturic acids have revealed no significant oxidation of these compounds to arylsulfates in the rat. Much confusion existed in the literature as the result of apparently conflicting data obtained by the administration to animals of inorganic sulfate or compounds which could give rise to inorganic sulfate (6, 9, 10, 11). The discrepancies may be explained in part by the different modes of administration of these compounds, as well as by differences in their handling by the kidney, liver, and gastrointestinal tract. In any case, the evidence that inorganic sulfate, whether of endogenous or exogenous origin, participates in arylsulfate synthesis seems to be definite. The experiments of Laidlaw and Young (12, 13), and of Dziewiatkowski (14) on the formation of S^{35}-labelled arylsulfates when S^{35}-inorganic sulfate was administered to rats simultaneously with the phenolic compound, showed conclusively that administered inorganic sulfate can partake in ethereal sulfate formation. That the source of sulfate used for conjugation can also be endogenous is indicated by the experiments of Binkley (15), and of Reed et al (16).

Some of the earliest work on the metabolism of phenols by tissue preparations was that of Herter and Wakeman (1899) (17) who were the first to find that tissue brei of liver and intestinal epithelium were the most efficient for disposing of phenols. That activation of sulfate is required before its transfer to various acceptors is suggested by the work of Bernheim and Bernheim (18) who showed that phenol could be conjugated with sulfate by guinea pig liver slices under aerobic conditions, and if free sulfate were present; cysteine or methionine would not act as sources of sulfate. Thermolabile enzyme systems were noted to be involved (18, 19).

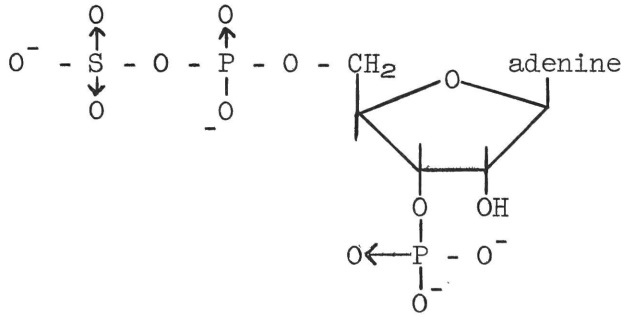
The Activation of Sulfate

The pioneering work of De Meio and his collaborators laid the groundwork for the subsequent elucidation of the enzymatic steps involved in the formation of the activated form of sulfate, as well as for the actual characterization of this compound. The inhibition of phenol conjugation by anaerobiosis or cyanide in tissue slices, and the inability of a liver brei to conjugate phenol led De Meio and his co-workers (20, 21) to believe that a source of energy was required for this conjugation, and that this was probably provided by a coupled oxidation. De Meio and Tkacz (22, 23) were the first to study phenyl sulfate formation in cell-free homogenates, and suggested that the mitochondria, by making high energy bonds available, were involved in the process. Bernstein and McGilvery (24), studying the conjugation of m-aminophenol, showed the complete synthetic system to be present in a high-speed supernate of the homogenate. The conjugation proceeded anaerobically with the supernatant fluid alone, provided that adenosine triphosphate (ATP) was present. By kinetic analysis of the system, these same workers (25) were able to resolve the question as to whether the sulfate or the phenol undergoes activation. When the enzyme preparation was incubated in a medium containing inorganic SO_4, ATP, Mg^{++} but not m-aminophenol, there was an accumulation of some intermediate which manifested itself by an initial rapid conjugation when m-aminophenol was added. The formation of the intermediate was shown to be dependent on the presence of ATP, Mg^{++}, and inorganic sulfate; no activation occurred if any of these were omitted. De Meio and his collaborators (26) also demonstrated the dependency of the soluble system on these compounds, and by heating and dialysis procedures were able to separate the system into two activities: the sulfate activating system, and the transfer system. Microsomes were found to have an inhibitory effect on the formation of phenyl sulfate by the rat liver supernatant fraction; this was attributed to the adenosine triphosphatase activity of the microsomes and not to sulfatase activity (27). Segal (28) extended the kinetic analysis of the system, and came to the conclusion that there was a two-step reaction involving first, activation, and then, transfer to the phenolic compound. He demonstrated the formation of estrone sulfate by the soluble enzymes of rat liver but not by spleen, heart, kidney, or butter-yellow induced hepatoma.

Isolation and Identification of Active Sulfate

A direct attempt to isolate the "active sulfate" was successfully made by Hilz and Lipmann (29). By alumina-gel fractionation of lamb liver supernatant fluid, they were able to obtain separation of the sulfate activating system from the p-nitrophenol transfer system. Incubation of ATP and radioactive sulfate with either a liver ammonium sulfate fraction or the extract of Neurospora sitophila led to the formation of a substance containing radioactive sulfate that, on paper electrophoresis and autoradiography, had a mobility just faster than ATP at pH 5.8. This compound was also shown to be labelled on incubation by carbon-labelled ATP, and could transfer its sulfate group to p-nitrophenol in the liver system. The stoichiometry indicated that inorganic pyrophosphate was probably a product of the reaction. This evidence, plus the fact that the compound had an absorption maximum at 260 mμ, led to the tentative conclusion that the substance was an adenyl sulfate derivative. In analogy with acetate activation, the sulfate activation was thought of as a reaction between ATP and sulfate to form an anhydride by pyrophosphate elimination.

Robbins and Lipmann (30, 31), using a purified phenol sulfokinase for assay, were able to prepare 50 μmole quantities of active sulfate with a purified liver sulfate activating system. The active sulfate could be isolated by chromatography on Dowex-1 columns. Quantitative analysis revealed that the compound contained two moles of phosphate for one mole of adenosine and sulfate. One phosphate was acid-stable and the other acid-labile. The lability was such as to suggest a 2'- or 3'-adenylic acid. This was supported by the absence of reaction with periodate. The specific 3'-nucleotidase prepared from rye grass seed liberated an equivalent of phosphate from the compound. From this data it was concluded that active sulfate is 3'-phosphoadenosine-5'-phosphosulfate, or PAPS:



This structure has been confirmed by chemical synthesis (32).

The Two Enzymatic Steps of Sulfate Activation

The presence of a phosphate in the 3'-position in addition to the 5'-phosphosulfate group suggested a two-step reaction. Bandurski and his collaborators (33, 34) made the observation that the sulfate activation in yeast could be split into two heat-labile fractions, inactive by themselves but active when combined. Bandurski et al (34), and Robbins and Lipmann (35) presented evidence that enzyme I, ATP-sulfurylase, catalyzes the formation of adenosine-5'-phosphosulfate (APS) which is then converted into PAPS by enzyme II, adenosine phosphosulfate kinase (APS-kinase), as shown in the reaction sequence:

$$\begin{aligned} ATP + SO_4^{-2} &\xrightarrow{\text{Enz. I}} APS + PP \\ APS + ATP &\xrightarrow{\text{Enz. II}} PAPS + ADP \\ \hline 2ATP + SO_4^{-2} &\longrightarrow PAPS + ADP + PP \end{aligned}$$

The first reaction catalyzes the displacement of inorganic pyrophosphate from ATP with the formation of APS. The second reaction is the phosphorylation by ATP of the 3'-hydroxyl group of APS to form PAPS. Bandurski and co-workers (34, 36) assayed for sulfurylase activity by substitution of certain group VI anions for sulfate. Pyrophosphate was then liberated from ATP by sulfurylase presumably by transient formation of an unstable anhydride between adenosine monophosphate (AMP) and the anion. Selenate, sulfite, chromate, tungstate, and molybdate led to increasingly active enzymatic cleavage of ATP to adenylic acid and inorganic pyrophosphate in the presence of sulfurylase. Stable adenylic acid-anion anhydrides were formed only with sulfate and selenate. Robbins and Lipmann (35) assayed for sulfurylase by measuring the reverse reaction by means of synthetic APS (37, 38). The chemically synthetic APS has been shown to be identical with that produced enzymatically.

The ATP-sulfurylase of yeast could be highly purified by ammonium sulfate precipitation followed by electrophoresis on an inert resin, Geon 426 (39). An electrophoretically and ultracentrifugally homogeneous protein was obtained. With the purified enzyme, equilibrium studies were performed that indicated an extremely unfavorable equilibrium for APS formation. At pH 8 and 37°, Robbins and Lipmann found an apparent equilibrium constant of approximately 10^{-8}; therefore, ΔF° equals + 11 kilocalories.

$$K_{app} = \frac{[\overline{APS}]\,[\overline{PP}]}{[\overline{ATP}]\,[\overline{SO_4}]} = 10^{-8}$$

APS-kinase was found to have a very high affinity for APS (40); furthermore, the initial rate of reaction was highest with the lowest concentration of APS that could be tested, 5×10^{-6} M. As noted by Lipmann (41), the amounts formed enzymatically are, under physiological conditions, probably of similar magnitude, and the little that is formed can be immediately phosphorylated by APS-phosphokinase and can thus be eliminated from the equilibrium which it inhibits. The reaction is pulled, therefore, in the direction of PAPS synthesis. The other product of the reaction, pyrophosphate, is removed by pyrophosphatase. Both of these reactions are strongly exergonic. Two energy-rich phosphates are used for the formation of bonds of higher group potential, the phosphosulfate anhydride. The phosphorylation of APS (-6 kilocalories), and the pyrophosphate hydrolysis (-5 kilocalories) approximately balance the initial endergonic step (+ 11 kilocalories). Robbins and Lipmann (39) also describe a yeast enzyme, adenosine diphosphate (ADP)-sulfurylase, which catalyzes the reaction of APS with inorganic phosphate or arsenate. This enzyme is very unstable and its significance remains obscure.

Brunngraber (42) has recently described an improved method for making 85-90% pure PAPS in substrate amounts by use of the activating system from rabbit liver and isolation by chromatography on Dowex-1 with sodium chloride elution.

Species and Tissue Distribution of PAPS Formation

The enzymatic system for forming PAPS has been found in a wide variety of species and tissues, including mammalian liver and Neurospora sitophila (29); chick embryo (43); baker's yeast (33); colonic mucosa (44); mouse mast cell tumor (45); and Fusarium solani (46). As a result of the experiments described in this report, the green, red, and brown marine algae, and the mucous gland of the marine snail, Busycon canaliculatum, may be added to this list (47).

A direct demonstration of the endogenous formation of PAPS in vivo has been made (45). Shortly after the injection of S^{35}-sulfate into the mouse, hot water extracts of liver, colon, and mast cell tumor were shown to contain PAPS.

On the basis of studies of the growth requirements of certain mutants of Neurospora, it has been postulated that the initial step in the biosynthesis of cysteine and methionine in this organism is the activation of sulfate (48). Ragland (49) has described two mutants which will grow on sulfite or

any more reduced organic sulfur source as well as a number of organic forms of sulfur. One mutant is completely lacking in ATP-sulfurylase activity, whereas the other has about three times the wild type activity and may be deficient in APS-kinase or PAPS-reductase. Spencer and Harada (50) have recently reported on a series of mutants of Aspergillus nidulans with blocks in the PAPS-synthesizing system.

PAPS and Sulfate Reduction

PAPS has been implicated in sulfate reduction by baker's yeast. Wilson and Bandurski (51) have shown that PAPS can be reduced by the reduced form of triphosphopyridine nucleotide (TPNH), and Hilz and Kittler (52) found that p-nitrophenylsulfate plus 3',5'-diphosphoadenosine (PAP) in the presence of phenolsulfokinase will substitute for the ATP requirement of the sulfate-reducing system. On the other hand, Peck (53) has investigated sulfate reduction in Desulfovibrio desulfuricans where sulfate serves as the terminal electron acceptor. An extract contained an active ATP-sulfurylase, and the stoichiometry of the reduction reaction showed that for each mole of hydrogen taken up, one mole of ATP was formed. APS would substitute completely for ATP. APS, therefore, appeared to be the substrate for reduction in this system. Whether the reactions for sulfate reduction in the case of assimilation differ from those involved in the respiratory reactions remains to be clarified. The finding of Ragland (49) of a Neurospora mutant requiring sulfite or any more reduced sulfur compound for growth and having an increased sulfurylase activity, suggests a direct way of studying whether or not PAPS is required for assimilatory reduction. If APS-kinase is found to be lacking and not the reduction system, then PAPS is implicated as the form of sulfate required for reduction.

The Sulfokinases

The role of PAPS as the common metabolic carrier of activated sulfate in nature has been repeatedly confirmed. The enzymes that catalyze the transfer from PAPS to various acceptor compounds have been termed sulfokinases (41). The term sulfokinase may be applied to a class of compounds, i.e., steroid sulfokinase, with the realization that this may represent a family of enzymes with more or less sharply developed specificity (54).

Phenol Sulfokinase

Phenol sulfokinase of rabbit liver has been studied extensively by Gregory and Lipmann (55), and by Brunngraber (42). The enzyme system, which has been partially purified, appears to have a rather broad specificity. Sulfate can be transferred from PAPS to a number of phenolic compounds. The system is, however, specific with respect to PAPS. The sulfate group potential in p-nitrophenyl sulfate was shown to be less than 2,000 calories below, and that in 3,5-dinitrophenyl sulfate to be only 850 calories below that of PAPS. The transfer of sulfate from p-nitrophenyl sulfate to other simple phenolic compounds is a PAP-dependent reaction. Transfer from p-nitrophenyl sulfate to the β-3'-hydroxyl groups of steroids in the presence of steroid sulfokinase from rabbit liver, however, could not be demonstrated (55). With the appearance of the color of p-nitrophenol as a measure of this reaction, an assay system sensitive to less than one millimicromole of PAP and PAPS was developed. The distribution of PAP in a number of tissues was determined, with the realization that the PAP might be, to a greater or lesser extent, the result of the autolytic hydrolysis of coenzyme A. Liver was found to have the highest concentration, and brain the lowest of the organs studied.

Brunngraber (42) reported a similar assay procedure in which m-aminophenol was acceptor, and the m-aminophenyl sulfate was determined colorimetrically. A nucleotidase that attacks PAP by hydrolysis of the 3'-phosphate linkage was found in the rabbit liver supernatant fraction. PAPS was attacked at a slower rate, but 3'-adenylic acid was not a substrate for the enzyme. PAP was shown not to be recycled in the system containing both activation and transfer abilities. In the system to which fluoride had been added to inhibit the nucleotidase action, PAP accumulated commensurately with m-aminophenol sulfurylation.

That the transfer system from PAPS to phenol may not be a simple one-step reaction is suggested by Brunngraber's extension of the earlier observations of Bernstein and McGilvery (25). These workers noted that when pre-incubation was carried out with sulfate, ATP, and Mg^{++} prior to phenol addition in the presence of fluoride and a low concentration of ATP (0.003 M), an anomalous and unexplained result was found. The addition of the phenol produced a rapid conjugation (expected, due to the accumulation of PAPS during the pre-incubation), which, however, slowed to an almost complete halt and then proceeded at about the same rate as the control. The existence of this inflection in the kinetic curves obtained in these experiments seemed to

indicate a delay for building up the concentration of the active sulfate intermediate after the initial accumulation was depleted. It was also possible that the intermediate contained a component which was added with the enzyme and, therefore, its concentration would limit the final level of the intermediate accumulation. This concept was supported by experiments in which reduction of the enzyme level by half lowered the inflection level by half, and increase in the concentration by half almost eliminated the inflection. Brunngraber's results ruled out the possibility that the esterification of phenols depended upon the recycling of PAP. He repeated the experiments of Bernstein and McGilvery but added isolated PAPS in the original incubation medium instead of permitting an accumulation of PAPS by means of a pre-incubation in the absence of phenol. The inflection in the course of phenol esterification was observed again. This inflection was also present in the curve that represented PAP accumulation. The concentration of PAPS was never limiting and was actually increasing during the lag period of phenol esterification. The suggestion was made that this anomalous behavior might indicate the presence of other, undisclosed enzymes or co-factors that play a role in the metabolism of sulfate. Another finding which may be pertinent is that some enzyme preparations (only in some preparations of lower activity from female rat liver) continued to shown an initial lag period despite the fact that PAPS had been included in the original incubation medium.

Tyrosine-O-Sulfate

Although tyrosine-o-sulfate is normally found in appreciable amounts in human urine (56), attempts to sulfurylate the phenolic amino acid, tyrosine, in the supernate of human or rat liver homogenate have been unsuccessful (54, 57, 58, 59). Segal and Mologne (57) tested a number of tyrosine derivatives as sulfate acceptors, using the assay of Gregory and Lipmann (55). Only those compounds in which the carboxyl group was absent or substituted, and in which the amino group was unsubstituted, underwent sulfurylation. These results suggested that an analogous type of derivative is the naturally occurring sulfate acceptor. Four-fold purification of the rat liver enzyme by ammonium sulfate fractionation failed to separate this activity from phenol sulfokinase; the same enzyme may be involved.

The quantities of tyrosine-o-sulfate found in urine by Tallan et al (56) represent a sizeable fraction of the total tyrosine excreted. If it arises

from the breakdown of peptide-bound material, it could suggest that the total pool of sulfurylated protein is larger than heretofore supposed, or that it undergoes a rapid turnover. It is quite possible, as suggested by Segal and Mologne (57), that a number of proteins occur naturally in a sulfurylated form, since in the usual procedure for the analysis of the composition of proteins the sulfate groups would be rapidly hydrolyzed, and sulfate is usually not sought among the hydrolysis products. A portion of the sulfur found to be associated with serum proteins by Dziewiatkowski and his collaborators (60, 61), and by Smith et al (62) may be in the form of peptide-linked sulfate.

Bettelheim (63) has found that the hydroxyl groups of the tyrosine residues in bovine fibrinogen are esterified with sulfate. Fibrinogen is converted to fibrin in the presence of thrombin. During this process, limited proteolysis of the bovine fibrinogen molecule occurs and two peptides, A and B, are released. Bettelheim found that peptide B from bovine fibrinogen contained tyrosine-o-sulfate. This finding has been confirmed by Blombäck and Vestermark (64), and Von Korff and Bronfenbrenner (65). The latter workers, however, found no evidence for the presence of tyrosine in the peptides liberated during the clotting of human fibrinogen. Recent experiments by Blombäck et al (66) show that $S^{35}O_4$ administered to rabbits can be found in tyrosine-o-sulfate in one of the fibrinopeptides released on clotting. It is of some interest to note that tyrosine-o-sulfate is not a substrate for a mammalian liver arylsulfatase (67).

In a recent report, Grimes (58) confirmed the presence of tyrosine-o-sulfate in normal human urine (also phenylsulfate, p-cresylsulfate, and indican), but was unable to detect any in the urine of the rat. When radioactive sulfate was administered to rabbits, rats, guinea pigs, and mice, it was possible to detect the above listed phenolic compounds but no labelled tyrosine-o-sulfate. It is of interest that in man, whose fibrinopeptides do not contain this compound, tyrosine-o-sulfate is found in large amounts in the urine, whereas in animals like the rabbit, which do contain the sulfurylated amino acid in fibrinogen, no tyrosine-o-sulfate is detected in the urine. Grimes, unlike Segal and Malogne (57), was not able to observe any sulfurylation of L-tyrosine amide or L-tyrosine methyl ester from inorganic sulfate in rat liver supernatant fractions assayed by paper chromatography. This apparent discrepancy may be due, at least in part, to the different pH's used for incubation by the two groups of workers. When tyramine was incubated

with this system a sulfurylated product was formed which did not have the same mobility on paper chromatography in three solvent systems as chemically-prepared tyramine sulfate. It was suggested that the conjugate might be an N-substituted sulfate.

Recently, Dodgson, Powell, and Tudball (68) injected tyrosine-o-S^{35}-sulfate into rats, and examined the urine for radioactive compounds. They found that only a small amount of desulfation had occurred but that the urine contained two radioactive esters which were not the same as tyrosine-o-sulfate by paper chromatography. The same results were found in rats maintained on antibiotics to avoid degradation by intestinal flora. The rapid conversion to these two principal metabolites was invoked as the reason for not finding tyrosine-o-sulfate in rat urine (58).

Sulfurylation of the Catechol Amine Hormones

The experiments of Richter (69) indicated that a large percentage of an administered dose of d- or l-epinephrine in man and in the rabbit was eliminated in the urine in the form of the sulfate conjugate of epinephrine in which one of the phenolic hydroxyls was esterified. Recently, Axelrod et al (70) have reported the finding of 3-methoxy-4 hydroxyphenylglycol sulfate as a new metabolite of epinephrine and norepinephrine. In rats, about 35% of administered epinephrine or norepinephrine was excreted as this sulfurylated metabolite, whereas in man, only 5% of the catechol amines was present in the urine as the conjugated glycol. Whether the sulfate moiety is esterified to the hydroxyl group of the aromatic ring or to the side chain has not been determined. Subjects with pheochromocytomas excreted from 3-10 mg of the conjugate daily. In rat liver slices and liver homogenate fractions, Vestermark and Boström (71) have recently obtained evidence for sulfurylation of epinephrine and norepinephrine starting with $S^{35}O_4$ and ATP.

Mention should also be made of the finding of sulfurylation of other phenolic compounds such as triiodothyronine and triiodothyroacetic acid (72, 73). Triiodothyronine sulfate has been found in the plasma and in the bile but not in the urine. This observations has stimulated speculation on the physiological role of this compound beyond that of an excretory product.

The question as to whether there is one phenol sulfokinase with a broad specificity, or separate enzymes for the different phenolic compounds, has not been resolved. Of possible significance in this regard is the finding by Nose and Lipmann (54) that a partial separation in activity for sulfuryla-

tion of p-nitrophenol and estrone (a phenolic steroid) was obtained by resin electrophoresis.

Steroid Sulfokinases

The synthesis in vitro of the sulfate conjugates of dehydroepiandrosterone was first observed by De Meio and Lewycka (74). Segal (28), using a similar soluble extract of rat liver, detected estrone sulfate formation. Roy (75) has presented other experimental evidence for the formation of dehydroepiandrosterone and other steroid sulfates. The requirements for such a synthesis appear to be the same as those found for the sulfurylation of the phenols. Direct evidence for the sulfurylation of dehydroepiandrosterone, testosterone, estrone, and estradiol-17β by these systems has been provided recently by De Meio et al (76). Bridgwater and Ryan (77) have obtained sulfurylation of a number of polyhydric steroid alcohols in frog liver homogenates. Schneider and Lewbart (78) found that of 32 steroids which were incubated with rabbit liver supernate, 14 were conjugated with sulfate. Two apparent relationships between the structures of the steroids and their ease of conjugation were noted. Among the 21-carbon steroids, those having the Δ^5-3'-β-hydroxy system were more extensively sulfurylated than a number of related, fully saturated steroids. In addition, conjugation appeared to occur more readily when the steroid substrate belonged to the allo series (A/B ring fusion trans). Androsterone, epiandrosterone, dehydroepiandrosterone, and androstanediol were readily conjugated, whereas etiocholanolone and etiocholanediol were conjugated to only a minor degree. Also, allopregnanediol was conjugated more readily than prenanediol. That esterification with sulfate was not limited to the hydroxyl group at the 3 position, however, is indicated by the evidence that testosterone and deoxycorticosterone are conjugated.

Nose and Lipmann (54) attacked the question as to whether the steroid sulfokinase and the phenol sulfokinase were the same enzyme. They showed that by combination of alumina gel absorption and ammonium sulfate fractionation, it was possible to separate the systems for transfer of sulfate from PAPS to phenols and steroids. Furthermore, by electrophoresis on Geon 426, they were able to separate estrone from dehydroepiandrosterone sulfurylating activities. Additional data suggested that the fractions obtained still represented mixtures of sulfokinases of greater specificity. The ease of conjugation with sulfate found for various steroids coincided with the

results of Schneider and Lewbart (78). The dehydroepiandrosterone sulfokinase also reacted with other steroids having a 3'-β-hydroxyl group, such as epiandrosterone and progesterone.

Arylamine Sulfokinases

Boyland et al (79) have shown that the administration of certain arylamines to rats or to rabbits is followed by the urinary excretion of the corresponding arylsulfamates. This was followed by the demonstration of the synthesis *in vitro* of 2-naphthyl sulfamate by rat liver preparations (80), and of phenyl sulfamate by sheep intestinal-mucosal enzymes (81). Roy (82) has extended these studies; 1-naphthylamine and aniline have also been found to be substrates for this enzyme system but benzylamine and glucosamine were not. The sulfate donor was shown to be PAPS but the enzyme differed from phenol sulfokinase in requiring magnesium ions for maximal activity; it, too, was inhibited by p-chloromercuribenzoate, and the inhibition was abolished by cysteine. A most interesting finding was that the synthesis of 2-naphthyl sulfamate by rat liver enzymes is increased by about 400% by 17-oxosteroids in concentrations of approximately 10^{-5} M. This was not so for guinea pig liver preparations. It was suggested that the mechanism of this activation involves the formation of steroid 17-enol sulfates and that these compounds would be a form of "active sulfate", having a high sulfate-group potential. Some support for this concept was provided by the lack of stimulation of synthesis of arylsulfamate by 16-hydroxy 17-oxosteroids, which cannot enolize. The chemical synthesis of such a 17-enol sulfate has thus far not been successful. The physiological significance of this observation remains to be determined.

Choline Sulfokinase

Since the first isolation of choline sulfate in *Aspergillus sydowi* by Woolley and Petersen (83), this compound has been found in *Penicillium chrysogenum*, and in lichens of the genus *Roccella*, and the red alga *Gelidium cartilagineum* (85). The role of choline sulfate is not clear. Kaji (86) has indicated that the substance might be a reservoir of carbon and sulfur since it can serve as a source for these elements in some microorganisms. The biosynthesis of choline sulfate in extracts of *A. sydowi* has been shown recently by Kaji and McElroy (87). Kaji and Gregory (88) have shown that PAPS is the sulfate donor in the reaction. The enzymatic activity was stimulated by

magnesium ions and cysteine. Attempts to show reversal of the reaction, from choline sulfate to PAP, were unsuccessful.

Spencer and Harada (50) have recently reported on the occurrence of choline sulfate in the mycelium of over 30 fungi grown in media containing radioactive inorganic sulfate. All of the Moniliales, apart from Torula utilis, accumulated choline sulfate in the mycelium but none of the Mucorales and Endomycetales nor any of eight bacteria produced this compound. Experiments with cell-free extracts showed the presence of the PAPS-synthesizing system and choline sulfokinase in all the fungi which produce choline sulfate in vivo. On the other hand, the PAPS-synthesizing system was present in all fungi which can utilize sulfate as the sole source of sulfur.

The Mucopolysaccharides of Higher Animals

In higher animals, polysaccharide sulfates are found principally in the connective tissues. These polysaccharides include the chondroitin sulfates (A, B, and C), keratosulfate, heparin, and the heparitin sulfates (89, 90), all of which contain either D-glucosamine or D-galactosamine. These compounds have been designated mucopolysaccharides (91, 92).

In the biosynthesis of the sulfated mucopolysaccharides two main mechanisms have been considered and have been subjected to experimentation:

1. Based primarily on the finding in tissues of polysaccharides sulfated in varying degrees or not at all (chondroitin), under conditions of isolation not expected to lead to desulfation, the suggestion was made by Meyer and his group (89, 93) that the polysaccharide chain is first synthesized and subsequently sulfurylated. If there were a chondrosulfatase in mammalian tissues, this might detract somewhat from their arguments. So far, however, such an enzyme has not been found in mammalian tissues (94), although shown to be present in certain microorganisms (95, 96), and the digestive organs of molluscs (97). Some liberation of $S^{35}O_4$ into the urine, however, has been observed following the feeding or injection of S^{35}-labelled chondroitin sulfate to rats (98, 99). The possibility of desulfation by organisms of the gastrointestinal tract has not been excluded, although rats pre-treated with antibiotics excreted radioactive inorganic sulfate in the urine when given the S^{35}-chondroitin sulfate parenterally. Some evidence, which is far from conclusive, has been presented that the enzyme elastase contains more than one component, and that one of these might be a chondrosulfatase (100, 101).

2. The other mechanism which has been postulated to occur, is based on the polymerization of small sulfurylated intermediates. Support for this view

had been previously provided by the finding by Strominger (102) of uridine diphosphoacetylgalactosamine sulfate in the hen oviduct. So far, however, no evidence has been presented for the participation of a sulfurylated nucleotide-bound carbohydrate intermediate in the formation of chondroitin sulfate.

The Chondroitin Sulfates

The incorporation of radioactive sulfate into cartilage has been studied in the whole animal and in tissue slices by Dziewiatkowski (103), and Layton (104), and by Boström and his group (105, 106). The first cell-free system successfully used for the biosynthesis of chondroitin sulfate was that of D'Abramo and Lipmann (43). PAPS was shown to be the sulfate donor to mucopolysaccharide in a high-speed supernatant fraction of extracts of embryonic chick or beef cartilage (107). No evidence for a low-molecular sulfurylated intermediary could be obtained, although the enzyme preparation appeared to have the system for making chondroitin sulfate de novo. Delbrück and Lipmann (108) have recently isolated an enzyme system from extracts of calf embryo cartilage by chromatography on DEAE-cellulose. This system could transfer sulfate from PAPS directly to various polysaccharide acceptors such as chondroitin sulfates A, B, and C, and the corresponding chemically desulfated compounds, as well as to heparitin. That this was not the result of an exchange reaction with the sulfate already on the polysaccharide was shown by the complete lack of reversibility when S^{35}-labelled chondroitin sulfate was used.

Adams (109), using the chick embryo extract of D'Abramo and Lipmann (43) found that chondroitin sulfate C isolated from umbilical cord stimulated sulfate incorporation, but chondroitin sulfate A from bovine trachea did not. The full meaning of this finding remains to be elucidated; the possibility of separate sulfokinases for A and C must be considered.

Suzuki and Strominger (110) have demonstrated the transfer of sulfate from PAPS to added mucopolysaccharide acceptors by a soluble enzyme system prepared from the isthmus of the hen oviduct. A number of mucopolysaccharides including chondroitin, chondroitin sulfates A, B, and C, and the heparin-like sulfated heptasaccharide from the liver of a patient with Hurler's syndrome were active in the system. Sixty-four percent of the sulfate from PAPS was transferred to mucopolysaccharide. Despite the presence of the sulfurylated uridine compound in this tissue, no evidence for any

intermediates in the transfer of sulfate from PAPS to chondroitin sulfate A could be obtained. In addition, it could be shown that this system transferred the sulfate directly from PAPS to monosaccharides, and oligo-saccharides containing N-acetyl galactosamine. The primary sulfurylation of N-acetyl galactosamine was catalyzed by the preparation as well as the introduction of a second sulfate to form an acetyl galactosamine disulfate. The system could also introduce a second sulfate into chondroitin sulfate.

Heparin

Spolter and Marx (111, 112) have shown the incorporation of sulfate from PAPS into heparin by mouse mast-cell tumor homogenates. Korn (113, 114) has demonstrated the incorporation of radioactive sulfate into heparin in tumor slices. The distribution between ester and amide sulfate was approximately equal. When the transfer of radioactive inorganic sulfate to heparin was examined in the soluble fraction of mast cells, most of the radioactivity had been fixed into ester linkages. It was not possible to tell whether the newly incorporated radioactive sulfate occupied sites that were not esterified before.

Mucopolysaccharides of Molluscs

In 1925, Levene (115) analyzed the mucoitin sulfuric acid of the mucous secretions of the snails, Helix aspersa and Helix pomatia. These studies were extended by Suzuki (116) who found the foot mucin of the snail to be lacking in sulfate, whereas the mucus mucin was composed of hexuronic acid, chitosamine, acetic and sulfuric acids, and galactose. He concluded that this was composed of a mucoitin sulfuric acid plus an acetyl chitosamine: galactose complex (1:1) - the exact chemical composition of the former is still in doubt.

A series of heparin-like compounds, the mactins, have been extracted from clam tissues (117). A sulfated polysaccharide composed of equivalent amounts of glucosamine and galactosamine has been described in the mucin secreted by the hypobranchial gland of Busycon canaliculatum (118, 119, 120). On the basis of infrared data alone it was concluded that the amino groups of the sugars were unacetylated and that the sulfate was not covalently bound. Chemical analysis is required. Recently, Lash and Whitehouse have reported the finding of polyglucose sulfate in the chondroid tissue (odontophore) of Busycon (121). Masamune and his co-workers (122, 123) have

characterized limacoitin sulfate, from the mucin of a Japanese snail, which contains ester sulfate, L-fucose, galactose, mannose, galacturonic acid, and glucosamine.

Polyglucose sulfate has been found in the mucin of the marine snail, Charonia lampas (124, 125). After injection, radioactive sulfate was shown to be localized in the mucous gland of this snail as the ethereal sulfate of charonin sulfuric acid (126, 127). Charonin sulfuric acid is a mixture of glucan polysulfates with different sulfur contents (1-20%). A part of the glucan has a cellulose structure, and the other part an amylose structure (125). Whereas radioactive sulfate could be incorporated into charonin-sulfate by slices of the mucous gland, no incorporation was observed in extracts of the acetone powder of the gland unless p-nitrophenyl-$S^{35}O_4$ was present as the sulfate donor (128). A purified arylsulfatase preparation of the liver of the snail was able to catalyze the incorporation of $S^{35}O_4$ from the p-nitrophenyl sulfate into the polysaccharide (129). This finding, plus the inhibition of the reaction by phosphate and fluoride, suggest an analogy to the ability of p-nitrophenyl phosphate to act as a non-specific phosphate donor with phosphatase (130).

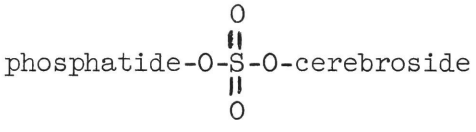
Sulfated Polysaccharides of Marine Algae

The marine algae are rich sources of sulfated polysaccharides. These are typified by the polygalactose sulfates of the red algae, Chondrus crispus (carrageenin) and Porphyra umbilicalis; the polyfucose sulfates of the brown alga, Fucus vesiculosus (fucoidins); the heteropolysaccharide containing rhamnose sulfate of the green alga, Ulva lactuca; and the mucilage from Dilsea edulis (a heteropolysaccharide containing D-galactose, glucuronic acid, xylose, 3:6-anhydrogalactose, and ester sulfate) (131, 132). The galactan sulfate of Porphyra umbilicalis has recently been shown to contain D-galactose, 6-O-methyl-D-galactose, and 3:6-anhydro-D-galactose (133). No work has been published to date on the biosynthesis of the sulfated polysaccharides in these organisms.

The Sulfolipids

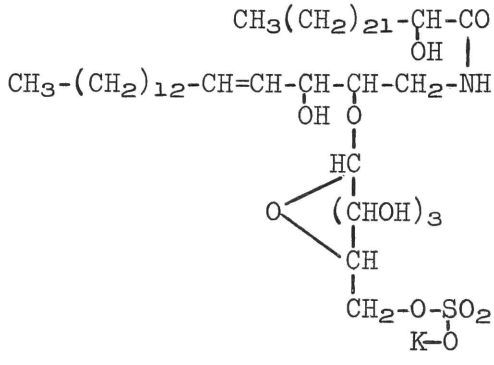
Thudichum (134,135, 136) reported in 1884 the presence of sulfur in a preparation of cerebrosides obtained from brain. The purified product had a sulfur content of 6.19%, which was of such magnitude as to preclude its being an impurity. Brain protagon (the white mass resulting after exhaustive

acetone-ether extraction of brain) was believed by many to represent a single lipid species with both sulfuric and phosphoric acids in the structure of its molecule. Thudichum, however, mainly on theoretical grounds, 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 isolated a sulfate-containing lipid from an ether-insoluble residue which contained 1.91% sulfur, 1.80% phosphorus, and 12.8% sugar (137). Koch was led to the conclusion that the sulfolipid contained an equimolar proportion of sulfuric and phosphoric 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. In studies on the lipids of beef brain, P. A. Levene (138) settled the problem by isolating 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 isolated a substance having only slight solubility in water, a positive orcinol test, 2.87% sulfur, and no phosphorus (139, 140).

In 1933, Blix (141) was able to isolate from human brain a sulfatide free of phosphorus which amounted to 20-25% of the total cerebrosides. Its constituents upon hydrolysis were cerebronic acid (hydroxylignoceric acid), sphingosine, galactose, and sulfate. The composition suggested a substance 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 structure he suggested was the following:

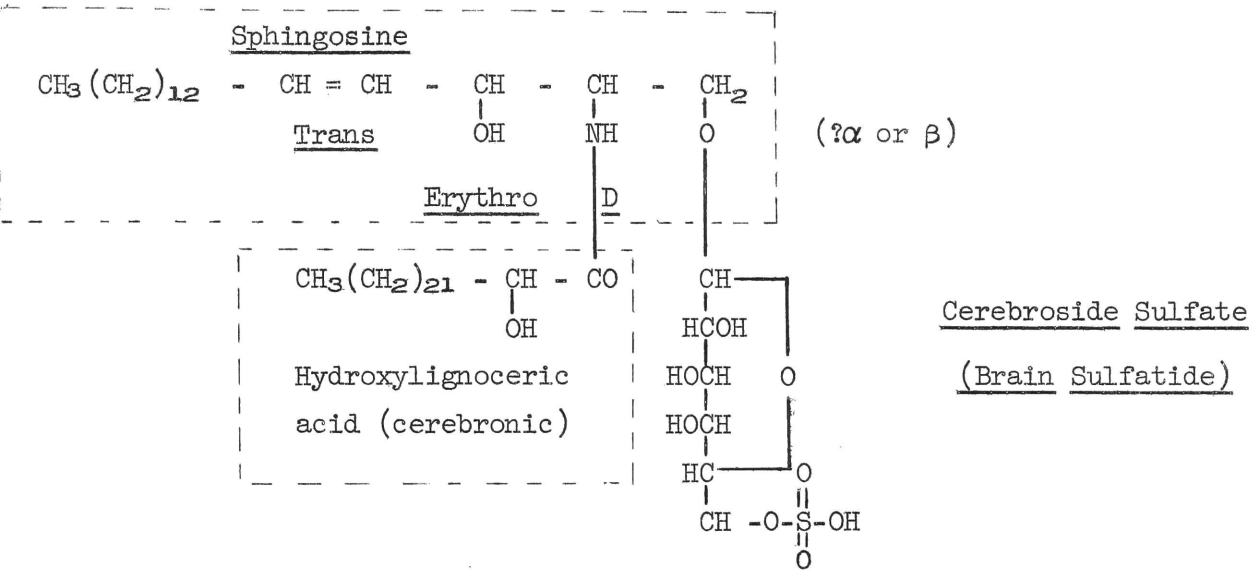


The structure written by Blix was in error - the sulfate was correctly located on the galactose moiety but the structure of sphingosine, and the place of attachment of the sugar to the lipid were not correct. It was not until several years later that the relative position of the functional groups of sphingosine and the point of attachment of the glycosidically-bound galactose in cerebroside were determined by Carter and his collaborators (142, 143, 144). Attempts to determine the configuration of the galactosidic bond have given contradictory results and this question remains to be settled. A direct proof of both the D-configuration of the amino carbon and the erythro configuration of the asymmetric system in sphingosine (and dihydrosphingosine) has been provided by this same group (145, 146). By use of Klenk's barium hydroxide procedure for alkaline hydrolysis (147) cerebroside was hydrolyzed (a process not expected to lead to inversion), and an excellent yield of the erythro isomer (no threo) of sphingosine was obtained. The trans structure of the double bond in sphingosine was shown by the infrared studies of Mislow (148), and of Marinetti and Stotz (149).

These results make it seem certain that sphingosine as it exists in the cerebroside molecule has the erythro configuration. Similar studies have not been carried out on sphingomyelin or sulfatide but it is assumed by these workers that only one isomer of sphingosine occurs in the various natural sphingolipids.. The enzymatic studies of Sribney and Kennedy (150) also indicate that the sphingosine moiety of sphingomyelin isolated from tissues is predominantly or entirely in the erythro configuration.

Little if any work had been done on the cerebroside sulfuric acid ester from the time of Blix's isolation and characterization (1933) until 1951 when Nakayama examined the problem of the position of the sulfate group (151). From his unsuccessful efforts to tritylate the cerebroside sulfate, he concluded that the primary hydroxyl of the galactose was esterified by sulfate. Thannhauser et al (152), 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 a relatively pure cerebroside sulfate ester isolated from beef brain (153), were able to methylate the compound, and the methyl galactose obtained after methanolysis was identified by paper chromatography with known methylgalactoses. That the sulfuric acid must be esterified to the primary

hydroxyl on carbon-6 of the galactose of the cerebroside sulfate was shown unequivocally. Based on the combined data of the above ~~ester~~ studies, a probable structure for cerebroside sulfuric acid ester may be written:



In addition to the finding of sulfolipid in horse kidney by Levene and Landsteiner (139, 140), there have been other reports of sulfur-containing lipids in tissues other than that of the nervous system. In 1907, Koch (154) reported the presence of sulfolipids in liver, testes, submaxillary glands, and muscle, although the highest concentrations were in the white matter of brain. Sammartino (155) has reported finding sulfur-containing lipids in the lung. Blix (141) has suggested that the sulfolipid reported to have been isolated from dog and rabbit liver, beef spleen, horse blood and muscle by Baldi (156), and from the adrenal of cattle and horse by Manasse (157), is the cerebroside sulfuric acid ester. These lipids, however, up to the present time have not been characterized.

One of the earliest studies on the incorporation of radioactive sulfur into the brain was reported in 1945 by Dziewiatkowski (158), who fould 0.02% of a dose of orally-administered S^{35}-sodium sulfide to be located in the brain. Boström and Odeblad (159) found the uptake of radioactive sulfate to be highest in the gray matter. The incorporation of parenterally-administered radioactive sulfate into isolated sulfolipids of rat brain has been demonstrated by Holmgärd (160). By using C^{14}-galactose, and S^{35}-sulfate administered to rats, Radin and his co-workers (161) measured the turnover

of isolated brain sulfatide and compared its metabolism with that of cerebroside. It was found that the rate of incorporation of C^{14}-galactose into sulfatide was slower than that into cerebroside. The rates were compatible with the cerebroside's being the precursor of the sulfatide. In confirmation of the results of Koch and Koch (162), who had found that sulfatides continued to accumulate for a considerable portion of the life of the rat, these workers showed that an old rat was still able to incorporate S^{35}-sulfate into the brain sulfatides. Recently, Green and Robinson (163) have compared the rates of turnover of injected $S^{35}O_4$ that was incorporated into brain mucopolysaccharides and brain sulfolipid. There was a fairly rapid turnover of the mucopolysaccharide sulfate but the sulfolipid accumulated in the brain, although apparently not in other tissues.

Radin et al (161) have described a P^{32}-labelled lipid which could not be separated by column chromatography from the $S^{35}O_4$-labelled sulfatide fraction. Recently Folch and his collaborators (164, 165) have obtained, by a new, simplified procedure for the preparation of brain sulfatides (based on the distribution of brain lipids between the two phases of a series of related solvent systems), evidence for two main sulfatide fractions. One fraction was essentially pure sulfatide; the other contained both sulfur and phosphorus but could be separated into a phosphorus-free sulfatide fraction and a phosphatide mixture by passage through a Florisil column. Analysis of the second sulfatide fraction revealed components which suggested to the authors that this fraction was composed of sulfatide plus non-phosphatide contaminants such as cerebrosides and cholesterol. Complete characterization remains to be done.

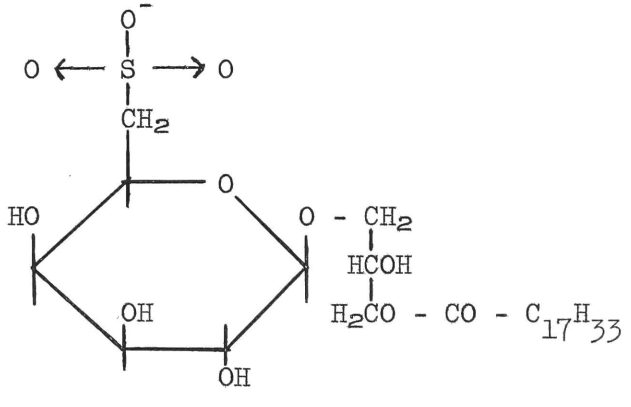
Sulfatides in Diffuse Metachromatic Sclerosis

Of considerable interest are the recent reports of Austin (166) on the finding of metachromatic-staining lipid granules in the urine of children with the so-called metachromatic form of diffuse cerebral sclerosis, a progressive demyelinating disease of the central nervous system (167,168,169, 170). By study of the solubility, staining, and electrophoretic characteristics, and the inhibition of the staining reaction with protamine, Austin concluded that the urinary substance was a "carbohydrate-lipid polyanion complex." Pathologically, the same substance is found distributed in many tissues outside the nervous system.

Jatzkewitz (171) and Svennerholm (172) have recently developed paper chromatographic methods for the separation of the sulfatides from the other sphingolipids. They have found that preparations of brain sulfatide produce two spots on paper chromatography. Both spots revealed on hydrolysis the presence of sulfate, galactose, sphingosine, and a long-chained fatty acid. In addition, the slightly faster moving spot also contained phosphate. This is of interest in view of the findings of Radin et al (161) and Lees et al (165) reported above. The sphingolipid fraction of the myelin sheaths from patients with diffuse metachromatic sclerosis showed marked increases in the two sulfatide components. Svennerholm found increased amounts of these substances in pathological specimens of tissues outside the nervous system, including liver, kidney, blood, and urine. He also has evidence for a sphingolipid that contains two sugar units per sulfate - glucose attached to the sphingosine moiety, and galactose sulfate attached to the glucose.

Sulfolipids in Plants and Tubercle Bacilli

Recently there have been reports of two new sulfur-containing lipids in plants and virulent tubercle bacilli. Benson and his co-workers (173, 174,175) have described a sulfolipid present in photosynthetic microorganisms and higher plants. Its concentration in Chlorella (4×10^{-3} M) exceeds that of the phosphatides. This compound has not yet been fully characterized, but its resistance to acid hydrolysis has suggested that it might be 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 (176) 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 preliminary form. There appears to be, however, about one 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 percent of the 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 comes from infrared spectrophotometry which indicates sulfur-oxygen vibrations.

Background for this Investigation

At the start of this research project, direct evidence favoring one of the two proposed mechanisms for mucopolysaccharide sulfurylation in mammals was not available (developed in the Historical Introduction). Only evidence of a negative sort was to be had. Because of their rather complex nature - with substituted aminosugar and uronic acid units - and the additional problems introduced by the necessity for polymerization, the biosynthesis of mammalian mucopolysaccharides represented a formidable problem. It was decided, therefore, to study similar or related reactions in systems that undergo a simpler but analogous metabolic sequence than that involved in the synthesis of chondroitin sulfate (41). Brain cerebroside sulfate appeared attractive from this point of view. It is a monomer with a simple galactosyl sulfate attached to an N-acyl sphingosine moiety. By study of the sequence of sulfurylation and formation of the glycosidic linkage, one might be able to infer the mechanism in the more complex molecules.

The feasibility of studying the biosynthesis of lipid molecules attached to polar components was emphasized by the elegant studies of Kennedy and Weiss (177, 178) on the role of the cytidine nucleotides in the biosynthesis of lecithin and phosphatidyl ethanolamine from added D-α,β-diglycerides. It seemed reasonable to suppose an analogous mechanism for the biosynthesis of cerebroside and cerebroside sulfate in the brain. Similar to the schemes proposed for chondroitin sulfate synthesis, it was possible to formulate the following ones for cerebroside sulfate synthesis:

1. N-acyl sphingosine+UDPGalactose $\longrightarrow$ cerebroside $\xrightarrow{\text{PAPS}}$ cerebroside sulfate

2. N-acyl sphingosine+UDPGalactose-sulfate $\longrightarrow$ cerebroside sulfate

That the N-acyl sphingosines (ceramides) might be intermediates in the biosynthesis of sphingolipids had been suggested by Thannhauser and Reichel (179). The ceramides have been isolated from tissues under conditions that would not be expected to have led to their formation by breakdown of the cerebrosides or sphingomyelin. Free lignoceryl sphingosine has been found in pig liver and beef spleen (180, 181) without prior hydrolysis.

The possible importance of the N-acyl sphingosine compounds as intermediates in the biosynthesis of the sphingolipids was suggested by the work of Zabin (183) on the biosynthesis of ceramide by brain homogenates of 15-20 day old rats, and by the work of Sribney and Kennedy (184) on the enzymatic synthesis of sphingomyelin from CDP-choline and a crude preparation of the

stereoisomers of N-acetyl sphingosine by chicken liver particles. At about this time, Burton and co-workers (185) found uridine diphosphogalactose to be involved in cerebroside formation by particles from brain of 10-20 day old rats.

Accordingly, experiments were designed to study the incorporation of radioactive sulfate into sulfolipid by cell-free preparations of the brains of 10-20 day old rats. Preparations active in this respect that were stimulated by added N-acetyl sphingosine and were dependent on either endogenous or exogenous PAPS could be obtained. In the brain, evidence was obtained by acid hydrolysis that the radioactive sulfate was probably being incorporated into a cerebroside sulfate-like compound. Because of the greater activity and the soluble nature of the enzyme systems, a more detailed investigation of sulfolipid formation was pursued using rat liver extracts. Although the initial intention was to study the mechanism of sulfurylation of cerebroside sulfate, it gradually became apparent in the course of the experiments that the reaction under study in the liver system was rather a direct sulfurylation of one of the hydroxyls of the N-acetyl sphingosine. The lack of requirement for nucleotide co-factors in purified enzyme preparations, and the results of chemical degradations of the isolated radioactive lipid provide strong support for such a reaction. Further support for this concept was obtained by experiments on the sulfurylation of the stereoisomers of chloramphenicol,which bear marked structural similarities to the ceramides (see detailed discussion in Experimental).

The generality of sulfate activation and transfer was extended by experiments in cell-free extracts of the marine algae, and the mucous gland of the marine snail, Busycon - tissues known to be especially rich in sulfated mucopolysaccharides. The search for sulfurylated intermediates was continued in these systems.

METHODS AND PREPARATIONS

Preparation of Cerebroside - Cerebroside was prepared by crystallization from glacial acetic acid of the sphingolipid powder obtained from calf brain by the method of Carter et al (186,187).

Preparation of Crude N-Acetyl Sphingosine - A crude preparation of sphingosine sulfate was obtained by the hydrolysis of cerebroside with methanolic sulfuric acid by the method of Carter et al (187). Dihydrosphingosine sulfate was removed by recrystallization from methanol. The sphingosine sulfate so obtained consists of a mixture of the isomers of sphingosine (188). Crude N-acetyl sphingosine was prepared from the crude sphingosine sulfate by conversion to the free base in ether, followed by acetylation in the presence of alkali (187).

Other Sphingolipids - Crude sphingosine base was prepared as above with omission of the acetylating reagent. D-erythro-dihydrosphingosine was the gift of Dr. B. Weiss. Samples of cerebroside, cerebronyl ceramide, and N-palmitoyl-erythro-trans-sphingosine were gifts of Dr. H. E. Carter. N-acetyl-DL-threo-trans-sphingosine, N-acetyl-DL-erythro-trans-sphingosine, N-octanoyl-DL-threo-trans-sphingosine, N-acetyl-DL-erythro-acetylenic sphingosine, and N-acetyl-DL-threo-acetylenic sphingosine were the gifts of Dr. E. P. Kennedy. N-acetyl-D-erythro-dihydrosphingosine was prepared from D-erythro-dihydrosphingosine by the procedure of Carter et al (187). Psychosine (galactosyl sphingosine) was prepared from cerebroside by the method of Carter and Fujino (189). A sample of psychosine was also the gift of Dr. H. E. Carter. Brain sulfatide was isolated from frozen calf brain by the method of Lees et al (164,165). Samples of sulfatide were also obtained from Drs. Lees and Folch, Dr. G. Schmidt, and Dr. H. Jatzkewitz. All samples of the sulfatides behaved similarly on paper chromatography in the solvent system of Jatzkewitz (171), giving rise to two lipid-staining areas of slow mobility (see Experimental).

Yeast PAPS-Generating System - The PAPS-generating system was prepared from bakers' yeast by the method of Nose and Lipmann (54). The enzyme preparation was dialyzed against 0.02 M Tris buffer, pH 7.6, before use.

Preparation of $PAPS^{35}$ - Radioactive PAPS was prepared enzymatically from inorganic sulfate by use of the yeast system. The reaction mixture consisted of 120 μmoles of ATP, 600 μmoles of Tris buffer pH 8.0, 60 μmoles of

$MgCl_2$, K_2SO_4 (amount depending on the desired activity), 2 mc. of $S^{35}O_4$ (2 ml.), and 4.0 ml. of the dialyzed enzyme preparation in a total incubation volume of 9 ml. After incubation at 37° for 90 minutes, the reaction was stopped by boiling for one minute, the precipitate was spun off and the supernatant fluid was streaked on paper (Whatman No. 31, double thickness) for electrophoresis in 0.05 M ammonium acetate buffer pH 6.2. in the Durrum apparatus. After electrophoresis sufficient to provide adequate separation of PAPS from ATP (usually 200 volts for 16 hours), the paper was dried with the aid of a cool air blower, and the radioactive PAPS was eluted with deionized distilled water. The eluate was lyophilized to ensure complete removal of ammonium acetate, and was redissolved in water, neutralized by the addition of solid potassium bicarbonate and maintained as a stock solution in the deep freeze.

Chemical Sulfurylation of Crude N-acetyl sphingosine - The method is based on that of Duff (190) and Soda (191). Crude N-acetyl sphingosine (25 mg.) was dissolved in anhydrous pyridine (1.0 ml., dried over calcium hydride). Chlorosulfonic acid (8.6 mg.) dissolved in chloroform (0.5 ml.) was added to the pyridine solution at 4° with stirring by means of a teflon covered bar and a magnetic mixer. The stirring was continued for one hour and the reaction allowed to stand an additional three hours at room temperature. Water and chloroform : methanol (2:1) were added and the sulfuric acid was removed as barium sulfate by the addition of saturated barium hydroxide. The sulfurylated lipid was converted to the magnesium salt by addition of 0.25 M $MgSO_4$. The chloroform layer was separated, concentrated *in vacuo*, and used for spotting on paper chromatography. Further purification or identification of the sulfurylated lipid has not been accomplished. This material, however, will be referred to as *sulfurylated crude N-acetyl sphingosine*.

A similar procedure was carried out with N-carbobenzoxydihydrosphingosine (gift of Dr. B. Weiss). A new material was produced which, on paper chromatography in the solvent system of Jatzkewitz (see Experimental), moved just ahead of the radioactive sulfolipid produced enzymatically in the liver system and the synthetic sulfurylated crude N-acetyl sphingosine. This material was not further characterized.

Chemical Sulfurylation of D-threo-Chloramphenicol (D-threo-1-p-nitrophenyl-2-dichloroacetamido-1,3-propanediol) - In the same manner as above, D-threo-chloramphenicol (97 mg.) in anhydrous pyridine (5 ml.) was reacted with chlorosulfonic acid (45 mg.) in chloroform (2 ml.). After completion of the reaction,water was added and the solution was neutralized with 1 N potassium hydroxide. This solution was used for application on paper electrophoresis without further treatment. Two anionic ultraviolet quenching spots were detected by paper electrophoresis at pH 5.6 and at pH 8, corresponding to the mono- and di-sulfuric acid esters of chloramphenicol.

Preparation of the base-HCl of D-threo-Chloramphenicol (D-threo-1-p-nitrophenyl-2-amino-1,3-propanediol - Chloramphenicol was hydrolyzed for 2 hours at 100° with 1 N hydrochloric acid and the product isolated as the free base by the method of Rebstock et al (192). The free base was converted to the hydrochloride by evaporation of a dilute aqueous hydrochloric acid solution of the base. The product was recrystallized from absolute ethanol.

Preparation of the base-HCl of DL-erythro-Chloramphenicol (DL-erythro-1-p-nitrophenyl-2-amino-1,3-propanediol) - The corresponding base of the erythro series was prepared in the same manner as above except that in this case the free base fails to crystallize from the alkaline solution. The base was extracted with ethyl acetate and the extracts were dried and evaporated to give the crystalline base (193).

Preparation of N-acetyl-Chloramphenicol (D-threo-1-p-nitrophenyl-2-acetamido-1,3-propanediol) - Chloramphenicol free base was acetylated with acetic anhydride by the method of Rebstock et al (192) to give a crystalline product with m.p. 126 - 126.5°. This procedure has been shown to cause no isomerization (based on the method of Edman (194)).

D-erythro-chloramphenicol was the gift of Dr. R. Monro of this laboratory. The other isomers of chloramphenicol and derivatives not listed above were the gift of the Parke-Davis Company, Inc.

Other O-Sulfates - Tyrosine sulfate was the gift of Dr. H. Tallan. S^{35}-labelled tyramine-o-sulfate was synthesized chemically by the method of Schmidt (195). The synthesis of glycolaldehyde $S^{35}O_4$ was based on the method of Suzuki et al (129) for p-nitrophenyl $S^{35}O_4$. Glycolaldehyde in pyridine/chloroform was sulfurylated by S^{35}-chlorosulfonic acid prepared from inorganic $S^{35}O_4$, sulfuric acid, PCl_5, and chlorosulfonic acid. On paper electrophoresis, the reaction mixture showed one major radioactive area

(excluding unreacted inorganic $S^{35}O_4$) which moved just ahead of ATP at pH 5.6, and just ahead of AMP at pH 7.5. The radioactive compound could be prepared by elution with water from the dried paper previously soaked in the volatile triethylammonium carbonate buffer at the latter pH. This solution was not stable even when kept in a frozen state. Re-electrophoresis showed that the compound had given rise to several weaker faster and slower moving radioactive materials, one of which was inorganic $S^{35}O_4$. The slower moving compounds might represent condensation products.

Emulsification of the Sphingolipids - For most experiments the sphingolipid was made 0.03 M with water containing 1-2% Tween 20 for emulsification. This mixture was made up in a screw cap vial and shaken on a Nossel shaker at about 25% full speed for 1-2 minutes. In the case of the crude N-acetyl sphingosine preparation, adequate emulsification could usually be obtained even in the absence of the Tween 20. In many experiments, however, in order to standardize the reaction conditions, Tween 20 was included. Even with the use of Tween 20, some sphingolipid preparations remained poorly emulsified. Because of marked inhibition of the enzyme system by concentrations of Tween 20 over 3 mg. per ml., comparable emulsification could not be obtained with all lipid preparations. This important factor must be taken into account in any interpretation of relative activities of these sphingolipid preparations.

Assay for Incorporation of Radioactivity into Sulfolipid - The incubation mixture for each experiment is described in detail under Experimental and Results. It is appropriate, however, to emphasize here the need for adequate controls. It has been found that the only type of control that approaches the ideal is the following: the reaction mixture is the same as that in the experimental tube with the undenatured enzyme system and the concentration of Tween 20 that is present in the experimental tube to which sphingolipid has been added. The control tube is incubated for the same length of time as the experimental and just before stopping the reaction (by boiling or trichloroacetic acid), the sphingolipid is added. This must be done for each particular sphingolipid under study. The presence of Tween 20 during the incubation, and the addition of lipid just before stopping the reaction are most important, otherwise falsely high or low "control values" are found. The control values are then subtracted from the experimental.

Extraction, Washing, and Counting Procedures (Sulfolipid) - Advantage was taken of the fact that complete extraction of brain lipid can be accomplished by the use of 19 volumes of chloroform : methanol (2:1) v/v per gram of tissue, and the fact that the non-lipid contaminates and strandin appear in the upper aqueous phase when this solution is washed with aqueous saline (196,197).

McKibbin and co-workers (198,199) in studies on the extraction and purification of tissue lipids found that repeated washes of the chloroform solution with 0.25 M $MgCO_4$ resulted in the removal of contaminant nitrogen with the loss of only 1-2% of the lipid nitrogen. When water alone was used as a washing medium, the loss of lipid was prohibitive - the fatty acid in the washings being calculated as monoaminophosphatide, the loss for liver was 3.9%, brain 26.5%, kidney 8.6%, muscle 14.4% for six washings, and heart 6.2% after seven washings. The superiority of magnesium salt solution to other washing media in minimizing lipid losses has been explained as a "salting-out" effect, or due to the formation of water insoluble complexes with several of the lipids. $MgSO_4$ had also been used by Folch and Van Slyke as a washing solution (200).

Two basic methods have been employed for stopping the reaction following incubation. Both have been used alternatively in these experiments with similar results:

1. The reactionwas stopped by immersing the incubation tube in a boiling water bath for one minute; the tube was cooled to room temperature and the contents were then transferred to a 40 ml. graduated conical extraction vessel with a ground glass stopper (Maizel-Gerson vessel) with 19 volumes of 2:1 chloroform : methanol. The vessel was then shaken vigorously by hand repeatedly over a 15-minute period; the insoluble material was removed by filtration and the extract collected in the same type of vessel. To the extract was added an equal volume of either 2 M KCl or 0.25 M $MgSO_4$ (the latter was used routinely in all but the earliest experiments), and the tube was stoppered and shaken vigorously. The phases were separated by low speed centrifugation and the aqueous-methanol layer removed by aspiration. The chloroform phase was washed 5 more times with about 3 volumes of the salt solution, and a 2 ml. aliquot was plated in a stainless steel planchet, dried, and counted in a windowless gas-flow counter under conditions of negligible self-absorption. Control experiments showed that this amount of

washing was sufficient to remove inorganic $S^{35}O_4$ and $PAPS^{35}$ from chloroform : methanol extracts containing the amount of added sphingolipid and protein used in these experiments. Further washing with the salt solution or distilled water (one time) resulted in no significant loss of radioactivity from the chloroform phase in the incubation experiments. This is the method used in most of the experiments.

2. The reaction was stopped by the addition of an equal volume of cold 10% trichloroacetic acid. The tube was centrifuged and the supernatant fluid discarded. The precipitate was then homogenized with cold 5% TCA (2 ml.) by means of a tight-fitting glass plumper. This was repeated once more. The suspension was centrifuged and the supernatant fraction discarded each time. The residue was extracted with 2:1 chloroform : methanol (19 volumes to 1 volume of the original incubation), filtered, and washed with the salt solutions as described above.

Protein was determined turbidimetrically with TCA, with bovine serum albumin as a standard (201), or was calculated from ultraviolet absorption by Kalckar's formula (202).

Preparation of Rat Brain Fractions - Albino rats (10-20 days post partum) were killed by decapitation with a sharp scalpel, the soft crania were peeled off, and the whole brains were rapidly removed and placed in ice-cold 0.25 M sucrose containing 0.001 M versene (disodium salt of ethylenediaminetetraacetic acid) pH 8. Fractionation of the brains was accomplished by modification of the method of Brody and Bain (203). Whole brains were homogenized with 5 volumes of the sucrose/versene solution in a Potter-Elvehjem homogenizer at 0°. The homogenate was strained through 3 thicknesses of cheese cloth, and centrifuged for 10 minutes at 1,000 x g to remove nuclei, whole cells, and other debris. The supernate was centrifuged at 8,000 x g for 20 minutes, and the pellet obtained was washed once with 9 volumes of the above solution and recentrifuged at 8,000 x g for 20 minutes. The supernate and washings from the "mitochondrial" preparation were then spun for 30 minutes at 105,000 x g in the Spinco Model L Ultracentrifuge to remove the "microsomal" fraction. All fractions were diluted with the sucrose/versene solution back to the original volume for studies on enzymatic activity. No effort was made to further characterize the subfractions and, in fact, most experiments were conducted on particulate and supernatant fractions obtained by a direct 105,000 x g centrifugation after the initial 1,000 x g spin.

EXPERIMENTAL AND RESULTS

Incorporation of $S^{35}O_4$ Into Lipid by Rat Brain Homogenate

Incubation of radioactive inorganic sulfate with rat brain homogenate in the presence of ATP, Mg^{++}, and the PAPS-generating system from bakers' yeast resulted in the incorporation of label into sulfolipid (Table I).

TABLE I

Incorporation of $S^{35}O_4$ into lipid by brain homogenate

The complete system contained 100 μmoles of Tris buffer pH 7.4, 10 μmoles of glucose, 10 μmoles of galactose, 5 μmoles of $MgCl_2$, 0.5 μmoles of UTP, 10 μmoles of ATP, 0.8 μmole of K_2SO_4, 40 x 10^6 c.p.m. of $Na_2S^{35}O_4$ (carrier-free), 0.25 ml. of 11 day old rat brain homogenate (after 1,000 x g spin), and 0.1 ml. of yeast PAPS-generating system. The total volume was 1.0 ml., and the tubes were incubated at 37° for 90 minutes. The lipid fraction was isolated and counted as described in Methods. Omission of various components from the incubation are as indicated.

Omitted	c.p.m. into lipid
-	634
ATP	72
Mg^{++}	145
UTP	418
PAPS-generating system of yeast	306

The marked decrease in incorporation of radioactivity into sulfolipid, which takes place upon omission of either ATP or Mg^{++}, points to PAPS as the sulfate donor in this system. The fact that incorporation falls to about one half when the yeast system is not included indicates that the brain homogenate contains the enzyme system for activation of sulfate but such that this step then becomes rate limiting. If cerebroside sulfate (Blix's Compound) were being formed in this system, one might expect a stimulation of incorporation of sulfate into sulfolipid by addition of uridine nucleoside polyphosphates. The increased incorporation noted in the presence of a small amount of UTP may be related to the formation of appropriate lipid acceptors for sulfate; this effect, however, was not consistent enough to warrant further speculation.

Requirement for Cation - The requirement for Mg^{++} is shown in Fig. 1. Comparable concentrations of Mn^{++} were not as effective. At the higher

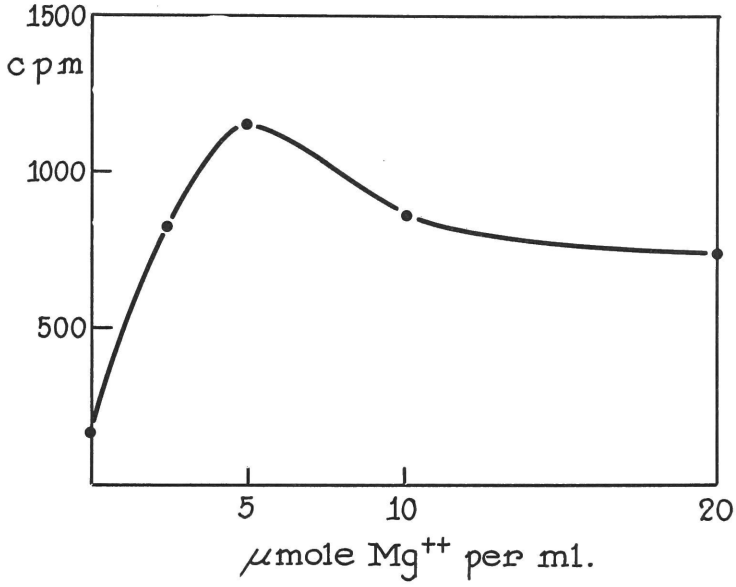


Fig. 1. Requirement for Mg^{++}. The incubation system was similar to that shown in Table I with rat brain homogenate, except for 0.4 μmole of K_2SO_4, so that the specific activity of $S^{35}O_4$ is twice that in Table I.

concentrations clumping of the particles and co-precipitation with ATP took place. The extent to which the cation concentration curve reflects solely the requirement of the PAPS-forming system cannot be told from these experiments.

Requirement for ATP - The effect of varying the amount of ATP is shown in Fig. 2. Again, the extent that this reflects only the requirement of the PAPS-generating system is not known. There was no advantage to adding an ATP-generating system such as carbamyl phosphate and its kinase.

Time Course of Incorporation - After an initially rapid incorporation of $S^{35}O_4$ into lipid, the rate falls off between 10 and 20 minutes to assume a new slope which is then maintained, so that after 90 minutes there is continued incorporation (Fig. 3). The availability of the lipid acceptor may determine the shape of the curve.

Intra-Cellular Localization of Activity - Enzymatic activity resides in the particulate fractions (Table II). Essentially complete activity could be recovered by combination of the "mitochondrial" and "microsomal" activities. The subcellular fractions were not examined microscopically for contamination with one another. Because of the difficulties inherent in brain homogenate fractionation, the exact localization of activity is avoided. Most experiments with brain were designed to differentiate only between particulate (non-cellular) and high speed supernatant (105,000 x g) fractions.

TABLE II

Intra-cellular localization of activity

The incubation system was similar to that shown in Table I, with 16 day old rat brain homogenate subfractions prepared as described above. The incorporation of $S^{35}O_4$ into sulfolipid is as indicated.

Fraction	c.p.m. into sulfolipid
Homogenate (after 1,000 x g spin)	550
Mitochondrial fraction	315
Microsomal fraction	153
Supernate	59
Mitochondria + supernate	360
Microsomes + supernate	189

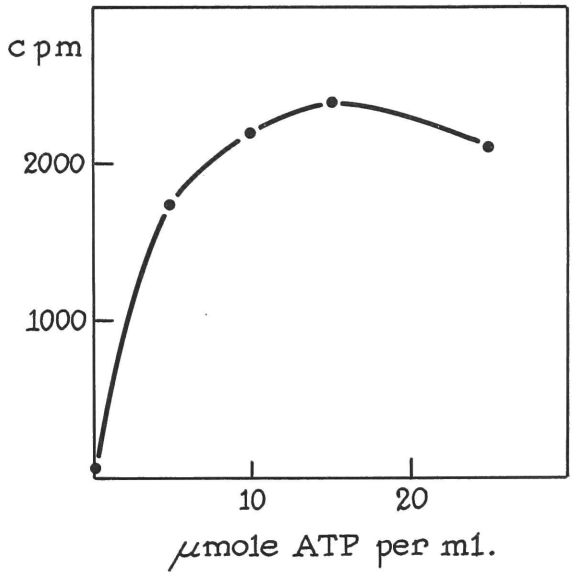


Fig. 2. Effect of varying ATP concentration. The incubation system was similar to that shown in Table I with rat brain homogenate, except for 0.4 μmole of K_2SO_4, so that the specific activity of $S^{35}O_4$ is twice that in Table I.

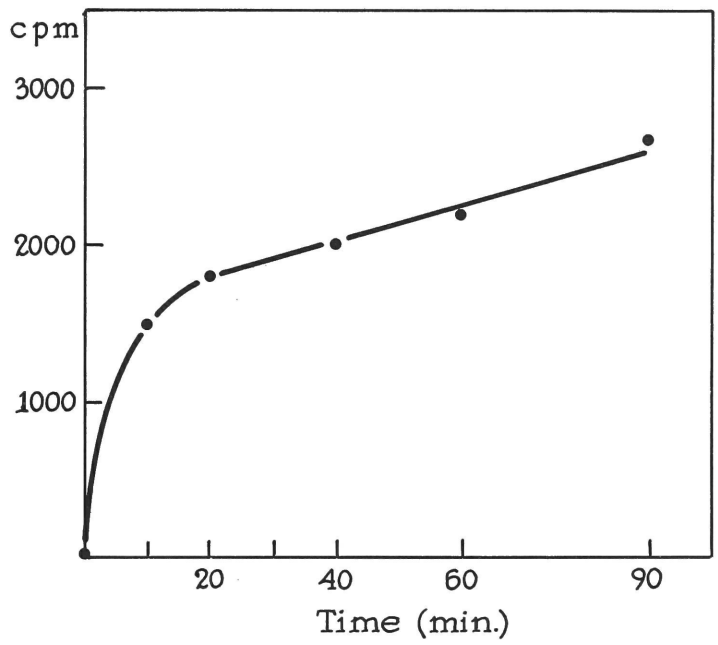


Fig. 3. Time course of incubation. The incubation system was similar to that shown in Table I with rat brain homogenate, except for 0.4 μmole of K_2SO_4, so that the specific activity of $S^{35}O_4$ is twice that in Table I.

Incorporation of Radioactivity From PAPS^{35} Into Sulfolipid By Rat Brain Homogenate

Addition of Sphingolipids - In subsequent experiments, PAPS^{35}, prepared enzymatically as described in Methods, was substituted for inorganic $S^{35}O_4$ and the yeast sulfate activating system. In addition, crude N-acetyl sphingosine and other sphingolipids were tested as possible sulfolipid precursors. The addition of 3.0 μmoles of crude N-acetyl sphingosine to a 1.0 ml. incubation volume resulted in a 2-4-fold increase in incorporation of $S^{35}O_4$ from PAPS^{35} into sulfolipid by rat brain homogenate (Table III).

TABLE III

Incorporation from PAPS^{35} into lipid by brain homogenate

The complete system contained 100 μmoles of Tris buffer, pH 7.4, 1 μmole of galactose, 1 μmole of glucose, 10 μmoles of $MgCl_2$ or 4 μmoles of $MnCl_2$, 5 μmoles of ATP, 3 μmoles of crude N-acetyl sphingosine, 0.25 ml. of 12 day old rat brain homogenate, 1 x 10^6 c.p.m. PAPS^{35} (60 x 10^6 c.p.m. per μmole). The total volume was 1.0 ml., and the tubes were incubated at 37° for 90 minutes. Omissions are as indicated.

Crude N-acetyl sphingosine	ATP	Mg^{++}	Mn^{++}	c.p.m.
-	+	+	-	671
+	+	+	-	2550
+	+	-	-	1203
+	+	-	+	2760
-	+	-	+	1356
+	-	+	-	1280

As will be noted later, the stimulation of incorporation by addition of this lipid is much less in brain, where the baseline incorporation is relatively high as compared to liver, presumably due to endogenous lipid precursor or acceptor. Other lipids such as cerebronyl ceramide and cerebroside, isolated from natural sources and emulsified by Tween 20, possessed no such ability to stimulate incorporation of radioactivity into sulfolipid. Since it is possible to conceive of the sulfurylation occurring on the galactose after it is attached to the sphingosine but before the latter is acylated, psychosine (galactosyl sphingosine) was tested in this system. The addition of psychosine did not lead to an increased incorporation into lipid-soluble material. Since the sulfurylated psychosine would probably be quite water-soluble, a

search for such a compound was made by paper electrophoresis of the aqueous incubation mixture. No such material was found.

In addition to its requirement in PAPS formation, when PAPS is added as such, magnesium is needed for optimal activity. This is so, even when more than saturating amounts of PAPS are made available. Contrary to the situation when endogenous sulfate activation is required, if ample PAPS is added, manganese is as good a cation as magnesium with added crude N-acetyl sphingosine. But manganese is better than magnesium if the ceramide is omitted (Table III). Manganese may be more effective than magnesium in the endogenous formation of the appropriate lipid precursors. When ATP is omitted, the incorporation falls to about one half. Whether this is indicative of further synthetic steps, or represents some non-specific action, has not been determined. It may be significant that addition of uridine nucleotides (UTP, UDPG) or galactose 1-phosphate had no consistent effect. This may not be too unexpected, however, inasmuch as the enzyme preparation was a crude one and the availability of such factors might not be limiting in the complex sequence of reactions.

The same subcellular sites of activity could be demonstrated with PAPS as sulfate donor as when endogenous sulfate activation was required (Table IV). The particulate fraction was also the one that was stimulated by the addition of crude N-acetyl sphingosine. Glutathione or nicotinamide had no effect on these systems.

TABLE IV

Influence of crude N-acetyl sphingosine on incorporation from $PAPS^{35}$ into lipid by brain fractions

The incubation system was similar to that shown in Table II, with 10×10^6 c.p.m. $PAPS^{35}$ (33×10^6 c.p.m. per μmole) and 18 day old rat brain fractions.

Fraction	Crude N-acetyl sphingosine	c.p.m.
Homogenate	+	1005
Homogenate	-	410
Supernate	+	15
Supernate	-	35
Particles	+	875
Particles	-	325

Identity of the Sulfolipid Produced by Brain Homogenate

Hydrochloric Acid Hydrolysis - Only a limited investigation of the nature of the brain sulfolipid has been pursued. It was thought that if the labelled lipid were related to cerebroside sulfate, it might be possible by partial acid hydrolysis to obtain some splitting of the glycosidic linkage before the sulfate ester was cleaved from the sugar. If proper hydrolysis conditions for such could be obtained, then the galactose-$S^{35}O_4$ that resulted could be easily identified with known material on paper electrophoresis.

The hydrolysis of brain sulfatide (gift of Drs. Lees and Folch) was performed as follows: 3 mg. of the sulfatide in a conical test tube was taken up in 0.2 ml. of chloroform, 0.2 ml. of the HCl solution (0.2 to 1.0 N were tested) was added to each tube, and the tubes were then placed in a boiling water bath. Care was taken to allow the chloroform to boil off, and the hydrolysis was continued, after stoppering the tube, for the prescribed time intervals for each tube. At the appropriate time the tube was placed in ice and the hydrolysate was neutralized with 5 N KOH. Chloroform (0.2 ml.) was added to each tube and the mixture was shaken to extract the unhydrolyzed lipid into the chloroform. Centrifugation was often performed to break an emulsion. The aqueous layer was used for application on paper for electrophoresis at pH 5.5. The adenosine nucleotides, galactose, and glucose 6-phosphate were used as markers. Reducing sugar was detected by the aniline phthalate spray (204). When 0.5 N HCl was used, by ten minutes of hydrolysis, a faint brown reducing-sugar spot appeared on the electrophoretogram in the region where a sugar sulfate would be expected to move (about the same mobility as glucose 6-phosphate at pH 5.5, slower at pH 8) (Fig. 4). At this time there was no reducing-sugar spot in the galactose region. After 20 minutes of hydrolysis, the intensity of the sugar sulfate spot increased and reducing-sugar appeared in the galactose region. The latter continued to increase so that it was strongest at the last time sampled - 60 minutes. The galactose sulfate spot, which was about as intense at 40 minutes as at 20, disappeared by 60 minutes - desulfation was complete. When 0.2 N NCl was used, there was still a sugar sulfate spot at 60 minutes. The data, therefore, show that under these conditions of hydrolysis it is possible to obtain splitting of the glycosidic linkage before cleavage of the sulfate has occurred.

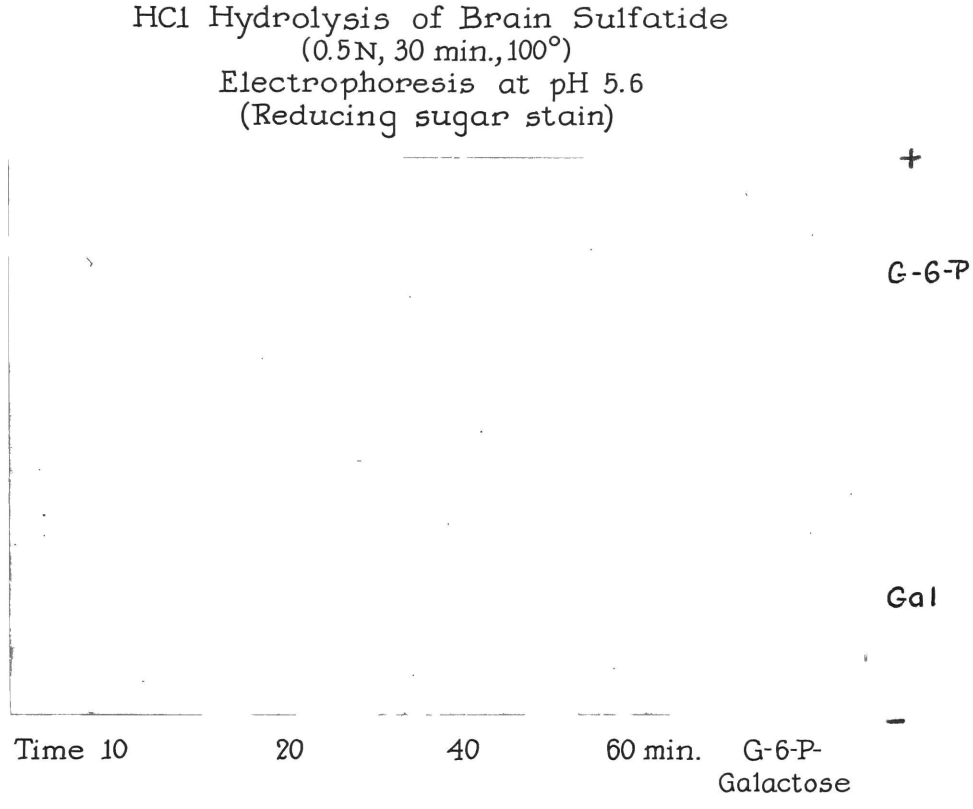


Fig. 4. See text for description.

The liberation of galactose-SO_4 by hydrolysis of brain sulfatide.

Experiments were then conducted on the hydrolysis of the enzymically-produced radioactive sulfolipid. The incubation and extraction were similar to that described before with rat brain homogenate, except that carrier-free $PAPS^{35}$ (1×10^6 c.p.m.) was used. Following washing of the chloroform layer, the solvent was removed on the rotary evaporator and the residue was redissolved in a small volume of chloroform. Aliquots of 0.2 ml. were distributed to test tubes containing 3 mg. of sulfatide and the acid hydrolysis (0.5 N HCl, 30 minutes at 100°) was performed as above. Paper electrophoresis of the hydrolysate showed, in addition to fast-moving inorganic $S^{35}O_4$, coincident radioactive and reducing-sugar spots with the mobility of a sugar sulfate (Fig. 5). When N-acetyl sphingosine had been included in the incubation, autoradiography of the so treated hydrolysate revealed a more intense radioactive spot in the sugar sulfate area; gal-1-P and the uridine polyphosphates had little, if any, effect. The anionic reducing-sugar substance and the coincident radioactivity produced by the acid hydrolysis could be absorbed onto charcoal directly from the HCl solution and eluted with ethanolic ammonia. Paper electrophoresis of the charcoal eluate again showed the two spots to be superimposable. The absorption of the presumed sugar sulfate by charcoal from an HCl solution is similar to that described recently by Crane (205) for the sugar phosphates.

The possibility of chemical transfer of $S^{35}O_4$ to galactose, or exchange of $S^{35}O_4$ with that of galactose 6-sulfate during the acid hydrolysis of the sulfatide was seriously considered. In extensive experiments it was shown that such was the case when relatively large amounts of $S^{35}O_4$ or $PAPS^{35}$ were substituted for the brain S^{35}-sulfolipid in the hydrolysis. In fact, galactose-$S^{35}O_4$ could be formed when galactose and $S^{35}O_4$ or $PAPS^{35}$ were heated at 100° for 30 minutes in 0.5 N HCl. Such was not the case, however, when the amount of radioactivity added was of the same order as that in the brain S^{35}-sulfolipid. The "control" in Fig. 5 comes from an experiment in which 3 mg. of sulfatide and an amount of lipid-bound $S^{35}O_4$ of the same magnitude as incorporated by the brain system (from an incubation of crude N-acetyl sphingosine and $S^{35}O_4$ with rat liver supernate - to be described in detail later) were hydrolyzed as above. There was no significant formation of the radioactive sugar sulfate compound. Likewise, when an equal amount of radioactivity in the form of $S^{35}O_4$ or $PAPS^{35}$ was substituted for the S^{35}-lipid and carried through the same procedure (hydrolysis with sulfatide included), again no formation of radioactive sugar sulfate was noted.

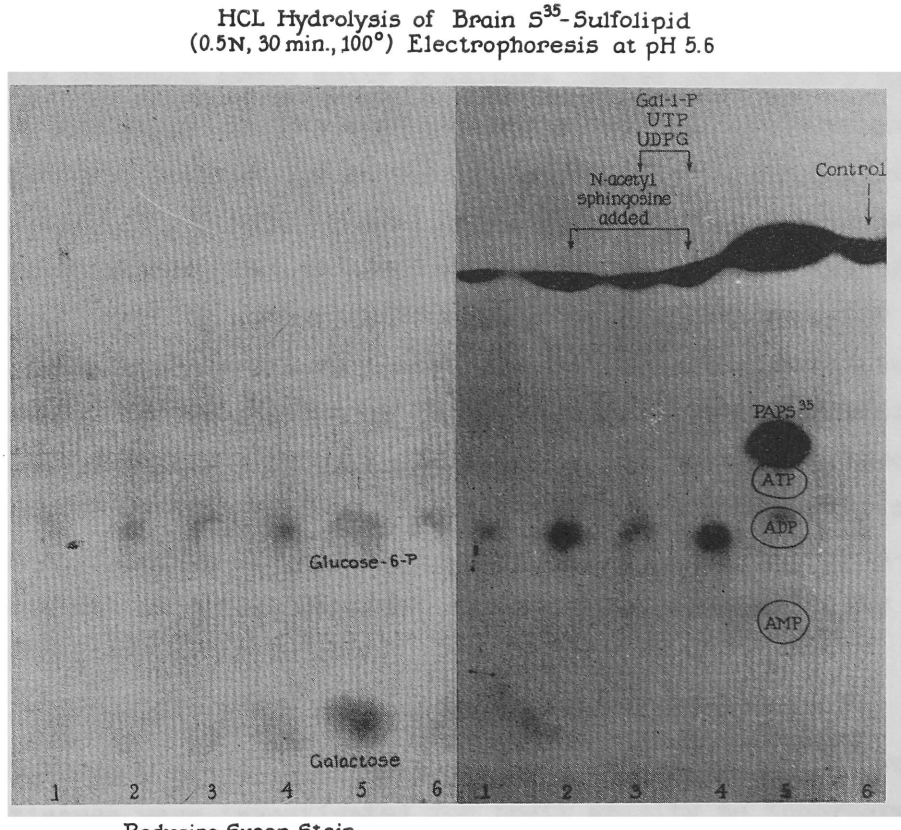


Fig. 5. See text for description.

The liberation of galactose-$S^{35}O_4$ by hydrolysis of brain S^{35}-sulfolipid.

The evidence suggests, therefore, that the $S^{35}O_4$ of the brain sulfolipid was enzymically fixed to the galactose of the sphingolipid during the incubation, and that the intact sugar sulfate was released during the acid hydrolysis. The enzymatic formation by the brain homogenate of a substance analogous to the Blix Compound seems quite possible. These preliminary experiments imply that the synthesis of this compound may go by way of the ceramide.

The results presented here on the acid hydrolysis of sulfatide differ from those recently reported by Lees et al (165). These workers found that hydrolysis with glacial acetic acid at 100° led to the complete release of inorganic sulfate by 90 minutes, at which time the cerebroside portion of the molecule was still intact. When the sulfatide was treated with glacial acetic acid : 10% sulfuric acid for 42 minutes at 100°, however, it was completely split into its component parts. The different hydrolysis conditions used might account for the different results. Since the amount of free sugar sulfate present at any time during the HCl hydrolysis represents only a relatively small fraction of the total, it might go undetected except for the sensitive paper chromatography technique.

Comparison of Rat Tissue Homogenates

In view of the finding of sulfolipids in tissues outside of the nervous system, other rat tissues were investigated for their ability to incorporate inorganic $S^{35}O_4$ into lipid. Various rat tissue homogenates were prepared in the manner previously described. In the absence of any added lipid, kidney and liver had the highest specific activities, almost four times that of brain (Table V). The yeast PAPS-generating system was added to each tube even though such tissue as liver has its own activation system. Young adult (6-8 weeks old) rat liver homogenates were more active than those of calf or pigeon.

TABLE V

Comparison of rat tissue homogenates

The incubation system was similar to that shown in Table I, with tissue homogenates of 13 day old rats, yeast PAPS-generating system, and 2 x 10^7 c.p.m. $Na_2S^{35}O_4$ (2 x 10^7 c.p.m./μmole).

Tissue	c.p.m. total	c.p.m./mg. protein
Brain	452	132
Liver	2430	434
Kidney	1008	500
Spleen	28	23
Heart	44	36

Intra-Cellular Localization of Activity in Rat Liver

Fractionation of young adult rat liver homogenates by the method of Hogeboom et al (206) showed that the activity, without added sphingolipid, was highest in the supernate after 105,000 x g centrifugation (Table VI). Since the particulate fraction has a weak or absent sulfate activation system, the yeast PAP-generating enzymes were added to the tubes containing this fraction. Nevertheless, the incorporation of $S^{35}O_4$ into lipid remained very poor with these fractions. The particles in these experiments were unwashed. Because of the high activity found in liver and the obvious advantages of a soluble enzyme system, the rat liver supernatant fraction was studied in greater detail.

TABLE VI

Intra-cellular localization of activity

The incubation system was similar to that shown in Table I, with fractions from homogenates of young adult rat liver, and 2 x 10^7 c.p.m. $Na_2S^{35}O_4$ (4 x 10^7 c.p.m./μmole).

Fraction	c.p.m. total	c.p.m./mg. protein
Homogenate	4750	392
Particles (+ yeast enzyme)	363	104
Supernate	4528	810

Effect of Added Sphingolipids on Incorporation by Rat Liver Fractions

The addition of either crude N-acetyl sphingosine or N-acetyl-DL-threo-trans-sphingosine in 2% Tween 20 resulted in a significant increase in incorporation of $S^{35}O_4$ into sulfolipid by homogenates of young adult rat liver (Table VII). Since Tween 20 depresses the endogenous incorporation, it is necessary to run control tubes without added lipid but incubated with 2 mg. of Tween 20. The greatest stimulation of activity is seen in the supernatant fraction, which has even more activity when freed from the particulate fraction. Recombination of these fractions usually resulted in the lower activity that was seen with the whole homogenate. As noted before, a similar effect had been found in the formation of phenyl sulfate by rat liver preparations; this has been attributed to the ATPase activity of the microsomes (27). In each instance, the crude N-acetyl sphingosine provides about twice

TABLE VII

Effect of sphingolipids on incorporation from inorganic $S^{35}O_4$ by liver fractions

The incubation system was similar to that shown in Table I, with fractions from homogenates of young adult rat liver, 2 x 10^6 c.p.m. $Na_2S^{35}O_4$ (1 x 10^6 c.p.m./μmole), and 0.1 ml. of yeast sulfate-activating system in those tubes containing only the particle fraction. The particles were not washed. 3.0 μmoles of lipid and 2 mg. of Tween 20 were added as indicated.

Fraction	Addition	c.p.m.
Homogenate	None	396
Homogenate	Tween, 2 mg	267
Homogenate	Tween, 2 mg + crude N-acetyl sphingosine	1900
Homogenate	Tween, 2 mg + N-acetyl-DL-threo-sphingosine	1102
Particles	None	146
Particles	Tween, 2 mg	7
Particles	Tween, 2 mg + crude N-acetyl sphingosine	136
Particles	Tween, 2 mg + N-acetyl-DL-threo-sphingosine	121
Supernate	None	366
Supernate	Tween, 2 mg	154
Supernate	Tween, 2 mg + crude N-acetyl sphingosine	3000
Supernate	Tween, 2 mg + N-acetyl-DL-threo-sphingosine	1800

the stimulation of incorporation as the purified threo compound. As will be seen later, the erythro isomer is much less stimulatory than the threo in this system. Whether these differences reflect the specificity of the enzyme system or are more dependent on solubility properties of the added lipid is not entirely clear. In many instances the ability to stimulate incorporation of $S^{35}O_4$ into lipid parallels the ease of emulsification of the lipid with or without Tween 20. This may account for the greater activity of the crude N-acetyl sphingosine preparation compared with the pure threo compound. The former actually requires no Tween 20 for maximal activity and adequate emulsification.

In another experiment, the crude N-acetyl sphingosine preparations of either brain or spinal cord origin (prepared from spinal cord sphingosine, provided by Dr. B. Weiss of the New York State Psychiatric Institute) were comparably active, whereas the threo compound was again about one-half as active and the erythro isomer much less active (Table VIII).

TABLE VIII

Effect of addition of various sphingolipids

Each tube contained 50 μmoles of K phosphate buffer pH 7.4, 2.5 μmoles of $MgCl_2$, 5 μmoles of ATP, 1.0 μmole of $Na_2S^{35}O_4$ (1×10^6 c.p.m./μmole), 0.05 ml. of 0.03 M sphingolipid emulsified in Tween 1% as indicated, and 0.125 ml. of rat liver supernate. The total volume was 0.5 ml., and the tubes were incubated at 37° for 90 minutes. Incorporation is expressed for 1.0 ml. incubation volume.

Lipid added	c.p.m.
Crude N-acetyl sphingosine (brain)	5810
Crude N-acetyl sphingosine (spinal cord)	4510
N-acetyl-DL-threo-sphingosine	2340
N-acetyl-DL-erythro-sphingosine	317
Crude sphingosine base	770
Tween, 1.0 mg.	86

No significant stimulation could be demonstrated with the following sphingolipids emulsified with Tween 20 (from 1-4%): N-octanoyl-DL-threo-sphingosine, N-palmitoyl-erythro-trans-sphingosine, cerebronyl ceramide, and cerebroside. Crude sphingosine base - prepared by alkaline treatment of the same sphingosine SO_4 used to make the crude N-acetyl sphingosine -

was about one-eighth as active as the acetylated base. It is not known whether this activity is due to the free base itself or to some contaminant lipid. The different solubilities and emulsifiabilities exhibited by these compounds, despite the addition of Tween 20, make comparisons of their relative activities rather hazardous. Psychosine was also tested as a sulfate acceptor in rat liver homogenate and supernatant fractions. Assay was both by extraction for lipid, and by paper electrophoresis of the incubation mixture. The results were negative.

Effect of Varying Concentrations of Crude N-Acetyl Sphingosine - In Fig. 6 is shown the concentration curve for crude N-acetyl sphingosine in the system consisting of rat liver supernate, inorganic radioactive sulfate, and the endogenous PAPS-generating system. No emulsifying agent was used. The rate of incorporation remains linear until a concentration of about 3.0 μmoles of the added lipid per ml. This value is of the same order as found in the sphingomyelin (188) and psychosine (207) synthesizing systems.

Time Dependence of Incorporation - The time dependence of incorporation is demonstrated in Fig. 7. There appears to be a time lag in the first 20-30 minutes which may represent the time required to generate optimal amounts of PAPS by the liver sulfate activating system. Even at 2 hours incubation, the rate appears to remain linear. This must be due to the presence of continuous PAPS generation for, as will be seen later, when a fixed amount of $PAPS^{35}$ is added as the only source for active sulfate, the incorporation into lipid reaches a plateau at about 40 minutes.

Optimal pH - The variation of activity with the pH of Tris-maleate buffer is shown in Fig. 8. The extent to which this is a reflection of the requirement of the endogenous PAPS-generating system cannot be told from these experiments.

Requirement for Cation - Fig. 9 indicates the requirement for divalent cations. Mg^{++} is much better than Mn^{++} even at low concentrations of the latter. This also may be related primarily to the requirement of the sulfate activation sequence rather than to the subsequent transfer steps.

Effect of Varying ATP Concentration - The effect of varying the amount of ATP added is shown in Fig. 10. The complete absence of incorporation when ATP is omitted indicates the necessity for activation of sulfate to PAPS before transfer. Because of the complexity of the system at this point (whole rat liver supernate), little can be said regarding other possible requirements for ATP.

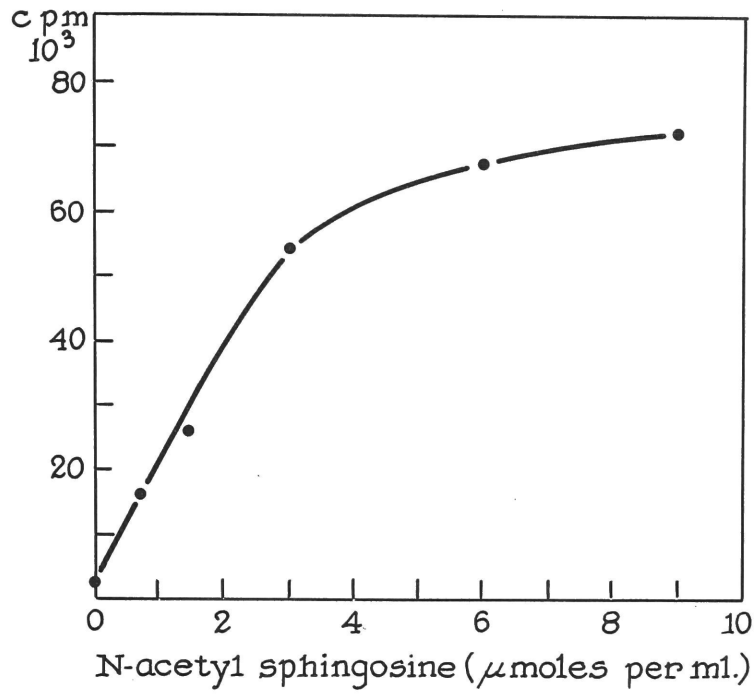


Fig. 6. Effect of varying concentration of crude N-acetyl sphingosine. The incubation system was similar to that used in Table VIII with rat liver supernate and inorganic $S^{35}O_4$, except that the incubation volume was 1.0 ml., and 2.0 μmoles of $S^{35}O_4$ (10 x 10^6 c.p.m./μmole) were used. N-acetyl sphingosine concentrations were varied by adding different amounts of a 0.03 M solution.

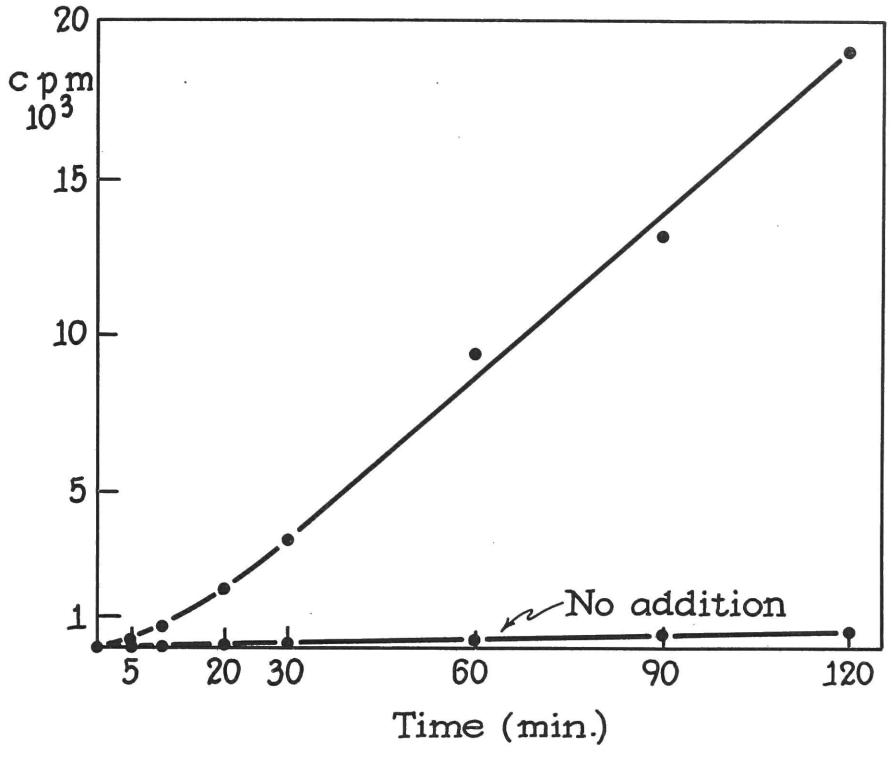


Fig. 7. Time course of incorporation with and without crude N-acetyl sphingosine, 6 μmoles. The incubation system was similar to that used in Table VIII with rat liver supernate and inorganic $S^{35}O_4$ except that the incubation volume was 1.0 ml. and 2.0 μmoles of $S^{35}O_4$ (2.5 x 10^6 c.p.m./μmole) were added.

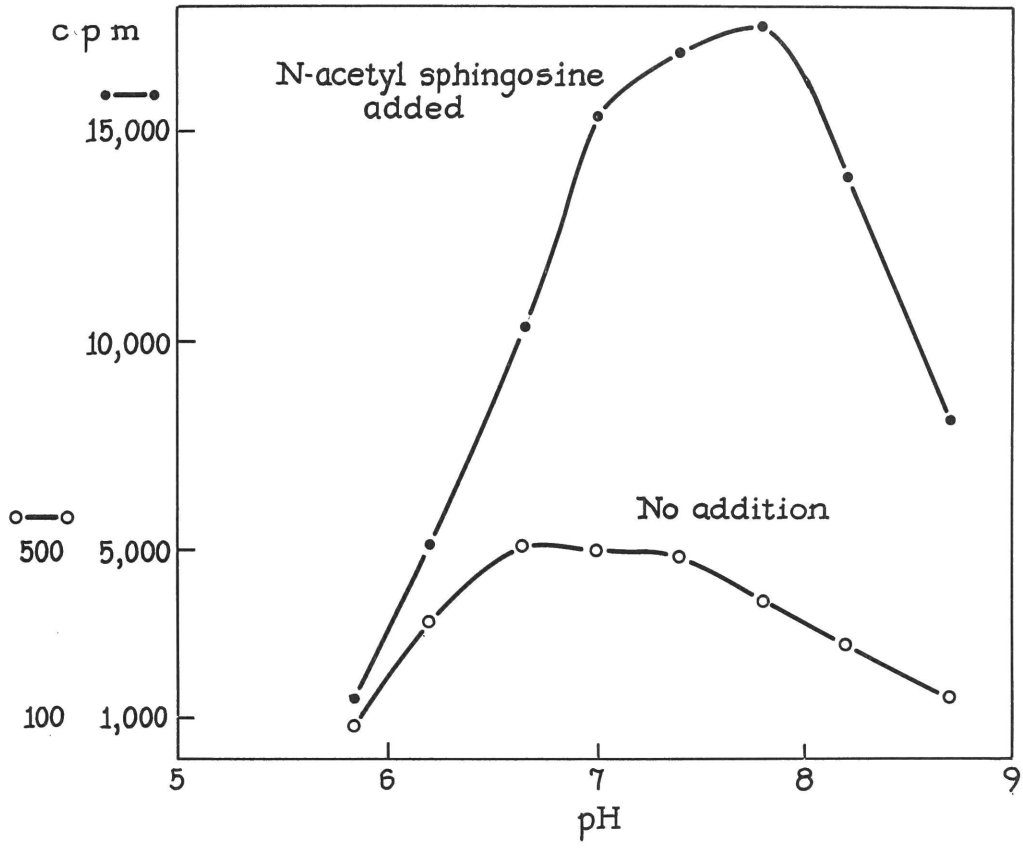


Fig. 8. pH profile. The incubation system was similar to that shown in Table VIII with rat liver supernate and inorganic $S^{35}O_4$, except that the incubation volume was 1.0 ml. and 2.0 μmoles $S^{35}O_4$ (2.5×10^6 c.p.m./μmole) were added. The pH of Tris-maleate buffer added to the system was varied as shown. If added, each tube contained 6 μmoles of crude N-acetyl sphingosine.

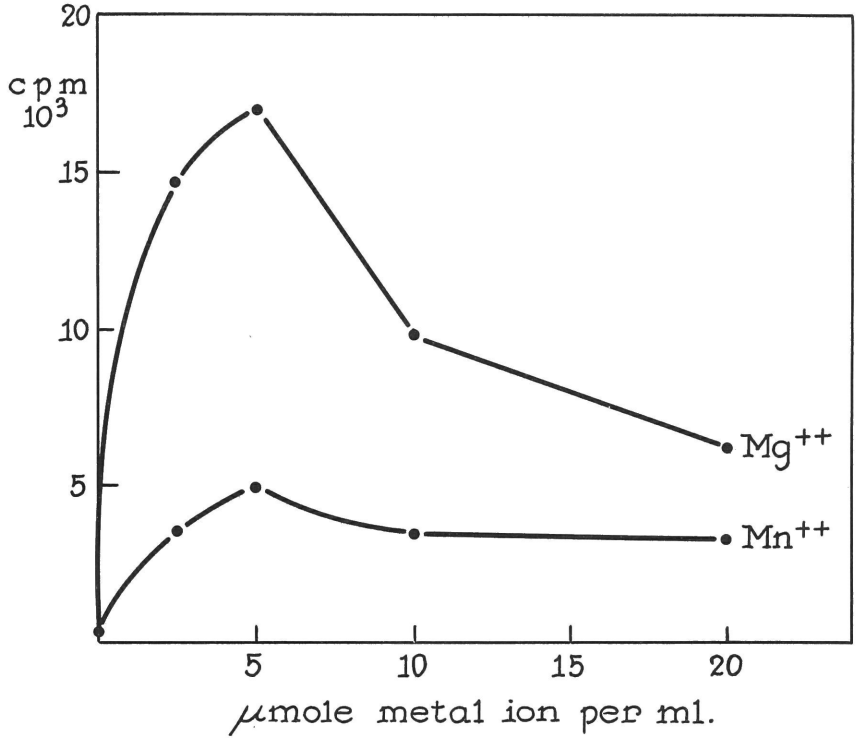


Fig. 9. Requirement for divalent cation. The incubation mixture was similar to that shown in Table VIII with rat liver supernate and inorganic $S^{35}O_4$, except that the incubation volume was 1.0 ml. and 2.0 μmoles of $S^{35}O_4$ (2.5×10^6 c.p.m./μmole) were added. Each tube contained 6 μmoles of crude N-acetyl sphingosine. Magnesium and manganese concentrations were varied as shown.

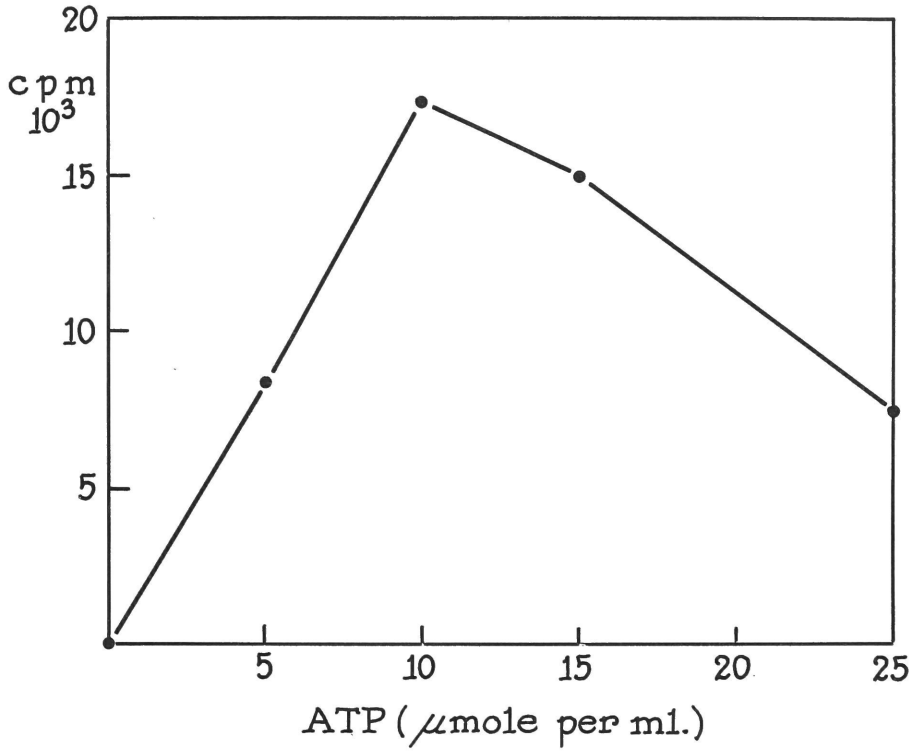


Fig. 10. Effect of varying ATP concentration. The incubation system was similar to that shown in Table VIII with rat liver supernate and inorganic $S^{35}O_4$, except that incubation volume was 1.0 ml. and 2.0 μmoles of $S^{35}O_4$ (2.5×10^6 c.p.m./μmole) were added. Each tube contained 6 μmoles of crude N-acetyl sphingosine. ATP concentration was varied as shown.

Effect of UTP, UDPG, Galactose 1-phosphate - In the complete system (10 μmoles of ATP per ml.) no stimulation of incorporation into lipid could be demonstrated by adding 0.5 to 1.0 μmole of UTP, UDPG, or galactose 1-phosphate (gal-1-P) either singly or in combination. In fact, as seen in Fig. 11, decrease in incorporation occurred with increasing concentrations of UTP. This inhibition might be non-specific in nature but could reflect the formation of a sulfurylated intermediate or product that would not be detected by the assay used in these experiments. Repeated efforts were made to find such possible compounds by paper electrophoresis of an aliquot of the incubation mixture with and without addition of the above compounds, but without success.

The Transfer from $PAPS^{35}$ in Liver Supernate

It was decided to study sulfate transfer from PAPS in greater detail. In order to explore the possibility that the N-acetyl form of cerebroside sulfate was being formed in this system from N-acetyl sphingosine, the effect of added uridine nucleotides, gal-1-P, and a uridinediphosphogalactose-generating system from calf liver were tested. If the Blix-type Compound was the one being formed in this system, then addition of these factors might be expected to lead to increased incorporation provided that any of these compounds had been previously limiting.

Effect of Uridine Nucleotides and Gal-1-P on the Transfer of $S^{35}O_4$ from $PAPS^{35}$ into Lipid (whole rat liver supernate) - When $PAPS^{35}$ was used as the sulfate donor with whole dialyzed rat liver supernate, a relatively low concentration of ATP (0.5 μmole per ml.) was required for maximal incorporation (Table IX). UTP (0.5 μmoles per ml.) resulted in partial recovery of activity when ATP was omitted, presumably due to nucleotide phosphokinase. When ATP was included, UTP resulted in no added stimulation. In other experiments, CTP was as good as ATP or UTP. UDPG and gal-1-P were without effect.

Addition of Gal-1-P Uridyl Transferase - Gal-1-P uridyl transferase prepared from calf liver acetone powder by the method of Maxwell (208) was pre-incubated with and without UDPG, gal-1-P, and TPN. After 30 minutes, $PAPS^{35}$, crude N-acetyl sphingosine, and rat liver supernate (dialyzed against 0.01 M Tris pH 7.4, versene 0.001 pH 8 for 6 hours) were added, and the incubation continued for another 30 minutes at 37°. There was no significant difference in incorporation of radioactive sulfate into sulfolipid between

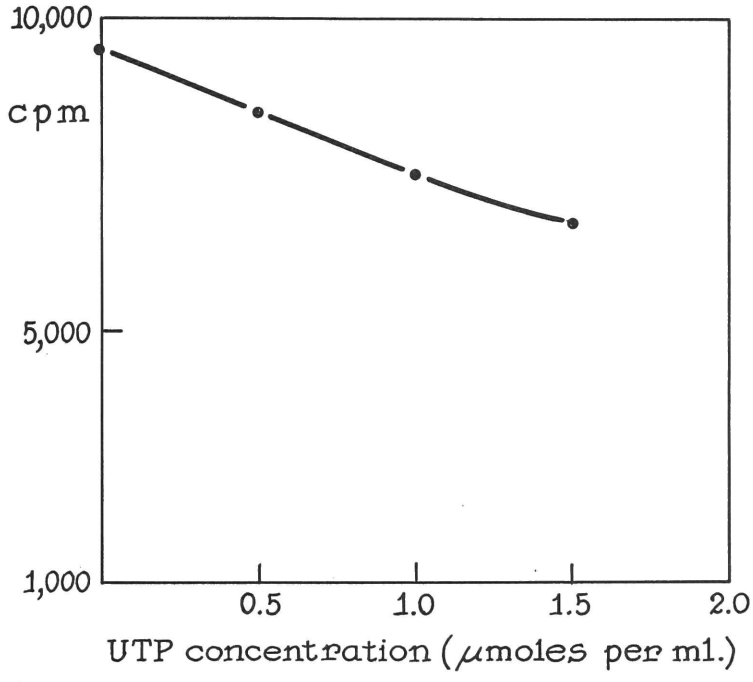


Fig. 11. Effect of added UTP. The incubation system was similar to that shown in Table VIII with rat liver supernate and 1.0 μmole of inorganic $S^{35}O_4$ (2.5 x 10^6 c.p.m./μmole). Each tube contained 3 μmoles of crude N-acetyl sphingosine. The amount of UTP added was varied as shown.

TABLE IX

Effect of Uridine Nucleotides and Gal-1-P
on transfer from $PAPS^{35}$ by liver supernate

Each tube contained 100 µmoles of Tris buffer pH 7.4, 5 µmoles of $MgCl_2$, 0.5 µmoles of the added compound, except as noted, 1 µmole of glucose, 1 µmole of galactose, 6 µmoles of crude N-acetyl sphingosine, 0.1 ml. of whole dialyzed rat liver supernate, 2.0×10^6 c.p.m. $PAPS^{35}$ (8×10^7 c.p.m./µmole). The total volume was 1.0 ml. and the tubes were incubated for 60 minutes at 37°. Additions were made as indicated.

Addition	c.p.m.
None	17,450
ATP	60,700
ATP (10 µmoles per ml.)	43,250
UTP	49,100
UDPG	20,800
ATP, gal-1-P, UDPG	52,700
Gal-1-P + UTP	50,500
Gal-1-P + UDPG	22,000

the tubes to which UDPG, gal-1-P, and TPN had been added, and those in which these had been omitted during the pre-incubation. These experiments, which indicate no effect on incorporation of radioactive sulfate into sulfolipid by added uridine nucleotide co-factors in the dialyzed enzyme preparation speak against cerebroside sulfate-like compounds as the substances being formed. This is supported by similar experiments performed on more purified enzyme preparations.

Omission of Magnesium - The omission of magnesium resulted in a fall in incorporation to less than one-third that of the complete system.

Fractionation of the Rat Liver Supernate - The pH of the rat liver supernate (in 0.25 M sucrose, versene 0.001 M, pH 8) was adjusted to 5.0 by the addition of 1.0 N acetic acid with magnetic stirring at 0°. The precipitate that formed was centrifuged off and dissolved in the original volume of 0.25 M sucrose, versene 0.001 M, pH 8, and neutralized by the addition of solid potassium bicarbonate. Any insoluble material was removed by centrifugation. The supernate from the pH 5 precipitation was returned to pH 7.4 by addition of solid potassium bicarbonate. The ability of these fractions to incorporate $S^{35}O_4$ starting with ATP, inorganic sulfate, and crude N-acetyl sphingosine was tested (Table X).

TABLE X

Incorporation into lipid from $S^{35}O_4$

by pH 5 fractions of liver supernate

Each tube contained 100 μmoles of Tris buffer, pH 7.4, 1 μmole of galactose, 1 μmole of glucose, 5 μmoles of $MgCl_2$, 10 μmoles of ATP, 2 μmoles of K_2SO_4 (2.5×10^6 c.p.m./μmole), 3 μmoles of crude N-acetyl sphingosine, and 0.25 ml. of the appropriate enzyme fraction. The total volume was 1.0 ml. and the tubes were incubated for 90 minutes at 37°.

Fraction	c.p.m.
Whole supernate	10,550
pH 5 supernate	158
pH 5 precipitate	824
pH 5 supernate + precipitate	3,685
pH 5 precipitate + boiled pH 5 supernate	634

Only very little of the original activity could be recovered when the two acid fractions were tested separately. Recombination, however, restored activity to about one-third of the original. The failure to reconstitute complete activity may be due to the known fragility of the sulfate activating system to acid treatment (209). It appeared that a separation of two or more enzymatic reactions had been accomplished by this fractionation. The small amount of residual activity in the acid precipitate can be accounted for by contamination with the acid supernate since no effort was made to wash the former entirely free of the latter.

$S^{35}O_4$ Transfer From $PAPS^{35}$ In Liver Fractions

To test the possibility that the fractionation had resulted in loss of activity due to the separation of the PAPS-generating system from the transfer system, the same type of experiment was carried out with added $PAPS^{35}$ (Table XI). In this experiment, the pH 5 precipitate was washed once with 0.02 M acetate buffer pH 5.0 before dissolving in the sucrose/versene solution and neutralization with solid potassium bicarbonate. It is evident from these experiments that the PAPS transfer system lies in the pH 5 supernate, whereas at least one of the two steps of sulfate activation resides in the pH 5 precipitate. A similar activity fractionation was found when N-acetyl-DL-threo-trans-sphingosine in 2% Tween 20 was used as the added lipid. In this system, which is no longer able to activate sulfate, PAPS is shown to be the sulfate donor.

TABLE XI

Incorporation into lipid from $PAPS^{35}$

by pH 5 fractions of liver supernate

Each tube contained 100 μmoles of Tris buffer pH 7.4, 1 μmole of galactose, 1 μmole of glucose, 5 μmoles of $MgCl_2$, 5 μmoles of ATP, 0.25 ml. of the enzyme solution prepared as described in the text, 3 μmoles of crude N-acetyl sphingosine, 5.0×10^5 c.p.m. $PAPS^{35}$ (2.0×10^7 c.p.m./μmole). The total volume was 1.0 ml. and the tubes were incubated for 90 minutes at 37°.

Fraction	c.p.m.
Whole supernate	15,560
pH 5 supernate	13,720
pH 5 precipitate	1,634
pH 5 precipitate + pH 5 supernate	14,230

Ethanol Fractionation of the pH 5 Supernate - The pH 5 supernate obtained by centrifugation after bringing dialyzed rat liver supernate to pH 5 was immediately cooled to -2° in a sodium chloride ice bath. The pH was maintained at 5 throughout the subsequent ethanol fractionation. Absolute ethanol (kept at -15° in the deep freeze) was added dropwise to the solution with magnetic stirring by a teflon covered bar. When the concentration of ethanol reached 10%, the temperature of the salt-ice bath was lowered to -5° and the slow addition of ethanol was continued to the desired concentration. By this procedure, precipitates at 10%, 20%, 30%, 40%, and 60% ethanol were obtained. These were dissolved in one-quarter of the original volume of 0.02 M Tris, pH 7.5. The solutions were transferred to Visking dialysis tubing, with the pH maintained at about 7.5, and were dialyzed against several changes of large volumes of 0.02 M Tris, pH 7.5, for 6 hours. These fractions were then tested for their ability to incorporate radioactivity from $PAPS^{35}$ into lipid in the presence of crude N-acetyl sphingosine. The experiments described in Table XII demonstrate that the enzymatic activity resides in two major ethanol fractions: 0-20% and 20-60%. The 0-20% fraction has about 50% of the total original activity, whereas the 20-60% fraction alone has no significant activity, but when combined with the other fraction, incorporation into sulfolipid is doubled. The most likely interpretation of these results is that the 0-20% fraction, I, is probably contaminated with the enzyme(s) present in the 20-60% fraction, II.

TABLE XII

Incorporation from $PAPS^{35}$ into lipid by ethanol fractions of the pH 5 supernate

Each tube contained 50 µmoles of Tris pH 7.4, 2.5 µmoles of $MgCl_2$, 2.5 µmoles of ATP, 1.5 µmole of crude N-acetyl sphingosine, 370,000 c.p.m. $PAPS^{35}$ (1.3×10^7 c.p.m./µmole), 0.03 ml. of each ethanol fraction (0-10%, 10-20%, 20-30%, 30-40%, 40-60%) or combinations of them as indicated (each fraction was in one-quarter of the original volume), 0.12 ml. pH 5 supernate (neutralized) as indicated. The total volume was 0.5 ml., and the tubes were incubated for 40 minutes at 37°.

Experiment No.	Fraction	c.p.m.
1.	Whole pH 5 supernate	5340
	0-20%	2555
	20-40%	136
	40-60%	117
2.	Whole pH 5 supernate	5260
	0-10%	1073
	10-20%	1812
	0-20%	2850
	0-60%	4840
	0-20% + liver kochsaft	1238
3.	0-20%	2105
	0-20% + boiled 20-60%	1955
	20-60%	136
	20-60% + boiled 0-20%	124
	0-20% + 20-60%	4570

Effect of Varying the Relative Amounts of the Two Fractions - Support for the outlined concept is provided by experiments shown in Fig. 12 where the concentration of fraction II is maintained constant and the amount of the fraction I is varied. At low concentrations of fraction I, stimulation of incorporation by addition of a constant amount of fraction II (which has no activity alone) is maximal. Presumable enzyme II, which is present as a contaminant in fraction I, is rate limiting at lower concentrations, but with increasing amounts of fraction I, the system becomes saturated with the second enzyme; further addition of fraction II is then without effect. Bovine serum albumin did not substitute for fraction II, indicating a specific effect. Both fractions could be stored in the deep freeze over a six-month period with about a 30% loss of activity.

The effect of increasing amounts of fraction II in the presence of a constant amount of fraction I, in the region where stimulation can take place, is shown in Fig. 13.

Heat Lability of the Ethanol Fractions - As can be seen in Table XIII, the activities of both ethanol fractions were destroyed by rapid heating to 55°. The test tube containing the enzyme solution and a thermometer was immersed in a water bath at 90° and kept there until the temperature of the enzyme solution approached 55°. It was then rapidly removed and immersed for one minute in a water bath maintained at 55°. The tube was removed and kept in ice until used. Heating of fraction I to 46° by the same procedure resulted in nearly no loss of activity.

TABLE XIII

Heat lability of the ethanol fractions

The incubation system was similar to that shown in Fig. 10 with 300,000 c.p.m. PAPS^{35} (1 x 10^7 c.p.m./μmole), crude N-acetyl sphingosine, 0.18 mg. of fraction I protein, 0.45 mg. of fraction II protein. The temperature of the protein solutions was varied as indicated and as described in the text.

Experiment No.	Fraction	c.p.m.
1.	I + II	3440
	I, 55°, 1 min.	22
	I, 55°, 1 min. + II	149
2.	I, 46°, 1 min.	1408
	I, 46°, 1 min. + II	2765
	I + II, 55°, 1 min.	1430
	I + II	2500

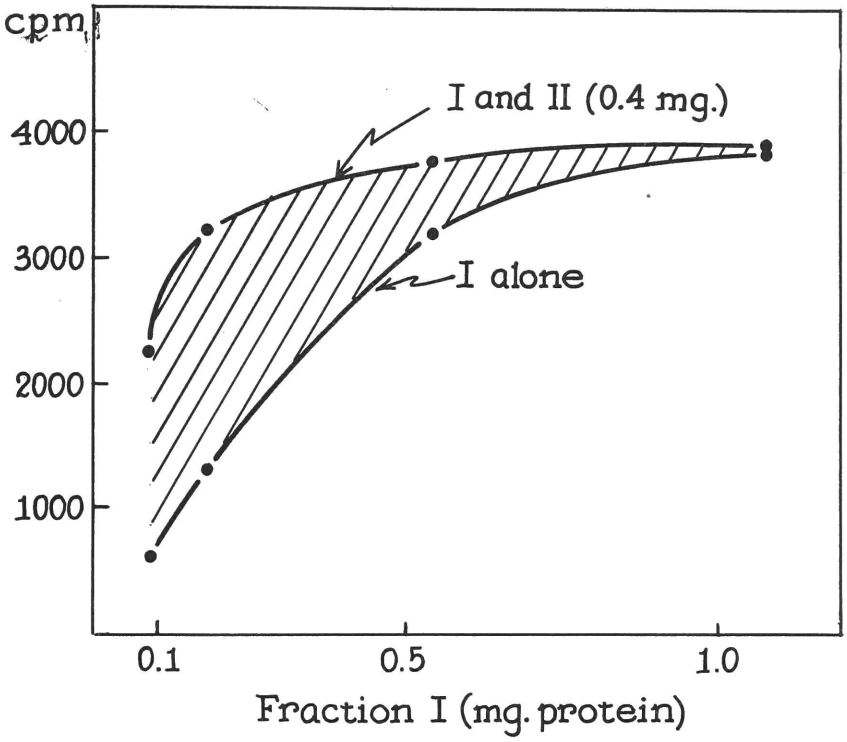


Fig. 12. Fraction I concentration curve and stimulation by a fixed amount of fraction II. Each tube contained 50 μmoles of K phosphate buffer, pH 7.4; 2.5 μmoles of $MgCl_2$; 2.5 μmoles of ATP; 1.5 μmole of crude N-acetyl sphingosine (plus Tween 20, 0.5 mg.); 3.7×10^5 c.p.m. $PAPS^{35}$ (1.3×10^7 c.p.m./μmole); 0.4 mg. of fraction II protein when added. The amount of fraction I added was varied as shown. The total volume was 0.5 ml. and the tubes were incubated for 30 minutes at 37°.

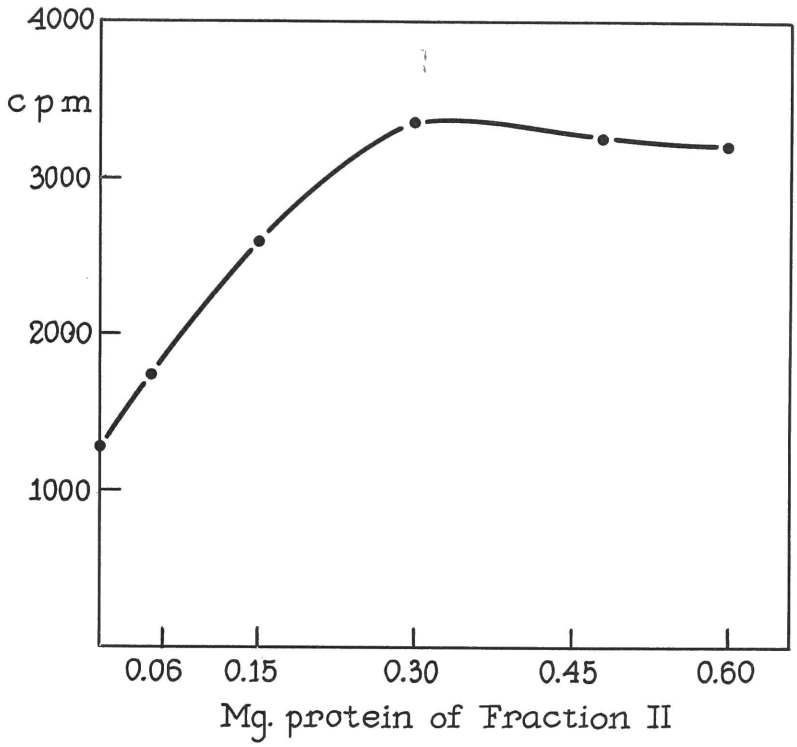


Fig. 13. Effect of varying amount of fraction II (constant amount of fraction I). The incubation system was similar to that shown in Fig. 12 with 3 x 10^5 c.p.m. of $PAPS^{35}$ (1 x 10^7 c.p.m./μmole), crude N-acetyl sphingosine, and 0.18 mg. of fraction I protein. The amount of fraction II added was varied as shown.

Effect of pH - The activities of fraction I alone and the two combined ethanol fractions were tested at three different pH's (6.2, 7.4, 8.2). As can be seen in Fig. 14, the pH optima for these fractions differed. This point was not explored further, but 7.4 was the pH used in all other experiments.

Dependence on ATP and Mg^{++} Concentration - The combined ethanol fractions were stimulated by both ATP and Mg^{++} for transfer from PAPS (Fig. 15). Manganese was not as effective as magnesium at the level of 5 μmoles per ml. The function of ATP in this system is not easily explained. It is significant that there is considerable activity in this system, starting with PAPS as sulfate donor, in the absence of added ATP. This fact makes it quite unlikely that additional synthetic steps are required in formation of the sulfurylated lipid. The same concentrations of UTP or CTP were as effective as ATP. It is possible that the ATP is acting by support of the PAPS added to the system. If the enzyme preparation (which is known not to possess the complete sulfate activating system) is contaminated with a 3'-nucleotidase and APS-kinase, the ATP might act to counter the effect of the former enzyme by means of the latter. This possibility was not pursued.

Effect of Pre-incubation With Either of the Two Fractions - Several experiments designed to detect the accumulation of an intermediate produced by pre-incubation with fraction I or II were unrevealing. Experiments were conducted by pre-incubating one or the other ethanol fraction in the presence or absence of $PAPS^{35}$ and crude N-acetyl sphingosine, together and separately. Neither total incorporation into lipid from $PAPS^{35}$ nor the kinetics of incorporation were altered by the pre-incubation.

Time Dependence of Incorporation by the Combined Ethanol Fractions - Fig. 16 demonstrates the time dependence of incorporation into lipid from $PAPS^{35}$ by the combined ethanol fractions. Incorporation is negligible when crude N-acetyl sphingosine is omitted from the incubation.

PAPS Saturation Curve - The concentration of $PAPS^{35}$ was varied as shown in Fig. 17. Saturation of the system was obtained at a level of about 0.07 μmole PAPS per ml.

Michaelis Constant for PAPS - PAPS is extremely tightly bound to the transfer enzyme. Since the reaction is essentially linear for the 30 minutes incubation period, these data were used to calculate an approximate Michaelis constant for PAPS. A double reciprocal plot (210) yielded a value of 1.7×10^{-8} M (Fig. 18).

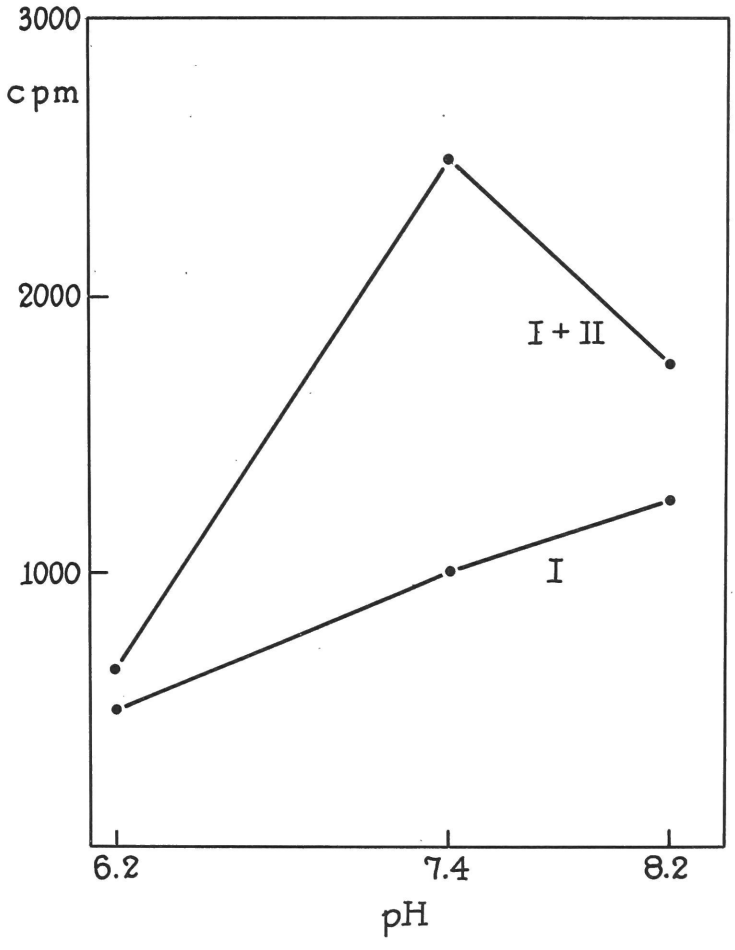


Fig. 14. pH profile with the ethanol fractions. The incubation system was similar to that shown in Fig. 12 with 3 x 10^5 c.p.m. of $PAPS^{35}$ (1 x 10^7 c.p.m./μmole), crude N-acetyl sphingosine, and 0.18 mg. of fraction I and 0.30 mg. of fraction II protein. The buffers used were Tris-HCl at pH 7.4 and 8.2, and Tris-maleate at pH 6.2.

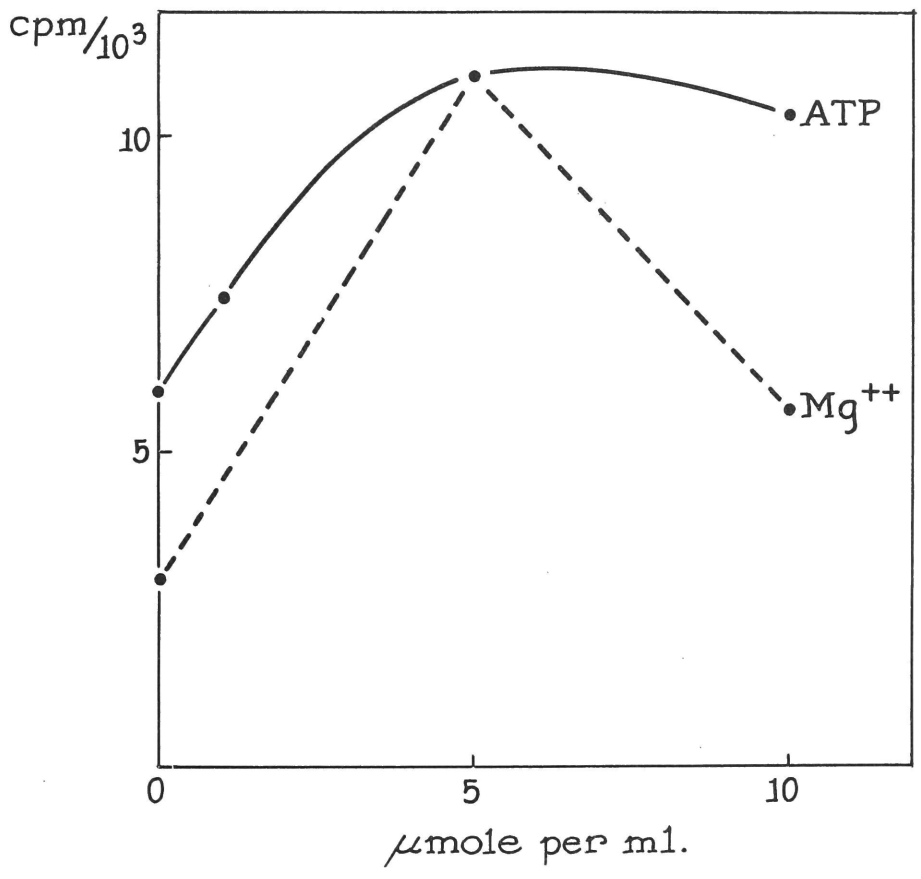


Fig. 15. Dependence on ATP and Mg^{++} concentrations. Each tube contained 50 μmoles of K phosphate buffer, pH 7.4; 2.5 μmoles of $MgCl_2$, except as noted; 2.5 μmoles of ATP, except as noted; 1 x 10^6 c.p.m. $PAPS^{35}$ (3.0 x 10^7 c.p.m./μmole); 1.5 μmole of crude N-acetyl sphingosine (+ 0.5 mg. Tween 20); 1.0 mg. of fraction I protein, and 0.45 mg. of fraction II protein. The ATP and Mg^{++} concentrations were varied as shown. The tube volume was 0.5 ml. and the tubes were incubated at 37° for 40 minutes.

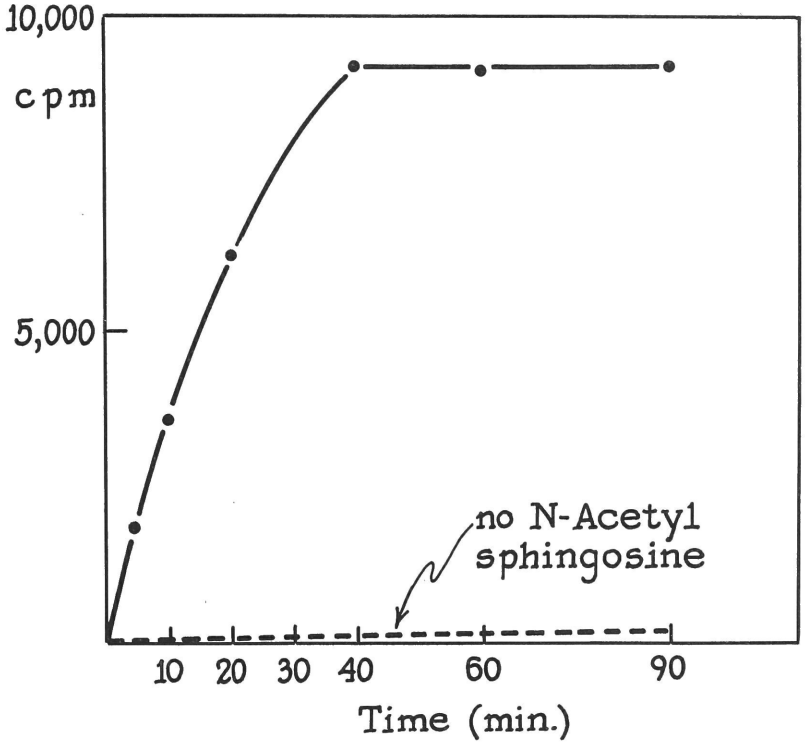


Fig. 16. Time dependence of incorporation by the combined ethanol fractions. The incubation system was similar to that shown in Fig. 15 with 3 x 10^5 c.p.m. of $PAPS^{35}$ (3.0 x 10^7 c.p.m./μmole); crude N-acetyl sphingosine; 1.0 mg. of fraction I protein; 0.45 mg. of fraction II protein. A similar experiment in which the added lipid is omitted is shown for comparison.

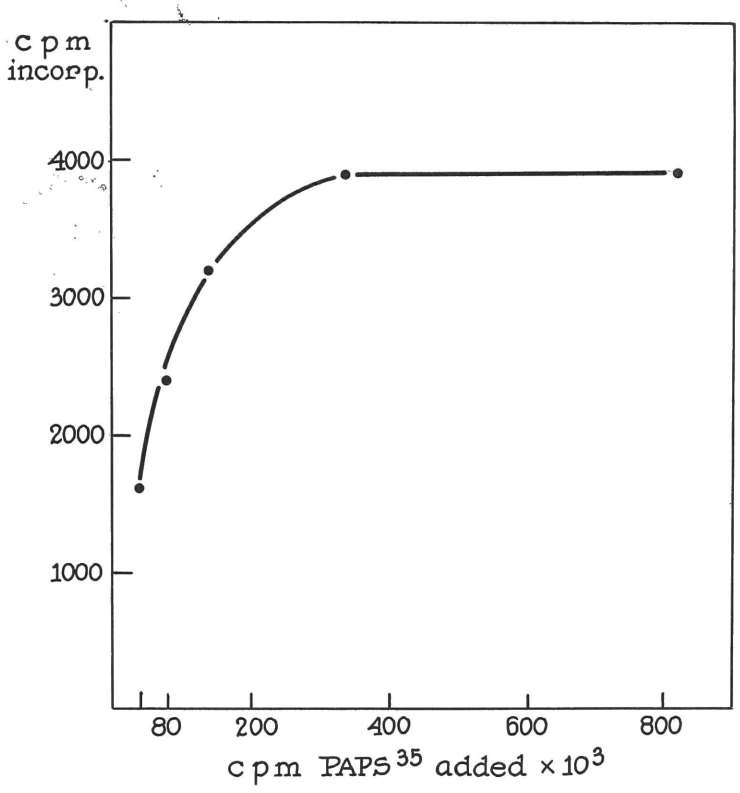


Fig. 17. PAPS^{35} saturation curve. Each tube contained 50 µmoles of K phosphate buffer, pH 7.4; 2.5 µmoles of $MgCl_2$; 2.5 µmoles of ATP; 1.5 µmole of crude N-acetyl sphingosine (+ 0.5 mg. Tween 20); 0.18 mg. of fraction I protein; 0.3 mg. of fraction II protein. The PAPS^{35} (1.0×10^7 c.p.m./µmole) was varied as shown. The total volume was 0.5 ml. and incubation was at 37° for 30 minutes.

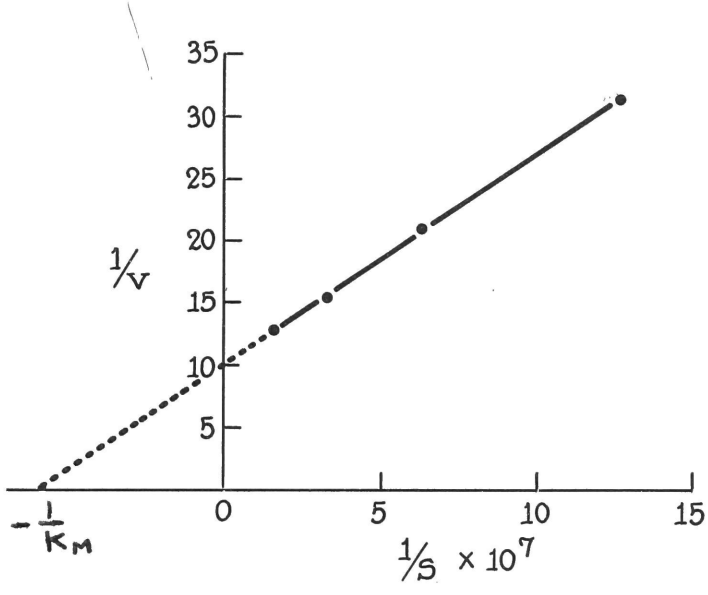


Fig. 18. Double reciprocal plot for PAPS binding. See text for details.

v = initial rate of reaction

s = substrate concentration ($PAPS^{35}$)

Further Purification

Attempts at fractionation by use of ammonium sulfate, calcium phosphate gel, and repeat ethanol fractionation resulted in little additional purification.

Chromatography on DEAE-Cellulose - DEAE-cellulose chromatography according to the method of Peterson and Sober (211) as modified by Boman (212,213), was carried out separately on both fraction I and II (Fig. 19). Columns with the dimensions of 2.5 x 20 cm. were prepared with DEAE-cellulose (0.86 m eq. per gm.) which had been washed with NaOH and finally equilibrated with 0.05 M Tris buffer pH 7.7. Elution was accomplished by step-wise increases in the concentration of Tris buffer at pH 7.7. As can be seen in Fig. 19, the activities associated with both fraction I and fraction II could be located in discrete protein peaks. It is of interest that both activities appear to be eluted from the column by the same concentration of Tris buffer, namely 0.2 M. This may account for the fact that activity can be elicited in fractions obtained from the chromatography of fraction I, even when fraction II is not included in the assay. The possibility remains, however, that an enzymic activity, as measured by incorporation from $PAPS^{35}$ into lipid in the presence of crude N-acetyl sphingosine, may exist in the absence of the component in fraction II. This aspect of the problem has not been further elucidated, but as will be seen later, stimulation by fraction II is also observed when other non-lipid sulfate acceptors are used.

Summary of Purification and Fractionation - Table XIV summarizes the purification and separation achieved by the above procedures. Because of the apparent complexity of the system, calculation of specific activities must be viewed with caution. Nevertheless, this has been done in order to provide a general picture of the results of the various purification procedures. On occasion, a recovery of activity as high as or greater than 100% of the original has been obtained. This is presumably due to removal of competing or interfering reactions present in the cruder material. That this may be so is indicated by the failure to find any requirement for ATP when the column eluate of fraction I is tested for activity in the presence or absence of fraction II. This finding rules out the possibility of additional synthetic steps being involved in the formation of the sulfurylated lipid in this particular system. The possibility of ATP being involved in the formation of other sulfolipids, activity for which has been lost by this purification procedure, has not been ruled out.

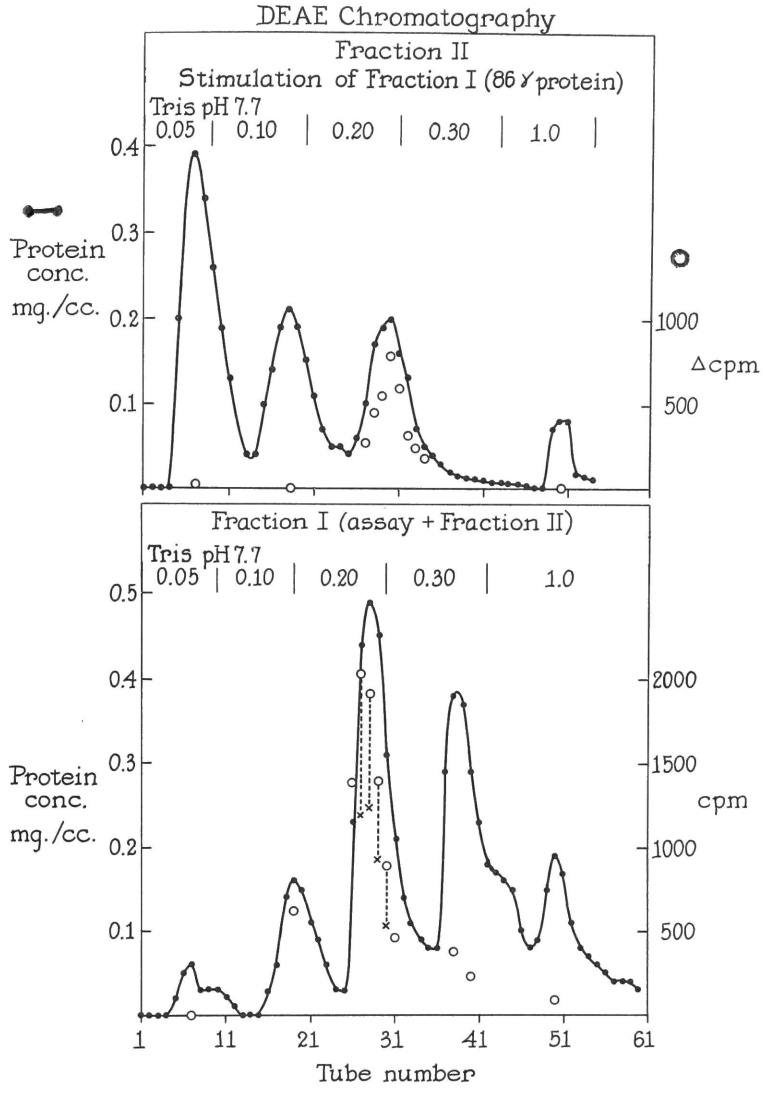


Fig. 19. DEAE chromatography of the ethanol fractions.
Top Diagram: Chromatography of fraction II (fractions assayed as stimulation of a constant amount of fraction I, 86 γ protein). The assay consisted of 50 μmoles of K phosphate buffer, pH 7.4; 2.5 μmoles of $MgCl_2$; 2.5 μmoles of ATP; 1.5 μmole of crude N-acetyl sphingosine (+ 0.5 mg. of Tween 20); 86 γ of fraction I protein; 0.3 ml. aliquot from each tube fraction; 1.5×10^5 c.p.m. $PAPS^{35}$ (5×10^6 c.p.m./μmole). Total volume was 0.5 ml. and the tubes were incubated at 37° for 40 minutes.
Bottom Diagram: Chromatography of fraction I (fractions assayed with and without addition of fraction II). The assay was as above except that 0.2 ml. aliquot from each tube fraction was tested. Open circles = assay to which 0.35 mg. of fraction II protein was added; X = fraction II omitted.

TABLE XIV

Enzyme purification and fractionation

The incubation system was similar to that shown in Fig. 10. Specific activity is expressed as c.p.m. per mg. of protein, and the recovery per ml. represents the total c.p.m. of radioactivity incorporated by 1 ml. of enzyme solution (corrected back to the original volume). Protein fractionation is described in the text.

Fraction	Specific activity	Recovery per ml.
Whole supernate	6,800	86,500
pH 5 supernate	9,860	76,400
pH 5 precipitate	1,720	9,050
Fraction I	7,100	35,000
Fraction II	294	1,000
Fractions I + II	15,400	76,000
DEAE-cellulose chromatography of fraction I*	45,600	20,100 **

* peak activity tube, assay + fraction II
** does not include adjacent tubes of the peak

Sulfurylation of Sphingolipids by Various Protein Fractions - As shown in Table XV, the activity for N-acetyl-DL-threo-trans-sphingosine is about one-third that for the crude N-acetyl sphingosine preparation; the ratio of activities with the different protein fractions remains roughly the same. Sphingosine base is about one-sixth as active as the acetylated compound, whereas cerebroside and N-octanoyl-DL-threo-trans-sphingosine have essentially no activity. Dihydrosphingosine and N-acetyldihydrosphingosine were only slightly active. The N-acetyl-erythro and threo acetylenic sphingosines were likewise poor in stimulation of incorporation into sulfolipid.

Direct Sulfurylation of N-acetyl Sphingosine - These results: 1) The lack of requirement for uridine nucleotide and carbohydrate co-factors in thoroughly dialyzed and partially purified enzyme, 2) the absence of need for ATP by active DEAE-cellulose column fractions, 3) the dependence on added N-acetyl sphingosine for incorporation from PAPS^{35} into lipid, imply that the reaction under study in the liver may be the direct sulfurylation by PAPS of one of the hydroxyl groups of N-acetyl sphingosine. The nature of the stimulation of incorporation produced by the second ethanol fraction

has not been clarified but, as will be seen later, qualitatively similar results have been found in the transfer of sulfate to other acceptors.

The results of experiments on the chemical degradation of the labelled lipid and on the chromatographic properties of this compound compared with known substances provide support for the direct sulfurylation mechanism.

TABLE XV

Sulfurylation of sphingolipids by various protein fractions

Each tube contained 50 μmoles of K phosphate buffer, pH 7.4, 2.5 μmoles of $MgCl_2$, 2.5 μmoles of ATP, 1.5 μmole of lipid added in 1% Tween 20 (0.5 mg. per tube), 300,000 c.p.m. $PAPS^{35}$ (1×10^7 c.p.m./μmole), 0.06 ml. of whole supernate, pH 5 precipitate, or pH 5 supernate, or 0.03 ml. of fractions I and II as indicated. The total volume was 0.5 ml. and the tubes were incubated for 30 minutes at 37°. Assay for incorporation of radioactivity into lipid is described in the text.

Protein fraction	Crude N-Acetyl Sphingosine	N-Acetyl-DL-threo-trans-Sphingosine	Sphingosine base	Cerebroside	N-octanoyl-DL-threo-trans-sphingosine
	c.p.m.	c.p.m.	c.p.m.	c.p.m.	c.p.m.
Whole supernate	2845	1050	488	70	50
pH 5 precipitate	635	290	-	-	-
pH 5 supernate	1635	840	-	-	-
Fraction I	2235	990	-	-	-
Fraction II	30	0	-	-	-
Fraction I + II	3975	1320	610	-	10

Identity of the Labelled Product (Rat Liver)

Radioactive sulfolipid was prepared by incubation of $PAPS^{35}$, combined ethanol fractions, and crude N-acetyl sphingosine as described in Table XII but on 10-20 times the scale.

Chromatography Procedures

I. Florisil column - The chloroform layer containing the radioactive sulfolipid (after $MgSO_4$ washes) was washed once with 3 volumes of distilled water, dried with anhydrous sodium sulfate, and taken to dryness on a rotary evaporator. The dried residue was redissolved in 1 ml. of chloroform:methanol (2:1) and passed through a column 2 cm. in diameter containing 5 gm. of Florisil which had been previously washed with this same solvent (161). The

Florisil was eluted with an additional 150 ml. of the above solvent and the effluent was evaporated. The Florisil column, which has been shown to remove phospholipids and gangliosides (161), did not withhold any of the radioactivity (Table XVI). The radioactive residue was then treated in either of two ways (II or III).

II. Mixed Ion Exchange Column - The residue was redissolved in a small volume of ethanol:chloroform:water (10:8:1) and placed on a mixed ion exchange column (Duolite A-7 and Dowex 50) according to the method of Radin et al (161). Elution was first accomplished with 150 ml. of the same solvent (eluate A) to remove neutral cerebrosides, and then with 80 ml. of 5% lithium acetate dihydrate in ethanol:chloroform:water (8:4:1) (eluate B), which has been shown to remove sulfolipids (Table XVI). The radioactive sulfolipid, therefore, behaved like an anionic lipid in a manner similar to that shown by Radin et al (161) for brain sulfatide.

TABLE XVI

Florisil and mixed ion exchange chromatography of radioactive sulfolipid

Chromatography was performed as described in the text.

Fraction	c.p.m.
Sulfolipids onto Florisil column	63,750
Chloroform:methanol (2:1) eluate of Florisil	61,000
Sulfolipid onto mixed ion exchange column	61,000
Eluate A of mixed ion resin	110
Eluate B of mixed ion resin	59,000 *

* corrected for self-absorption

III. Silicic Acid Column - After passage through the Florisil column, the labelled sulfolipid was redissolved in 0.2 ml. of chloroform:methanol (2:1) and placed on top of a silicic acid column (10 x 2 cm.) which had been previously washed with chloroform until translucent. Chromatography was performed by the method of Weiss (214) using gradient elution with increasing concentrations of methanol in chloroform. At about 16-20% methanol in chloroform, a single symmetrical radioactive peak appeared (Fig. 20). Large

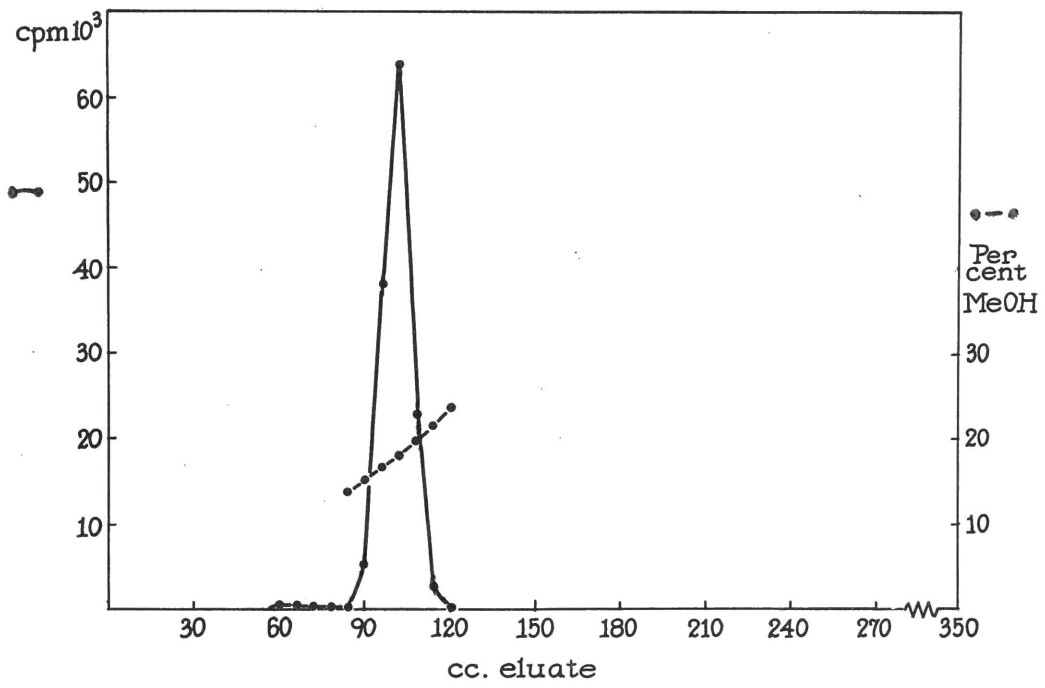


Fig. 20. Silicic acid chromatography of S^{35}-sulfolipid. Methanol in chloroform gradient elution. See text for description.

volumes of either 100% chloroform or petroleum ether resulted in no displacement of this peak. It remained as such on rechromatography on silicic acid but with further runs a second more polar radioactive peak appeared. Rechromatography of the two peaks indicated that the first peak was giving rise to the second, which remained stable. The second peak was eluted with about 30% methanol in chloroform, whereas, pure inorganic $S^{35}O_4$ or $PAPS^{35}$ required much higher concentrations of methanol for elution.

An effort was made to determine the presence or absence of carbohydrate material in the radioactive peaks by the anthrone reaction (215). The reaction was usually complicated by the production of a reddish-brown color, presumably due to interfering non-carbohydrate-containing materials, possibly aldehydic in nature. Spectra of the anthrone-reaction mixture were similar to those of Mallov et al (199), produced by the reaction of n-heptaldehyde with the anthrone reagent. This interfering color was found by these workers in anthrone reactions with the lipids of beef heart and dog liver. Attempts at removal of the interfering material by addition of ammonium reineckate, as used by them were not successful. No convincing evidence for the presence of sugar-containing material in the radioactive peaks could be obtained.

Paper Chromatography

1. The radioactive sulfolipid obtained after passage through Florisil was spotted onto silicic acid-impregnated paper and chromatographed in a solvent of methanol in chloroform (216). The R_f was 0.6 in 15% methanol in chloroform, and the radioactive sulfolipid moved close to the solvent front in 20% methanol in chloroform.

2. Because of the possibility, suggested by the enzymatic studies, of the radioactive lipid being the product of a direct sulfurylation on one of the hydroxyls of N-acetyl sphingosine, chemically synthetic "sulfurylated N-acetyl sphingosine" was compared chromatographically on paper with the enzymatic labelled product. Chromatography of the Florisil-treated enzymatic sulfolipid and other sphingolipids on Schleicher and Schüll paper No. 2045 B (washed with 5% acetic acid) in the solvent system of Jatzkewitz (171) - upper phase of isoamyl alcohol : n-butanol : water (27:2.5:10) - produced the following R_f's: radioactive sulfolipid, 0.38; cerebroside sulfate (prepared by the procedure of Lees and Folch (164,165) and gifts of Dr. G. Schmidt and Dr. H. Jatzkewitz), 0.17 and 0.10; cerebroside (prepared by procedure of Carter et al, and gift of Dr.H. E. Carter), 0.60; crude N-acetyl sphingo-

sine, 0.88; and chemically prepared "sulfurylated crude N-acetyl sphingosine", 0.38 (Fig. 21). The marker lipids were identified by staining the paper with 0.001% Rhodamin B and observing for fluorescence under ultraviolet light (217). The cerebroside preparation is noted to be contaminated with the sulfatide material.

Paper Electrophoresis.

The radioactive sulfolipid exhibits no electrophoretic mobility when applied to paper in either 0.05 M trisodium citrate solution or 0.05 M citrate buffer at pH 5.6. Separation from contaminating inorganic $S^{35}O_4$ or $PAPS^{35}$ may be easily obtained. The complete absence of movement from the point of application on the paper is due to its insolubility in the aqueous buffer.

Acid Hydrolysis of the S^{35}-Sulfolipid - The radioactive sulfolipid was shown to be an ester sulfate by acid hydrolysis. The dried lipid was dispersed in 0.25 ml. of distilled water and 0.25 ml. of 2 N HCl was added. The tubes with screw cap tops were immersed in boiling water for various periods of time, cooled, and the contents taken to dryness on the rotary evaporator. The dried residue was taken up in a small volume of methanol and electrophoresed on Whatman No. 31 paper in 0.05 M citrate buffer at pH 5.6. After 90 minutes, all of the radioactivity associated with the lipid appeared as inorganic $S^{35}O_4$. When the hydrolysis was carried out in methanolic HCl, a synthesis of methyl sulfate was observed.

Unlike the compound formed in the brain system, no appearance of galactose-$S^{35}O_4$ could be demonstrated with 0.5 N HCl for 30 minutes at 100°, under conditions such that transfer or exchange were ruled out.

Alkaline Hydrolysis of the S^{35}-Sulfolipid - By alkaline hydrolysis, it was possible to obtain evidence suggesting that the radioactive sulfolipid has an acylated amido-group and is, therefore, most probably a ceramide derivative. The acyl group could be removed from the amino function without cleavage of the sulfate from the lipid.

The dried S^{35}-sulfolipid was treated with saturated barium hydroxide according to the method of McKibbin and Taylor (198,218). After barium hydroxide hydrolysis, 87-100% of the original radioactivity was found in the chloroform layer. When the same procedure was carried out with 0.25 N NaOH and reflux for 4 hours, however, only 15% of the radioactivity remained in the chloroform layer; the rest was in the aqueous phase. This was identified

Paper Chromatography and Autoradiography of Sphingolipids

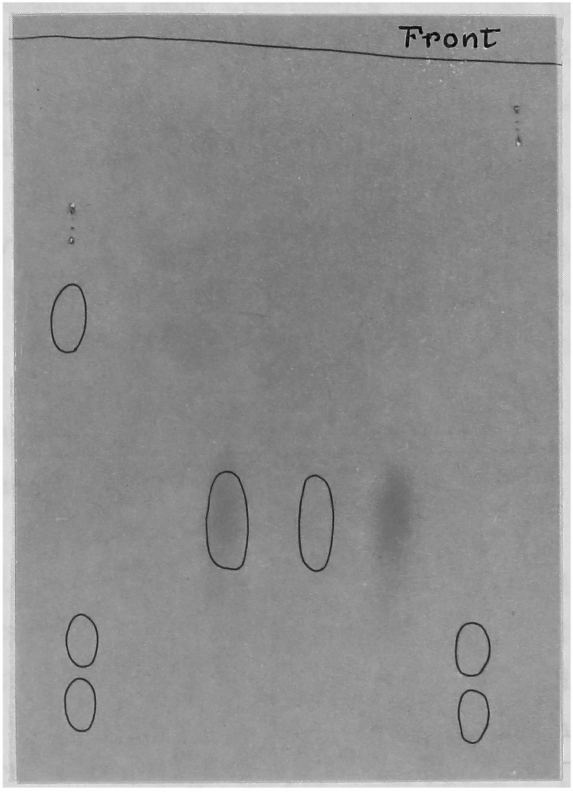


Fig. 21. See text for description.

as inorganic sulfate by paper electrophoresis. Reflux for 50 minutes in 0.1 N NaOH resulted in 37% of the radioactivity going into the aqueous layer, whereas a control treated in the same manner but without alkali lost only 3.6% of the radioactivity to the water. However, when the radioactive lipid was taken up in 0.5 N methanolic NaOH and kept at room temperature for 18 hours, the labelled lipid maintained its chloroform solubility.

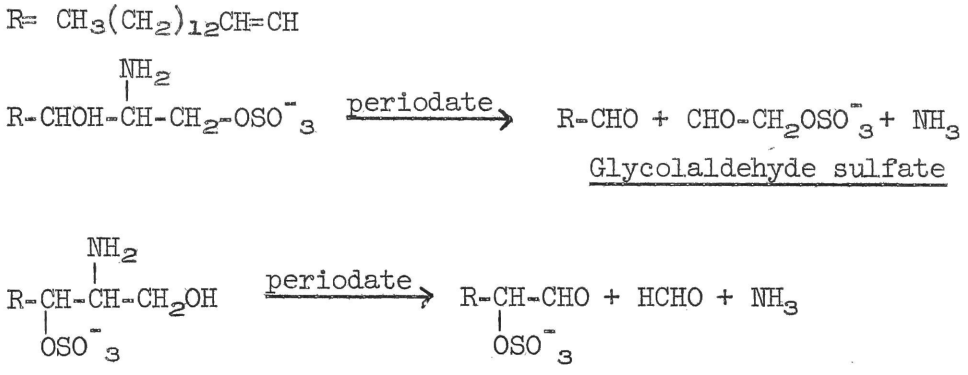
That the $Ba(OH)_2$ or methanolic NaOH treatments resulted in hydrolysis of the acetamido;group was indicated by the behavior of the hydrolyzed radioactive lipid on Dowex-50 cation exchange resin. Activated Dowex-50-hydrogen form resin was made up in chloroform:methanol:water (2:1:0.03) to form a column 1 x 5 cm. The lipid present in the chloroform after alkaline treatment was taken to dryness by rotary evaporation, and taken up again in 2 ml. of the above solvent. This was placed on the Dowex-50 column and washed with 75 ml. of the same solvent. Before alkaline hydrolysis, 100% of the radioactivity was washed through the column by this method, whereas after alkaline treatment, 83-92% of the radioactivity was retained on the column. This suggests that a protonated amino group has been made available by the hydrolysis.

Recently, Lees et al (165) have studied the barium hydroxide hydrolysis of brain sulfatide. They found that whereas there was a relatively slow appearance of free amino nitrogen, there was a relatively rapid cleavage of the galactosidic linkage of the sulfatide. More than 80% of the galactose was destroyed in 4 hours. These findings differ from Klenk's (147) observations on the hydrolysis of cerebrosides where psychosine is the product.

The experiments described above, therefore, show that the labelled sulfolipid behaved differently from brain sulfatide to barium hydroxide treatment. The acylamide linkage is apparently more labile in the former than that of the sulfate ester. These results are compatible with the direct sulfurylation of one of the hydroxyl groups of the added ceramide in the enzymatically produced substance.

Periodate Oxidation of the Alkali-Treated Radioactive Lipid - If the sulfate is attached directly to the sphingosine moiety (as suggested by the enzymatic and chromatographic data as well as that of the alkali-degradation studies), it might be conjugated through either the primary or the secondary hydroxyl of the sphingosine. After liberating the free amino group by alkaline hydrolysis, oxidation with periodate would be expected to yield glycolaldehyde

sulfate from the compound, sulfurylated on the primary hydroxyl, whereas a lipid-soluble compound would result from oxidation of the compound sulfurylated on the secondary hydroxyl. This is shown below:



Glycolaldehyde sulfate should be water-soluble and readily recognized by comparison with the chemically synthetic marker on paper electrophoresis. The periodate reaction carried out without prior alkaline hydrolysis should not cause an oxidative splitting.

The chloroform layer containing the alkali-treated lipid (washed once with distilled water) was taken to dryness by rotary evaporation and the dried lipid (13,700 c.p.m.) redissolved in 0.1 ml. of methanol. To this solution was added 0.2 ml. of freshly prepared 0.15 M sodium metaperiodate. The solution was mixed by swirling at room temperature for 30 minutes, at which time the excess periodatewas converted to iodate by addition of solid glucose. The contents were taken to near dryness by rotary evaporation, and 0.15 ml. of distilled water was added. The aqueous solution was applied to Whatman No. 31 paper for electrophoresis in 0.05 M triethylammonium carbonate buffer, pH 7.5. Chemically prepared glycolaldehyde sulfate (S^{35}) was run as a marker (Fig. 22). Several water-soluble radioactive areas were detected by autoradiography (3 weeks). One of the strongest has a mobility in this system similar to that of synthetic glycolaldehyde sulfate. Electrophoresis in 0.05 M citrate buffer, pH 5.6, showed the same correspondence of mobilities. (The evidence for sulfurylation of a primary hydroxyl group rests in large part on experiments described on page 67.)

Periodate oxidation was also carried out by the method of Carter et al (219) using methanolic periodic acid with results similar to above. When the periodate reaction was attempted by the method of Rouser et al (220)

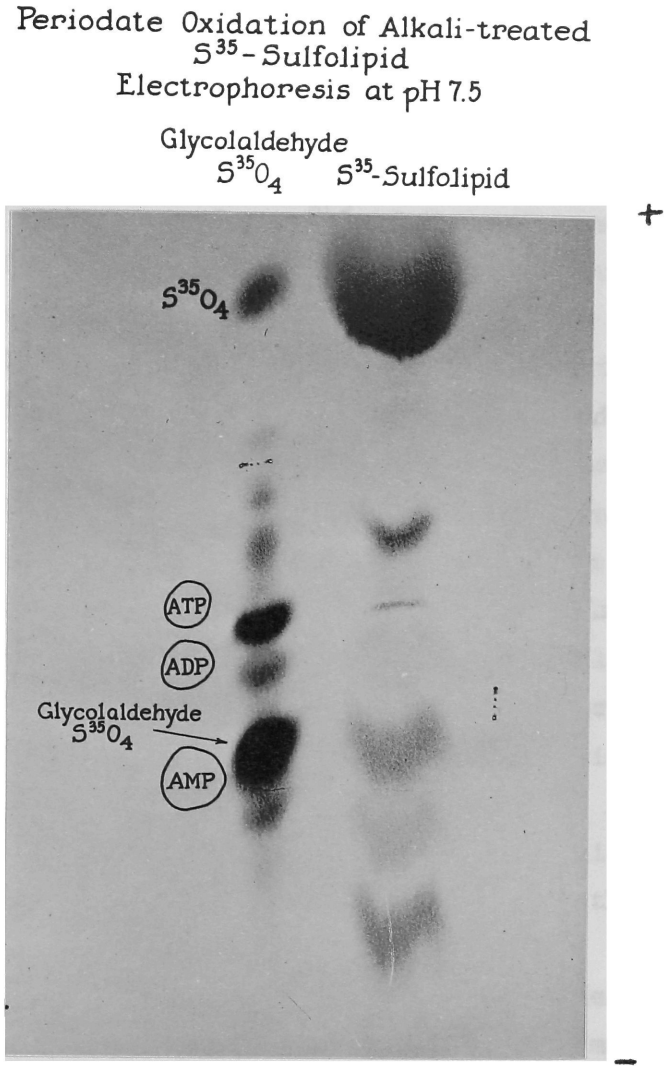


Fig. 22. See text for description.

The liberation of a radioactive fragment from the S^{35}-sulfolipid having electrophoretic properties of glycolaldehyde $S^{35}O_4$. The multiplicity of radioactive spots seen in both preparations is accentuated by the long time of autoradiography (3 weeks).

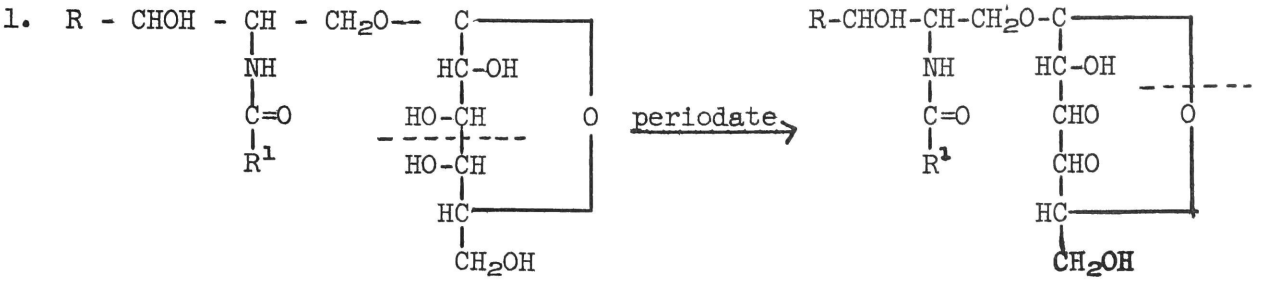
with vigorous shaking for several hours of the aqueous sodium metaperiodate and the chloroform solution of the lipid, an interphase precipitate formed which had most of the original radioactivity. This was insoluble in water and alcohol. As suggested by Carter et al (219), this may be the insoluble iodate or periodate salt of the sphingosine base.

Periodate treatment of the alkali-reacted sulfolipid resulted in the production of radioactive fragments which had a greater solubility in water than in chloroform. After such a procedure, 84% of the recoverable radioactivity (only about 25% of the total original radioactivity could be recovered) was found in the aqueous phase upon equilibration with the chloroform layer. When the alkaline step was omitted and the periodate reaction was carried out directly, 86% of the original activity was found in the chloroform (80% in a control without periodate). Prior alkaline treatment, therefore, is required to free the amino group of the sphingosine moiety for periodate reaction. These experiments also suggest that the sulfate is not attached to the lipid by means of a carbohydrate group, which would undergo periodate oxidation itself with the formation of water-soluble fragments (in case an oxygen-linked dialdehyde formed which remained attached to the lipid, treatment with weak acid and base was used after the periodate reaction).

The above results lend strong support to the idea that a direct sulfurylation of N-acetyl sphingosine by PAPS has occurred during the enzymatic reaction.

Periodate Treatment of Crude N-Acetyl Sphingosine - In order to rule out the possibility that the sulfate acceptor activity in this material was due to trace contamination with 1) a sugar-containing sphingolipid, or 2) a sphingolipid in the free base form, the crude N-acetyl sphingosine was treated with sodium metaperiodate by the method of Rouser et al (220). In either of these two cases, periodate oxidation would occur and lead to destruction of the lipid precursor. N-acetyl sphingosine should not be affected, as shown below:

1.



2. $R\text{-}CHOH\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}CH_2OH \xrightarrow{\text{periodate}} RCHO + NH_3 + HCOOH + HCHO$

3. $R\text{-}CHOH\text{-}\underset{\underset{\underset{\underset{CH_3}{|}}{C=O}}{|}}{\underset{\underset{NH}{|}}{CH}}\text{-}CH_2OH \xrightarrow{\text{periodate}}$ No reaction

N-acetyl sphingosine

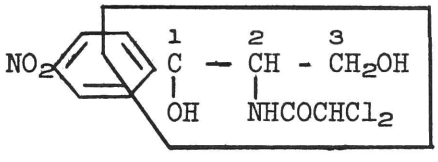
R- = $CH_3(CH_2)_{12}\ CH{=}CH$-

R^1 = aliphatic group

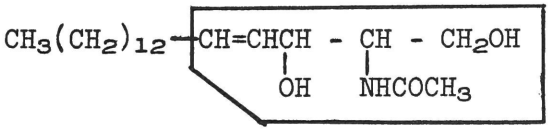
The chloroform layer was washed with distilled water after the reaction, and the lipid was re-isolated and emulsified with 1% Tween 20 in water (0.03 M). This treatment usually resulted in little loss of activity. Only when the manipulations resulted in a marked change in solubility properties of the lipid preparation was there a significant loss of activity. Dialysis of the emulsified crude N-acetyl sphingosine against 0.02 M Tris, pH 7.4, resulted in no loss of activity.

The Enzymatic Sulfurylation of Chloramphenicol, Its Stereoisomers, and Related Compounds

The concept of the direct sulfurylation of N-acetyl sphingosine is supported by experiments on the sulfurylation of the chloramphenicols by these same preparations. The chloramphenicols and N-acetyl sphingosine have striking structural and optical similarities:



CHLORAMPHENICOL



N-ACETYL SPHINGOSINE

Both molecules possess two asymmetric centers and a 2-amino-1,3-propanediol backbone. In chloramphenicol there is a benzylic hydroxyl and a dichloracetamido group, whereas in N-acetyl sphingosine there is an allylic hydroxyl and an acetamido group. It seemed reasonable to suppose that if this enzymatic system were sulfurylating N-acetyl sphingosine on its primary hydroxyl, then chloramphenicol might undergo a similar reaction. This has proved to be so.

Chloramphenicol (D-threo-1-p-nitrophenyl-2-dichloracetamido-1,3-propanediol), its stereoisomers, and related compounds were tested for sulfate acceptor activity using $PAPS^{35}$ and the enzyme fractions found to be active with the acetylated sphingosine.

Assay - Each tube contained 20 μmoles of K phosphate buffer pH 7.4, 1 μmole of $MgCl_2$, 0.2 μmole of sulfate acceptor, 0.025 ml. of fraction I, 0.05 ml. of fraction II, and 200,000 c.p.m. $PAPS^{35}$ (5×10^6 c.p.m./μmole). The total incubation volume was 0.2 ml., and the tubes were incubated for 60 minutes at 37°. The reaction was stopped by immersing the test tube in a boiling water bath for one minute. The precipitate that formed was centrifuged off, and an aliquot (60-70λ) was used for paper electrophoresis on Whatman No. 31, double thickness, in either 0.05 M citrate pH 5.6, or 0.05 M triethylammonium carbonate pH 7.5. After electrophoresis in the Durrum apparatus, the paper was dried and the newly formed radioactive compound (compared with the control in which no sulfate acceptor was added) was located, and counted by means of a Geiger-Mueller hand counter and scaler-rate meter. The radioactive areas were confirmed by subsequent autoradiography on Kodak no-screen X-ray film. In those experiments in which the incorporation of radioactive sulfate into a new material could not be demonstrated by this method, the same experiment was repeated using carrier-free $PAPS^{35}$ (200,000 c.p.m.).

Steric, Structural, and Electronic Requirements for Sulfurylation - As can be seen in Fig. 23, when the natural D-threo-chloramphenicol was added to the incubation, a new radioactive area with a mobility just ahead of AMP at pH 5.6 appeared. This substance has the same electrophoretic mobility at pH 5.6 and 8 as the chemically synthesized chloramphenicol monosulfate. Fig. 24 illustrates the relative sulfate acceptor abilities of the stereoisomers of chloramphenicol. These activities, plus those of derivatives and of related compounds have been tabulated (Tables XVII, XVIII, and XIX). The sulfate acceptor ability of D-erythro-chloramphenicol is taken as 100.

The erythro series is much more active than the threo; D-erythro-chloramphenicol is 20-times better as an acceptor of sulfate from PAPS than is the natural D-threo form. When there is asymmetry at both carbons 1 and 2, blocking of the hydroxyl function on carbon 3 (succinate ester) or reduction of carbon 3 to methyl results in complete loss of activity. This strongly implicates the primary hydroxyl function as the one involved in sulfate esterification. When, however, the primary hydroxyl is left intact

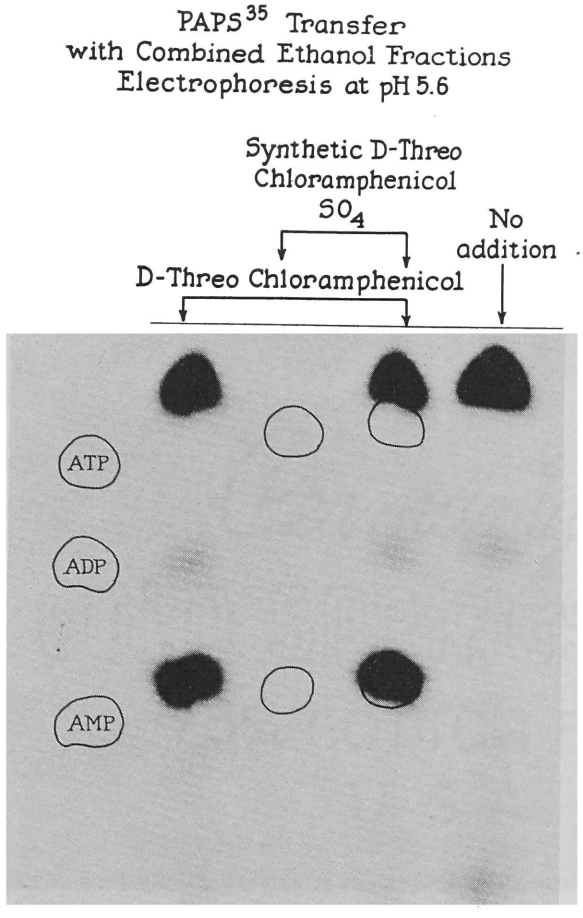


Fig. 23. See text for description.

Autoradiogram illustrating the enzymatic formation of chloramphenicol monosulfate.

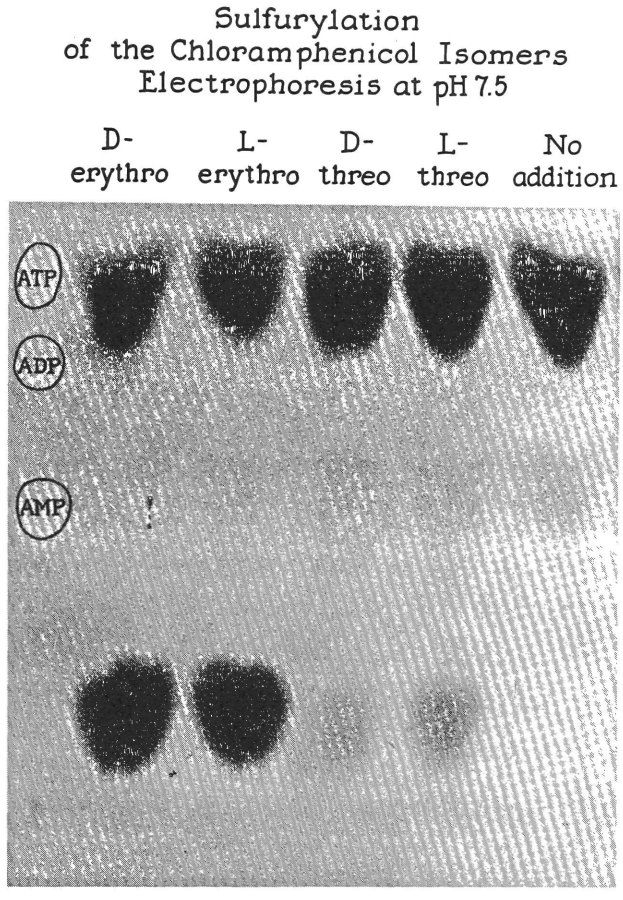
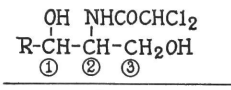
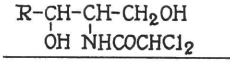
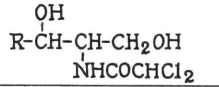
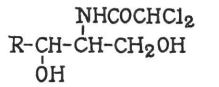
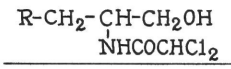
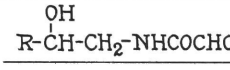
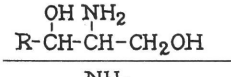
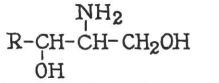
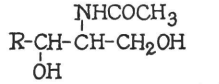
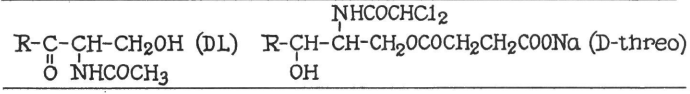
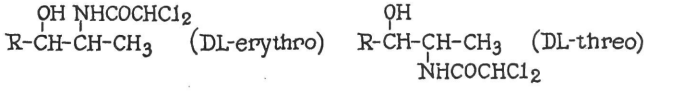


Fig. 24. See text for description.

Autoradiogram illustrating the relative abilities of the chloramphenicol isomers to accept sulfate from PAPS^{35}. The erythro series are much more active than the threo.

TABLE XVII

The Relative Sulfate Acceptor Activities of Chloramphenicol Sterioisomers and Derivatives

Compound			Per cent activity
	(D-erythro)	Chloramphenicols	100
	(L-erythro)		95
	(L-threo)		13
	(D-threo)		5
	(DL)		23
			6
	(D-erythro)		5
	(D-threo)		3
	(D-threo)		2
			0
			

and the secondary hydroxyl is oxidized to a keto function and the dichloracetic acid moiety on the nitrogen is changed to an acetyl group, activity is lost (DL-1-p-nitrophenyl-1-keto-2-acetamido-3-propanol). Although the interpretation may be complicated, this finding is not surprising. It is known from the D-threo series that substitution of acetyl for dichloracetyl results in significant loss of activity. In addition, loss of asymmetry at carbon 1 (DL-1-p-nitrophenyl-2-dichloracetamido-3-propanol) produces a much poorer sulfate acceptor. For these reasons, and whatever the keto function may do to impair the sulfate-acceptor ability of the molecule, this apparent exception does not detract from the probability of primary hydroxyl group sulfurylation in the chloramphenicols.

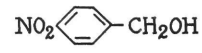
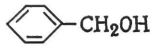
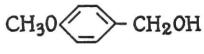
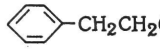
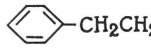
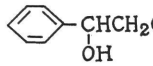
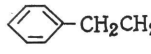
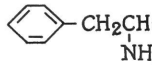
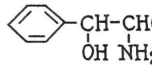
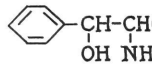
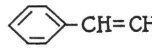
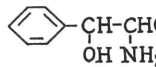
The relative sulfate-acceptor ability of the erythro and threo series, and the compound lacking the secondary hydroxyl (DL-1-p-nitrophenyl-2-dichloracetamido-3-propanol) are very similar to what has been recently described by Cleland and Kennedy (207) in the enzymatic synthesis of psychosine from sphingosine compounds. These workers have found that the addition of a hydroxyl group on carbon 1 of the 3-hydroxy-2-amino hydrocarbon (the numbering system used here is the reverse of that used by Cleland and Kennedy) doubles the activity if the orientation is erythro, and halves it if it is threo.

The free base of D-threo-chloramphenicol is somewhat less active than the parent compound, whereas in the erythro series removal of the dichloracetyl group results in a marked loss of activity. That the free bases and not some associated contaminant act as sulfate acceptors was proved by identification of the sulfurylated compounds by electrophoresis at acid and alkaline pH's.

The ability of the secondary hydroxyl function of the 2-dichloracetamido, 1-ethanol derivatives of chloramphenicol to accept sulfate (where asymmetry of carbon 2 is lost) is consistent with what is seen in the aliphatic alcohols with aromatic side chains (Table XVIII). Here, provided that carbon 2 is not asymmetric (as in the norephedrines or ephedrines), the secondary hydroxyl can act as a sulfate acceptor (1-phenyl-1-propanol). Addition of an amino group on carbon 2 of 3-phenyl-1-propanol leads to loss of activity. Whether the loss in activity is actually related in any way to the production of the new asymmetric center or is somehow associated with the amino function itself is not known. In the aliphatic alcohols with aromatic side chains, activity increases as the aliphatic group attached to the aromatic moiety

TABLE XVIII

The Relative Sulfate Acceptor Activities of Aromatic Derivatives of Aliphatic Alcohols Compared with D-erythro Chloramphenicol as 100%

Compound	Per cent activity
	25
	7
	0
	10
	30
	30
	24
	0
 (DL-erythro) (DL-norephedrine)	0
 (L-erythro) (Ephedrine)	0
	0
 (D, L)	0

increases to three carbons. The molecule with the five carbon chain is also active but somewhat less so than that with three.

Electron withdrawing substituents on the aromatic nucleus aid sulfurylation. A Hammett plot (221,222) of initial rates of reaction of para-substituted benzyl alcohols (to date only three compounds have been tested) reveals a ρ value of approximately +1 (Fig. 25). Because of the insufficient number of points on the curve, interpretation is to be avoided, but suffice it to say that it is a bit surprising to find the reaction, which presumably proceeds by a nucleophilic displacement of the hydroxyl function on the sulfur of PAPS, being aided by electron withdrawal from the oxygen. It is possible, however, that by making the oxygen less negative, proton release and, therefore, anion formation is facilitated in a manner analogous to the phenols. The oxide anion would then be a better attacking group on the sulfur. It is also conceivable that the observed effect is due, instead, to some charged center on the enzyme, either at some distance from the active center or close enough to allow for binding of a basic group on the enzyme with the facilitated proton of the hydroxyl function.

Compounds lacking the aromatic substituent were completely inactive at these concentrations (Table XIX). Unfortunately, the cyclohexyl derivatives were not available for testing, but the pyridine analogues had little if any activity.

From these experiments it cannot be decided with certainty whether the described activities are due to the action of a single enzyme or to more than one enzyme. The available data, however, are compatible with there being one enzyme whose specificity, or lack of it, is such as to encompass the chloramphenicols and the aliphatic alcohols with aromatic side chains, and even the sphingosine compounds. Another point which requires proof is that the activities attributed to the various compounds, obtained from commercial sources without further purification or characterization, are not due to contaminating substances.

In order to eliminate the possibility of reduction of the nitro group of chloramphenicol with subsequent sulfamation (these enzymes are known to be present in liver supernate (82,223,224,225)), p-nitro aniline and m-dinitrobenzene were tested as sulfate acceptors. Both exhibited no activity.

A series of unsaturated aliphatic alcohols, analogous to the sphingosine compounds, were also examined (Table XIX). Only 5-hexene-1-ol appeared to have some activity, although the possibility of this being due to some

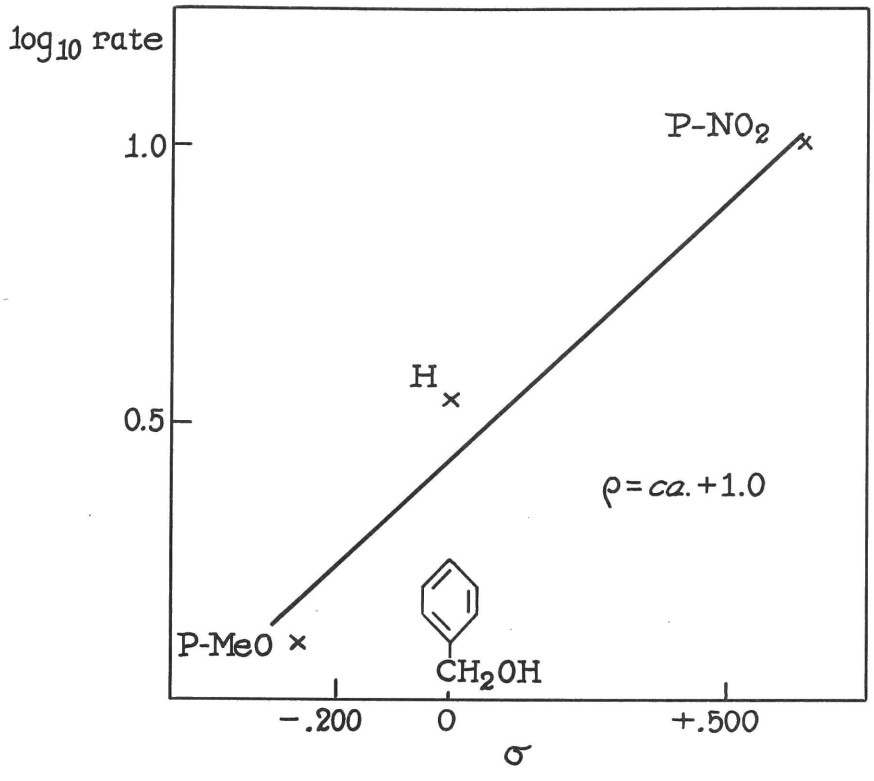


Fig. 25. Hammett plot of para substituted benzyl alcohol. See text for details.

TABLE XIX

Activity as Sulfate Acceptors from PAPS[35]

Compounds with no activity:

Serine; serinol; serine ethyl ester; glycerol; ethanolamine; 1,3-propanediol; 1,6-hexanediol; 2,4-hexadiene-1-ol; 2,4-hexadiyne-1,6-diol; 3-hexen-1-ol; 2-propen-1-ol; 4-penten-1-ol; 3-(2-pyridine)-1-propanol; 3-(4-pyridine)-1-propanol.

Compounds with slight activity:

(about 5 per cent of D-erythrochloramphenicol)

5-Hexen-1-ol; 3-(3-pyridine)-1-propanol.

impurity in the preparation has not been ruled out. 4-pentene-1-ol was inactive. Of some interest in this regard is the fact that cinnamyl alcohol was also inactive.

Effect of Varying Concentration of D-Erythro-Chloramphenicol - The concentration of D-erythro-chloramphenicol was varied as shown in Fig. 26. Saturation was obtained at a level of 0.5 μmole per ml.

Time Course of Sulfurylation of D-Erythro-Chloramphenicol - Fig. 27 demonstrates the time course of sulfurylation of D-erythro-chloramphenicol. The rate remains rather linear for the first 30 minutes.

Inhibition of Incorporation into Sulfolipid by D-Erythro-Chloramphenicol - If it can be shown that the same enzyme system is involved in the sulfurylation of both N-acetyl sphingosine and D-erythro-chloramphenicol, the case for the direct sulfurylation of the former compound would be substantially strengthened. Accordingly, D-erythro-chloramphenicol was tested for its ability to compete with crude N-acetyl sphingosine for incorporation of radioactivity from PAPS under such conditions that PAPS never becomes limiting for either reaction (Table XX). In the presence of 5×10^{-4} M D-erythro-chloramphenicol, the incorporation into sulfolipid was decreased to about one-third of its value in the absence of the chloramphenicol. The same type of experiment with p-nitrophenol instead of N-acetyl sphingosine revealed no chloramphenicol inhibition of sulfate incorporation into p-nitrophenyl sulfate. It seems probable, therefore, that the same enzyme system is concerned with the sulfurylation of both D-erythro-chloramphenicol and N-acetyl sphingosine.

Effect of Sulfhydryl Compounds on Inhibitor - Although there was no stimulation of sulfate acceptor activity by addition of cysteine or glutathione, this activity was completely abolished by 5×10^{-4} M p-chloromercuribenzoate.

Dependence on Magnesium and Both Ethanol Fractions - When magnesium was omitted from the incubation mixture, the uptake of sulfate by D-erythro-chloramphenicol decreased significantly (Table XXI). As was noted with the sulfolipid, fraction II was essentially inactive alone, but when combined with fraction I led to some stimulation of the formation of the sulfurylated chloramphenicol. Experiments in which the protein concentration of fraction I has been varied to allow maximal stimulation by the other fraction have not been done.

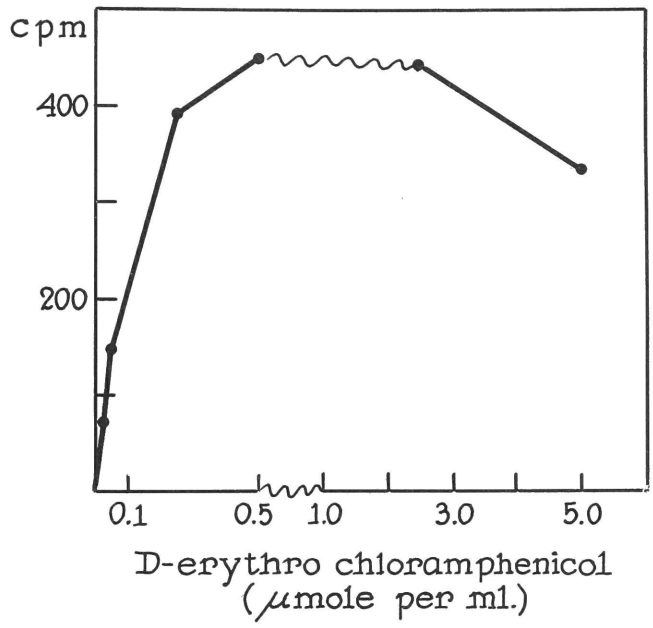


Fig. 26. Effect of varying concentration of D-erythro-chloramphenicol. The incubation system and assay were similar to that described in the text with the combined ethanol fractions and $PAPS^{35}$. The concentration of D-erythro-chloramphenicol was varied as shown.

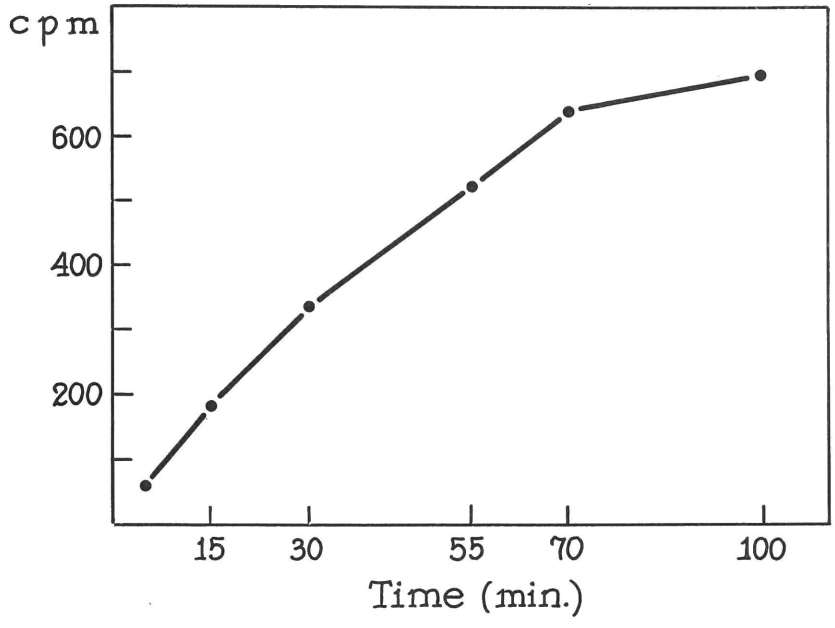


Fig. 27. Time course of sulfurylation of D-erythro-chloramphenicol. The incubation system and assay were similar to that described in the text with the combined ethanol fractions and PAPS^{35}.

TABLE XX

Inhibition of incorporation into lipid

by D-erythro-chloramphenicol

Each tube contained 25 μmoles of K phosphate buffer pH 7.4, 1.0 μmole of $MgCl_2$, 0.2 mg. of Tween 20, 400,000 c.p.m. $PAPS^{35}$ (5×10^6 c.p.m./μmole), 150 μg. of fraction I protein, and 102 μg. of fraction II protein. Addition of 0.1 μmole of D-erythro-chloramphenicol and 0.75 μmole of crude N-acetyl sphingosine was as indicated. Incubation was for 30 minutes at 37° in a total volume of 0.2 ml. Just before application onto paper, crude N-acetyl sphingosine was added to each tube that lacked it during the incubation. Without stopping the reaction, assay consisted of direct application of 60 λ of the incubation volume onto Whatman No. 31 paper, wet with 0.05 M triethylammonium carbonate buffer at pH 7.5 for electrophoresis. After electrophoresis, the paper was dried and the areas at the points of application were cut out (area that of the planchet) and placed in stainless steel planchets for counting in a windowless gas-flow counter. The radioactivity remaining in the PAPS region was recorded with a Geiger-Mueller tube connected to a scaler-rate meter (efficiency about 10-20% of that of gas-flow counter).

Addition	c.p.m. incorporated into sulfolipid area (gas-flow)	c.p.m. remaining in PAPS area (G-M tube)
D-erythro-chloramphenicol	28	725
D-erythro-chloramphenicol + crude N-acetyl sphingosine	144	592
Crude N-acetyl sphingosine	400	953
None	28	996

TABLE XXI

Dependence on magnesium and both ethanol fractions

The complete incubation system and the assay were similar to that described in the text with $PAPS^{35}$ and D-erythro-chloramphenicol. Magnesium and the ethanol fractions were omitted as indicated.

Additions	c.p.m.
Complete	523
- Mg^{++}	400
- Fraction II	410
- Fraction I	19
- Mg^{++} and Fraction II	260

Presence of Phenol and Steroid (Dehydroepiandrosterone) Sulfokinases in Various Protein Fractions - P-nitrophenol and dehydroepiandrosterone were tested as sulfate acceptors from $PAPS^{35}$ using the same incubation system as for D-erythro-chloramphenicol with the various protein fractions previously described (Table XXII).

TABLE XXII

Comparison of D-erythro-chloramphenciol, p-nitrophenol, and dehydroepiandrosterone as sulfate acceptors with various protein fractions

The incubation system and assay were similar to that described in the text with 1.0 μmole of sulfate acceptor per ml., and $PAPS^{35}$. Where indicated, 0.03 ml. of supernate and pH 5 fractions, or 0.015 ml. of the alcohol fractions were used in each tube. The dehydroepiandrosterone was added in 0.02 ml. of propylene glycol.

Fraction	D-erythro-chloram-phenicol	p-nitro-phenol	Dehydroepi-androsterone
	c.p.m.	c.p.m.	c.p.m.
Liver supernate (dialyzed)	466	361	79
pH 5 precipiate	135	148	32
pH 5 supernate	412	270	52
Fraction I	338	280	54
Fraction II	4	18	0
Fractions I and II	456	336	71

The protein fractionation has resulted in little, if any, separation of the three activities. Although not investigated in as great detail as with lipid sulfurylation, it can be seen that Fraction II, especially in the case of the chloramphenicol and the steroid, has a stimulatory effect beyond its own activity. That the chloramphenicol sulfokinase is distinct from the phenol and dehydroepiandrosterone sulfokinases is suggested by the following:

1). There was definite activity for p-nitrophenol in fraction II, but none, or much less, for D-erythro-chloramphenicol.

2). Dialyzed supernate of the mucous gland of the marine snail, Busycon, (to be described later) had activity for the phenol and the steroid but none for chloramphenicol (Table XXIII).

TABLE XXIII

Sulfurylation by dialyzed supernate of Busycon mucous gland

The incubation system and assay were as described in the text with 0.5 μmole of sulfate acceptor per ml., 0.01 ml. propylene glycol, PAPS^{35}, 20 μmoles of cysteine per ml., and 0.10 ml. of dialyzed supernate of Busycon mucous gland. Experiments done with the chloramphenicols without added propylene glycol gave similar results.

Compound	c.p.m.
p-nitrophenol	300
dehydroepiandrosterone	45
D-erythro-chloramphenicol	0
D-threo-chloramphenicol	0

3). D-erythro-chloramphenicol would not substitute for phenol in the p-nitrophenol sulfate transfer reaction using PAP as co-factor (55).

4). In some protein fractionations, the ratio of activity for p-nitrophenol and D-threo-chloramphenicol varied from 3.1 to 21.8.

5). Rat brain homogenates had activity for p-nitrophenol but no apparent activity for D-erythro-or D-threo-chloramphenicol. The low level of sulfurylation of these compounds might not be detected with this assay, however, since rat brain homogenate produces a weak radioactive substance in the same area on paper electrophoresis without addition of any sulfate acceptor.

6). Phenol sulfokinase, which was a gift of Dr. J. D. Gregory and was obtained by protamine precipitation, gel absorption and elution, ammonium sulfate fractionation, and dialysis in 0.01 M Tris buffer pH 7.8, had some activity for D-erythro chloramphenicol and dehydroepiandrosterone, but with different ratios than those in other protein fractions (Table XXIV).

TABLE XXIV

Sulfurylation by various enzyme preparations

The incubation system and assay are as described in the text with 0.5 μmole of sulfate acceptor per ml., 0.01 ml. propylene glycol, $PAPS^{35}$, 500 μg of phenol sulfokinase, 60 μg protein of DEAE-cellulose column peak tube (in deep freeze 10 mos.), 215 μg. of fraction I protein, 125 μg of fraction II protein (both 10 mos. old), and 254 μg of dialyzed whole liver supernate protein.

Enzyme Preparation	A D-erythro chloram-phenicol	B Dehydroepi-androsterone	C p-NO_2 phenol	Ratios A:B:C
Phenol sulfokinase	134	88	414	1:0.66:3
DEAE-column	145	44		1:0.30
Fractions I + II	314	118		1:0.35
Whole supernate	446	212	393	1:0.47:0.88

Chemical Characterization of the S^{35} Linkage in Chloramphenicol Sulfate

Alkaline Hydrolysis and Periodate Oxidation - As described in the studies on the identification of the sulfolipid, alkaline hydrolysis and periodate oxidation were performed on the sulfurylated D-erythro-chloramphenicol to localize the hydroxyl group to which the sulfate is esterified. The radioactive area on paper electrophoresis in 0.05 M triethylammonium carbonate pH 7.5 - corresponding to sulfurylated D-erythro chloramphenicol - was eluted, concentrated by lyophilization, treated with 0.5 N NaOH for 20 hours at room temperature, neutralized with sulfuric acid (6 N), and treated with increasing concentrations of sodium metaperiodate. This is a modification of the method of Glazko et al (226) for chloramphenicol-glucuronate. After 30 minutes at room temperature, the excess periodate was quenched by addition of solid glucose, and the solution was streaked on paper for electrophoresis in citrate at pH 5.6 (Fig. 28), and in triethylammonium carbonate at pH 7.5. With increasing concentrations of sodium metaperiodate, the sulfurylated base, which had no electrical mobility at pH 5.6, was oxidized to give a radioactive fragment having the mobility of glycyolaldehyde sulfate.

Periodate Oxidation of Alkali-treated
D-Erythro Chloramphenicol - $S^{35}O_4$
Electrophoresis at pH 5.6

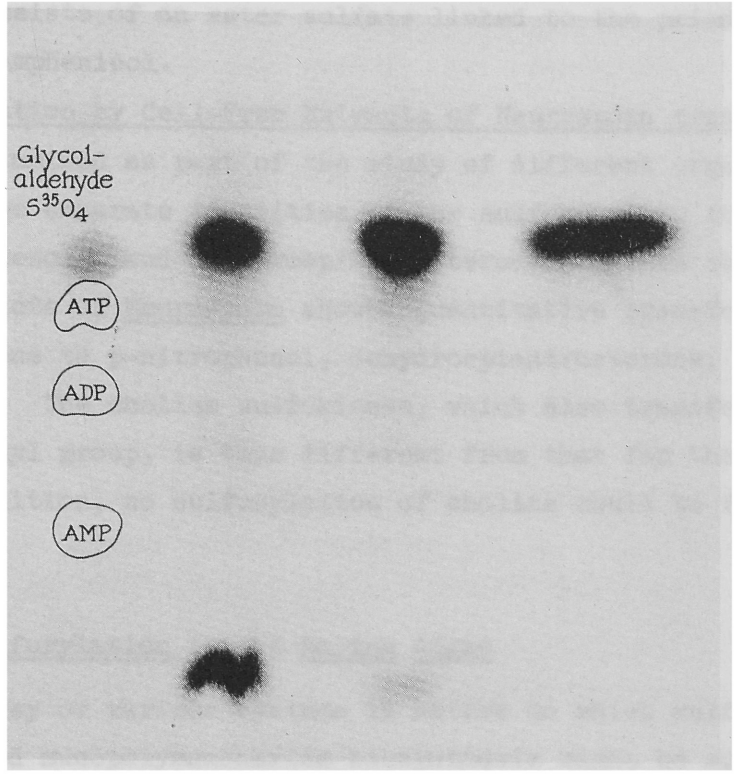


Fig. 28. See text for description.

The liberation of a radioactive fragment from chloramphenicol-$S^{35}O_4$ having electrophoretic properties of glycolaldehyde-$S^{35}O_4$.

Acid Hydrolysis of the Sulfurylated Chloramphenicol - Hydrolysis of the eluted radioactive chloramphenicol sulfate in 1 N HCl for 1 hour led to the quantitative production of radioactive inorganic sulfate (identified by paper electrophoresis).

As a result of these studies it seems most probable that the enzymatically-formed compound consists of an ester sulfate linked to the primary hydroxyl group of chloramphenicol.

Enzymatic Sulfurylation by Cell-Free Extracts of Neurospora crassa - Neurospora crassa was examined as part of the study of different organisms in which evidence for the separate identities of the sulfokinases for the chloramphenicols, the phenols, and dehydroepiandrosterone might be obtained. Dialyzed cell-free extracts of Neurospora showed quantitative transfer from $PAPS^{35}$ to choline but none to p-nitrophenol, dehydroepiandrosterone, or D-erythro-chloramphenicol. The choline sulfokinase, which also transfers sulfate to a primary hydroxyl group, is thus different from that for the chloramphenicol. In addition, no sulfurylation of choline could be found in the liver system.

Sulfurylation in the Marine Algae

Continuing the survey of various systems in nature in which sulfurylated intermediates of sulfated mucopolysaccharide biosynthesis might be sought, it was decided to investigate extracts of the marine algae for their ability to activate sulfate and transfer it to polysaccharide. Possible advantages of such organisms are their ability to produce in vivo large quantities of mucopolysaccharide sulfates, and the fact that some of these polysaccharides are composed of relatively simple sugar units - polyglucose sulfate, polyfucose sulfate, etc. Sulfurylated intermediates were not to be found using cell-free extracts to which $PAPS^{35}$ had been added, but such preparations were able to form PAPS from ATP and inorganic sulfate, and to transfer the sulfate to polysaccharide.

Formation of PAPS by Cell-Free Extracts of Green, Brown, and Red Marine Algae - Extracts of Chondrus crispus and Porphyra umbilicalis (Rhodophyceae or red algae), Fucus vesiculosus (Phaeophyceae or brown algae), and Ulva lactuca (Chlorophyceae or green algae) were prepared from seaweed freshly collected on the tidal rocks near Woods Hole, Massachusetts, August, 1958. The algae were kept in cool sea water upon collection, and brought to the

laboratory for experimentation the same day. After washing the seaweed under the tap to remove sea water, the material was immediately placed in ice-cold buffer of either 0.5 M KCl, 0.05 M Tris pH 7.4, and 4×10^{-3} M EDTA, or 0.25 M sucrose, 0.1 M K phosphate pH 7.5, and 10^{-3} M EDTA. Only the growing parts of the plant that were free of contaminants were cut into smaller pieces, weighed, and ground with the aid of washed sea sand in 1-1/2 volumes per weight of the same cold buffer. All operations were carried out at 4°. The extract was squeezed through four layers of cheese cloth, and the filtrate was centrifuged at 800 x g for 5 minutes. The supernate from this centrifugation was used in these experiments.

Each incubation tube contained 7.5 μmoles of $MgCl_2$, 100 μmoles of K phosphate buffer pH 7.4, 15 μmoles of ATP, 1.0 μmole of UDPG, 1.0 μmole of UTP, 2.0 μmoles of glucose 1-phosphate, 15 μmoles of cysteine, 20×10^6 c.p.m. $Na_2S^{35}O_4$ (carrier-free), and 0.7 ml. of the algae extract. The tube volume was 1.5 ml. and the incubation was for 3 hours at 25°. The reaction was stopped by immersing the tube in a boiling water bath for 1 minute. The supernate was used for paper electrophoresis at pH 5.6 or 8.0 as previously described. The adenosine nucleotides and $PAPS^{35}$ were used as electrophoresis markers. Radioactive areas were identified by autoradiography. Mucopolysaccharides were stained by the method of Leitner and Kerby (227).

With all four algal extracts the incubation produced a radioactive substance having the mobility of PAPS at both pH's. Elution and hydrolysis in 0.1 N HCl for 5 minutes at 100° led to the quantitative formation of inorganic radioactive sulfate (identified by paper electrophoresis). The electrophoretic mobility, acid lability, ability to be absorbed on charcoal, and chromatography in the systems of Suzuki et al (129) and of Bergkveist and Deutsch (228), made it most likely that the main radioactive compound formed during the incubation was PAPS. Control tubes in which the enzyme preparation had been heated to 90° for 1 minute prior to incubation produced no such material.

In addition to the radioactivity in the PAPS region, on several occasions faint but definite radioactivity could be found associated with the metachromatic-staining material, presumably mucopolysaccharide, remaining at the origin. In addition, in Porphyra extracts there also appeared to be radioactivity associated with a metachromatic band moving slightly ahead of

PAPS. This was not charcoal absorbable. An attempt was made to assay incorporation from $PAPS^{35}$ or $S^{35}O_4$ into mucopolysaccharide by the ethanol or cetavalon precipitation methods (43), but without success. It was not possible to get consistent washing out of non-polysaccharide radioactivity by these techniques. This may be due to the relatively large amount of mucopolysaccharide in these preparations. Although it seems likely that incorporation of sulfate into polysaccharide was taking place, this was not pursued further.

Assimilation of $S^{35}O_4$ by Photosynthesizing Porphyra umbilicalis - The light-dependent uptake of $S^{35}O_4$ has been demonstrated in Ulva pertusa (229) as well as the assimilation of $C^{14}O_2$ by a photosynthesizing red alga, Iridophycus flaccidium (230). It was thought of interest to study the in vivo assimilation of inorganic $S^{35}O_4$ by a marine alga. The transfer of sulfate to mucopolysaccharide and other compounds - perhaps sulfurylated intermediates of polysaccharide synthesis - could be studied in relation to time sequence and the requirement for light.

Experiments were conducted on freshly collected growing Porphyra umbilicalis which was immediately placed in sea water and brought to the laboratory. Circular sections of the flattened leaf (one cell thick) having a radius of 2 cm. were cut out, washed with cold artificial sea water containing no sulfate (from the formula used at the Marine Biological Laboratory, Woods Hole), and placed in a beaker containing the same cold solution for 15 minutes to leach out inorganic sulfate. Each section was then transferred to a 25 ml. Ehrlenmeyer flask containing 5 ml. of the same medium, to which was added 40×10^6 c.p.m. of carrier-free $Na_2S^{35}O_4$ in 0.1 ml. of solution. For incubations done in the dark, the flask was completely enclosed in aluminum foil prior to addition of the tissue section. The incubation was carried out at 20° on a rotatory shaker enclosing a battery of fluorescent lamps so that the leaf section remained fully unfolded on the bottom of the flask. At appropriate time intervals, the incubation was stopped by removal of the tissue, blotting it with filter paper, and immediate immersion in boiling 80% ethanol. Extraction of the alcohol-soluble compounds and preparation for paper electrophoresis were done by the method of Bean and Hassid (230). The alcohol-insoluble residue was extracted with 0.5 N NaOH for 1 hour at room temperature. The polysaccharide material was precipitated out of the alkali by addition of acetone, and redissolved in a small volume of water (0.5 ml.) for paper electrophoresis in citrate at pH 5.6. Autoradio-

graphy revealed the pattern of radioactive substances that were formed at various time intervals in light and dark (Fig. 29). A zero-time control showed only the presence of contaminating inorganic $S^{35}O_4$ from the medium.

Because the tissue had not been washed free of contaminating medium before being placed in the boiling ethanol, the influence of light on the uptake of inorganic $S^{35}O_4$ was not studied. It can be seen, however, that band B can be detected by autoradiography after only 5 minutes in the light, and is relatively strong by 15 minutes, whereas no such band is present at 15 minutes in the dark (Fig. 29). But by 2 hours in the dark, it is almost as strong as that in the light. Band A is weak in the light experiments and does not increase in intensity with time. In the dark, however, band A is moderately strong at 15 minutes and becomes intense by 2 hours. Band C is also much stronger in the dark experiment by 2 hours. Bands A and B could be absorbed onto charcoal from a 5% TCA solution and eluted with 0.5% ammonia in 25% ethanol.

Hydrolysis of the alcohol-soluble material in 0.5 N HCl at 100° led to the disappearance of band A within 2 hours of hydrolysis, but band C was not diminished (Fig. 30). Band B appeared to increase in intensity somewhat before practically disappearing by 2 hours. It is possible that band A gave rise to band B during the hydrolysis. Such an interpretation, however, must be viewed with caution because of the ease of sulfate transfer under acid conditions.

Autoradiography of the electrophoretogram of the alcohol-insoluble fraction reveals the presence of radioactivity associated with metachromatic-staining material at 5 minutes in the light (Fig. 31). There is an increase in intensity up to at least 2 hours in the light. At 15 minutes in the dark there is no such radioactivity, but by 2 hours the polysaccharide-associated radioactivity has become very strong.

It is doubtful that the uptake of the infinitesimal quantity of carrier-free $Na_2S^{35}O_4$ was of a limiting nature in the dark experiments. In any case, this would not account for the different pattern of labelling of the alcohol-soluble fraction in the presence or absence of light. The rate of formation of sulfurylated polysaccharide is also quite different in the presence of light.

These experiments are of a preliminary nature but do indicate: 1) the sulfurylation of a polysaccharide that occurs at a faster rate in the light,

S^{35}-Labelled Compounds in Alcohol Extract of Porphyra at Various Times in Light and Dark Electrophoresis at pH5.6

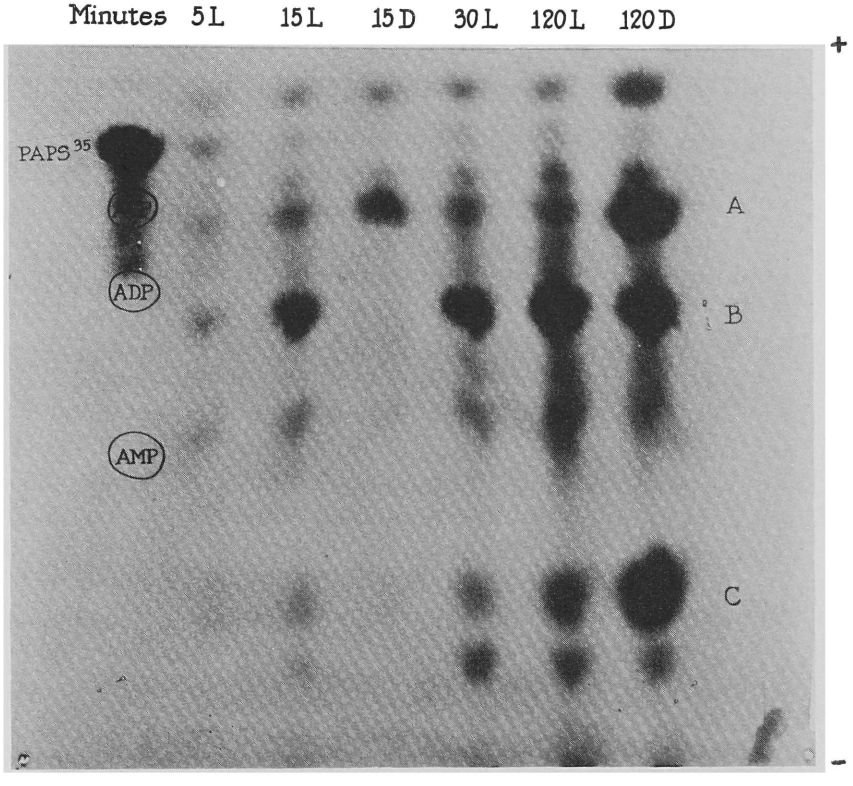


Fig. 29. See text for description.

Acid Hydrolysis of Alcohol Extract
of Photosynthesizing Porphyra

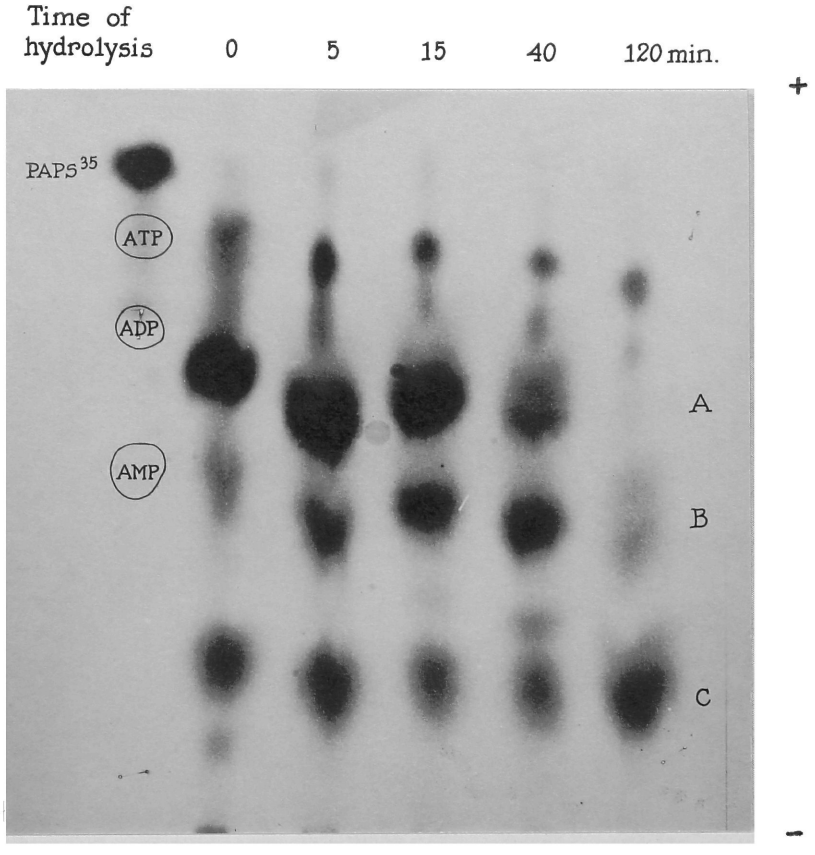


Fig. 30. See text for description.

Autoradiogram illustrating presence of esterified and non-esterified sulfur in alcohol soluble compounds.

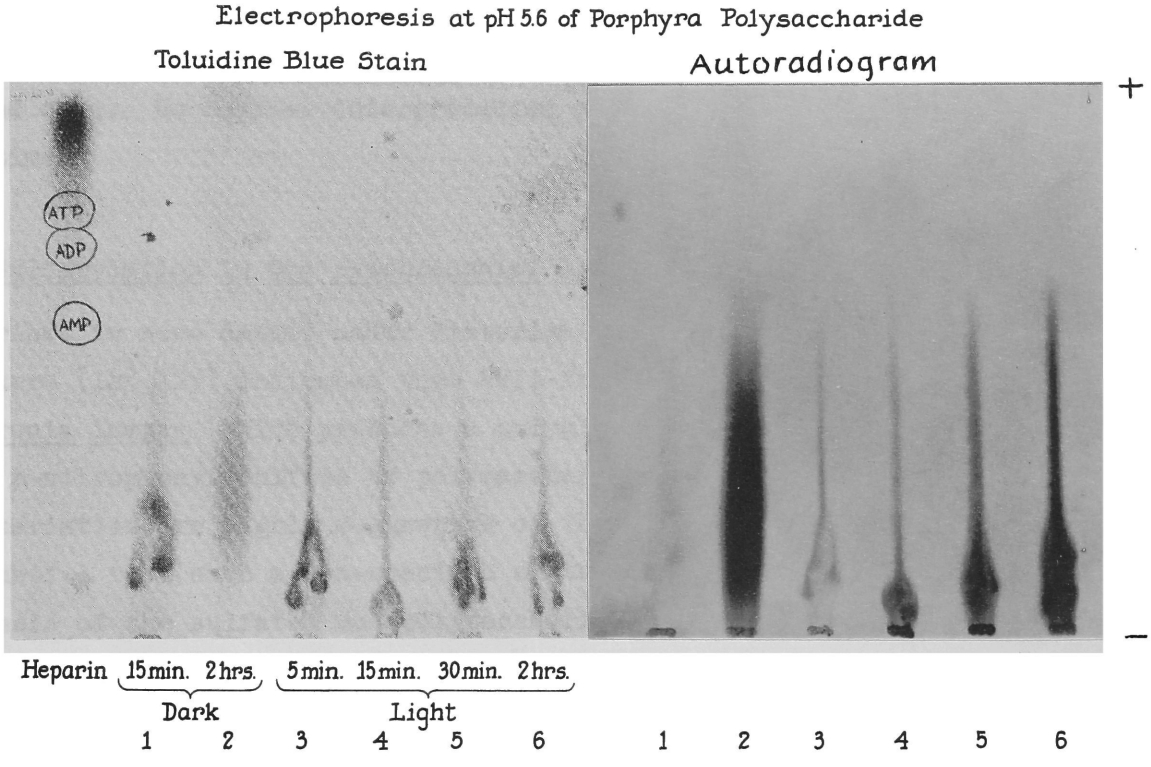


Fig. 31. See text for description.

The incorporation of $S^{35}O_4$ into polysaccharide by photosynthesizing and non-photosynthesizing alga.

2) the formation of several sulfurylated compounds, which are ester sulfates and charcoal-absorbable, and one of which may be produced by acid hydrolysis of the other, 3) the formation of an alcohol-soluble sulfate ester to a greater extent in the dark than in the light, and 4) the formation of an acid-resistant, charcoal-non-absorbable sulfated compound which becomes more intense in the dark. No further interpretation of these findings is warranted at this time.

Sulfurylation in the Hypobranchial Gland of Busycon

As described in some detail under Historical, the investigations of the Japanese workers (128,129) indicated that cell-free extracts of the mucous gland of Charonia lampas (which produces a polyglucan sulfate) can transfer sulfate from p-nitrophenyl sulfate to polysaccharide by an enzymatic reaction whose characteristics are highly suggestive of the action of a sulfatase. It seemed doubtful that such a non-specific system would be responsible for the biosynthesis of the sulfated mucopolysaccharide in nature. The role of PAPS in such a scheme was not clear. It was desirable, therefore, to clarify the mechanism of sulfurylation of the mucopolysaccharide, and to establish the generality of PAPS as the carrier for activated sulfate in such a system. For these reasons, as well as the opportunity to continue the search for possible sulfurylated intermediates, the American counterpart of the Japanese snail was subjected to study.

It was possible to demonstrate the synthesis of PAPS in cell-free extracts of the mucous gland of the marine snail, Busycon canaliculatum (see Historical for chemistry of the polysaccharide). In addition, transfer of sulfate from PAPS, and not from APS, to mucopolysaccharide, to an endogenous sulfate acceptor, and to added aminoalkyl phenols was observed.

Preparation of a Cell-Free Extract of the Hypobranchial (Mucous) Gland of Busycon canaliculatum - Busycon, a large marine snail, is readily available during most of the year by dredging off the shores of Cape Cod and Rhode Island. The early experiments were performed on animals collected at Woods Hole, Massachusetts, in August, 1958. Later experiments were done in New York on live animals purchased at a west side fish market. To obtain the fresh mucous gland, the outer whorls of the shell were cracked with a hammer and the adjacent whorls were pried off with an axe blade. The intact snail could then be unscrewed from the remaining shell. The pallial organs

including the hypobranchial gland could be seen through the translucent roof of the pallial cavity. The portion of the mantle bearing the hypobranchial gland was excised and placed in a beaker containing the following solution: 0.15 M KCl, 0.05 M Tris pH 7.4, 10^{-3} M EDTA pH 8. The friable mucosae of the glands (containing the metachromatic-staining cells which produce the mucus (231)) of several animals were scraped off with a scalpel and placed in a beaker containing 5 volumes per weight of the same buffer. The mixture was stirred by means of a magnetic mixer for 1-1/2 hours at room temperature. Over this period there was a significant decrease in the viscosity of the mixture. The mixture was homogenized in a Potter-Elvehjem glass homogenizer and the homogenate was pressed through three layers of cheese cloth and centrifuged for 30 minutes at 25,000 x g to give supernate I. The sediment obtained was resuspended in twice the volume of the same buffer solution and recentrifuged at 400 x g for 5 minutes. This supernate was then centrifuged at 25,000 x g for 30 minutes. Most of the mucopolysaccharide was associated with the sediment from this centrifugation (sediment III). Sediment III was homogenized in the original volume of buffer.

Some gland extracts were prepared by performing all operations at 4°, with direct homogenization of the cell suspension in cold buffer in a Potter-Elvehjem glass homogenizer and centrifugation as above. All protein solutions were stored at -15° in the deep freeze. It was found later that enzyme activity could be much better preserved in the frozen state when the homogenizing solution contained 0.01 M cysteine. Significant activity remained after as long as six months in the deep freeze.

Formation of PAPS - Incubation of the Busycon mucous gland homogenate, or supernate I, prepared by either of the two methods, with ATP, $MgCl_2$, inorganic $S^{35}O_4$, and phosphate buffer, led to the formation of strongly labelled PAPS (Fig 32). The incubation concentrations and the procedure for identification of PAPS were the same as described for the algae. When the same concentration of Tris buffer at pH 7.4 was substituted for the phosphate buffer, most of the radioactivity - other than the unreacted inorganic $S^{35}O_4$ - was in a substance having the chemical, electrophoretic, and chromatographic characteristics of APS. The phosphate buffer probably inhibits the activity of a phosphatase in the snail extract, which is able to cleave the 3'-phosphate of PAPS. In addition, when APS rather than PAPS accumulates during the incubation, little if any radioactivity is transferred

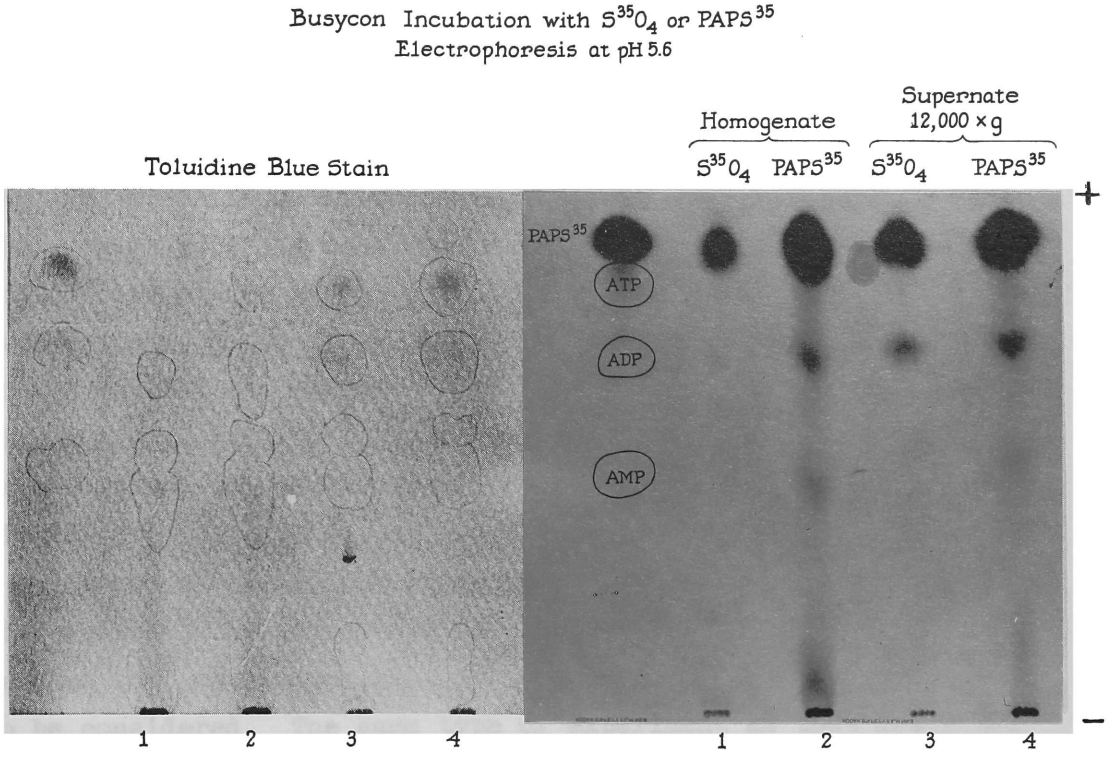


Fig. 32. See text for description.

The enzymatic formation of PAPS and the incorporation of $S^{35}O_4$ into mucopolysaccharide by Busycon preparations.

to sulfate acceptors present in the extract (mucopolysaccharide, artificial sulfate acceptors, the natural sulfate acceptor). The role of PAPS as the biological sulfate donor is emphasized by these experiments.

Incorporation into Mucopolysaccharide - On paper electrophoresis in citrate at pH 5.6, metachromatic-staining material remains at the point of application of the aliquot of the incubation mixture. Incubation of the mucous gland supernateI and sediment III with ATP and inorganic $S^{35}O_4$ or $PAPS^{35}$ in phosphate buffer leads to the formation of a radioactive substance which corresponds exactly to the metachromatic-staining material. (In Fig. 32 is shown the correspondence of the metachromatic-staining material and the radioactive substance produced by the whole homogenate or a 12,000 x g supernate.) Both fractions are needed for maximal incorporation; both are heat-labile (Table XXV). Since most of the mucopolysaccharide is associated with the sediment, it may be, as is probably the case with the muscle glycogen synthesizing-enzyme (232), that the enzyme systems involved here are bound in some manner to the mucopolysaccharide. The possibility of a primer function for the sediment has not been eliminated. That some particulate fraction other than that associated with polysaccharide has enzymatic activity must also be considered. The same type of experiment as that described in Table XXV with the combined supernate I and sediment III fractions, $PAPS^{35}$, phosphate buffer but no added ATP, was performed and assayed for incorporation of radioactivity into polysaccharide by the cetavalon technique as used by D'Abramo and Lipmann (43) (Table XXVI).

TABLE XXVI

Incorporation from $PAPS^{35}$ into polysaccharide by snail mucous gland fractions

The incubation system was similar to that described in Table XXV but no ATP was added. The assay was by use of the cetavalon procedure (43).

Fraction	c.p.m. into polysaccharide
Boiled control	159
Supernate I + Sediment III	2650

Effect of Other Nucleotides - No consistent stimulation in incorporation from $PAPS^{35}$ into mucopolysaccharide could be demonstrated by the addition of 1.0 μmole per ml. of UTP, UDPG, UDP-N-acetylglucosamine, UDP-Glucuronic acid, diphosphopyridine nucleotide alone or in combination.

TABLE XXV

Association of radioactivity with metachromatic-staining material

Each tube contained 2.5 μmoles of $MgCl_2$, 1 μmole of ATP, 25 μmoles of K phosphate buffer at pH 7.4, 1 x 10^6 c.p.m. $PAPS^{35}$ (carrier-free), 0.075 ml. of sediment III solution, and 0.15 ml. of supernate I prepared as described in the text and added to the incubation as indicated. The total volume was 0.5 ml. and the tubes were incubated for 2 hours at 37°. The reaction was stopped, and an aliquot of the supernate prepared for paper electrophoresis in citrate at pH 8.0 as described for the algae. The radioactivity remaining at the origin was measured with a Geiger-Mueller tube attached to a Scaler-rate meter.

Experiment	Fraction	c.p.m. at origin
1.	Supernate I	75
	Boiled Supernate I	0
	Sediment III	475
	Boiled Sediment III	8
	Sediment III + Supernate I	720
2.	Supernate I	25
	Sediment III	194
	Sediment III + Supernate I	450
	Sediment III + boiled Supernate I	110
	Supernate I + boiled Sediment III	85

Omission of ATP or Magnesium - When ATP or magnesium were omitted from the incubation system, incorporation fell to about one-half. The role of ATP in this crude system, to which $PAPS^{35}$ had been added, is not apparent. The lack of effect of the uridine nucleotides and other co-factors on the incorporation of sulfate into mucopolysaccharide may indicate that a direct transfer is taking place.

Sulfurylation of Aminoalkyl Phenols and Endogenous Material in Busycon Mucous Gland Supernate - A sulfate-acceptor compound, which became esterified with a considerable proportion of the radioactivity of the added $PAPS^{35}$, appeared to be present in the supernatant fraction of the autolysate and, to a lesser extent, of the cold-prepared extract (Fig. 33). The radioactive substance, which was formed enzymatically, had a mobility on electrophoresis at pH 5.6 much slower than that of AMP - in fact, because of movement caused by evaporation in the Durrum apparatus, this may not represent any electrical mobility. The sulfate acceptor is dialyzable, is present in a kochsaft of the gland extract, and is not absorbed by Dowex-1 but is absorbed by Dowex-50. Paper electrophoresis in 0.05 M carbonate pH 9.6 resulted in a significant increase in the mobility of the radioactive material to the anode. These facts suggested the presence of a free amino function on the sulfurylated substance. Supporting evidence was provided by treatment of this material with flurodinitrobenzene by the method of Kent et al (233), and chromatography of the DNP-derivative in the solvent system used by these workers. A radioactive DNP-derivative was formed which had a faster mobility than the untreated material. In addition, treatment of the radioactive compound with sodium nitrite resulted in the appearance of a new radioactive substance which now moved on paper electrophoresis at pH 5.6 between ADP and AMP. The enzymatically-formed compound is a sulfate ester. Hydrolysis of the sulfurylated compound in 1 N HCl for 1 hour at 100° resulted in the quantitative conversion of the radioactivity into inorganic $S^{35}O_4$.

Several aminoalkyl phenols and other related compounds were tested as sulfate acceptors in this system in an attempt to identify the natural acceptor with one of these. The addition (10 μmoles per ml.) of such compounds as tyramine, serotonin, tryptamine, 5-OH-tryptophan, tyrosine, tryptophan methyl ester, noradrenaline, and phenylalamine led to the formation of one or more sulfurylated substances (Figs. 34 and 35). The possibility of sulfurylation of the parent compound as well as its metabolic products

Busycon
Electrophoresis at pH 5.6

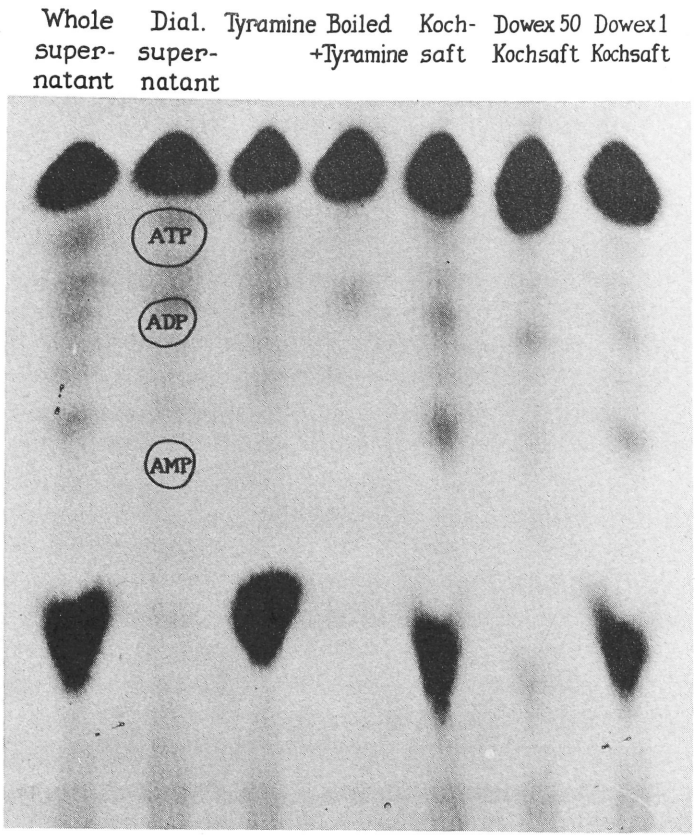


Fig. 33. See text for description.

The sulfurylation of an endogenous material in Busycon mucous gland supernate.

Busycon - Dialyzed
Electrophoresis at pH 5.6

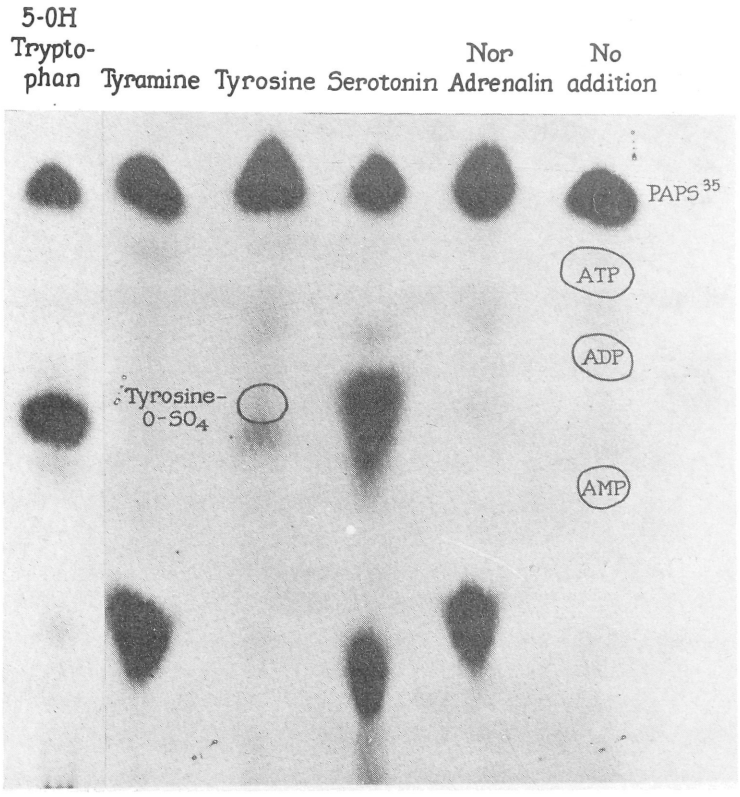


Fig. 34. See text for description.

The sulfurylation of various aminoalkyl phenols and related compounds.

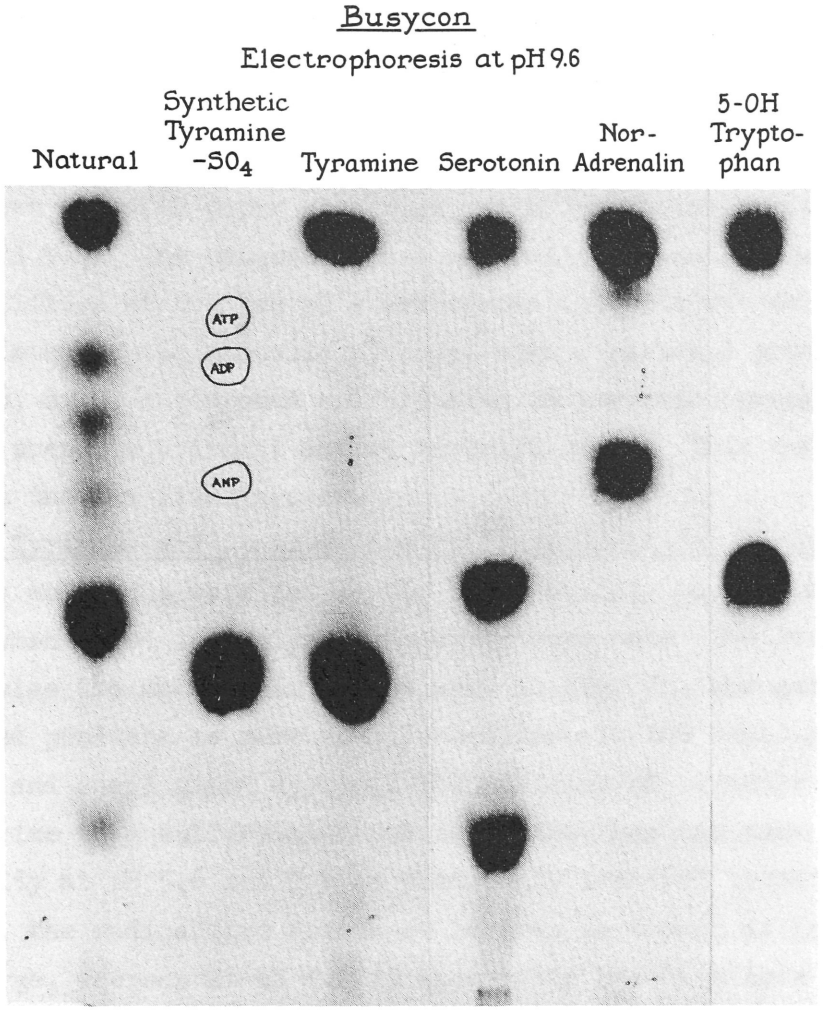


Fig. 35. See text for description.

The sulfurylation of various aminoalkyl phenols and related compounds.

must be considered. None of these sulfurylated compounds, however, had mobilities identical to those of the sulfate-conjugate of the endogenous compound at the two pH's. The great increase in mobility on paper electrophoresis at pH 9.6 of the sulfurylated compound produced by the addition of noradrenaline to the incubation is probably due to both the ionization of the catechol hydroxyl function and the suppression of the amino protonation. The addition of tyrosine to the incubation led to the formation of a sulfurylated compound which moved on paper electrophoresis just below tyrosine-o-sulfate at pH 5.6 and 9.6. The nature of this metabolite is not known, but the different mobilities at the two pH's are compatible with either: 1) a compound sulfurylated on the phenolic hydroxyl with a carboxyl group and a free amino group, or 2) a compound sulfurylated on the amino group with an unsubstituted phenolic hydroxyl and no carboxyl group. This compound was not found in the rat liver system.

Sulfurylation of Tyramine and Other Aminoalkyl Phenols in Rat Liver Supernate - These same compounds were tested for their ability to form sulfurylated substances when added to dialyzed rat liver supernate (the incubation system was otherwise the same). As can be seen in Fig. 36, the general pattern of sulfurylated products is more complicated than in the snail gland. In both the rat liver and snail gland systems, the addition of tyramine to the incubation gives rise to a sulfurylated substance that has the same electrophoretic mobility at pH 5.6 and 9.6 as chemically prepared tyramine-o-sulfate. At pH 5.6, the radioactive substance behaves as though it has no net electrical charge, whereas at pH 9.6 it appears to have a single net negative charge. Such behavior is compatible with sulfurylation of the phenolic hydroxyl and the presence of an unsubstituted amino group. That this enzymatically-formed compound is identical with tyramine-o-sulfate cannot be said with certainty, but the above behavior is consistent with this possibility. In addition, on tyramine addition in the rat liver system but not in the snail extract, there was another strong radioactive material with a mobility as fast as that of ATP at pH 5.6. It is possible that this is the disulfate of tyramine or the monosulfate with an ionized carboxyl group. It is of interest that of the several sulfurylated substances to which tyrosine gives rise, none is identical electrophoretically with tyrosine sulfate.

Rat Liver Supernatant - Dialyzed

Electrophoresis at pH 5.6

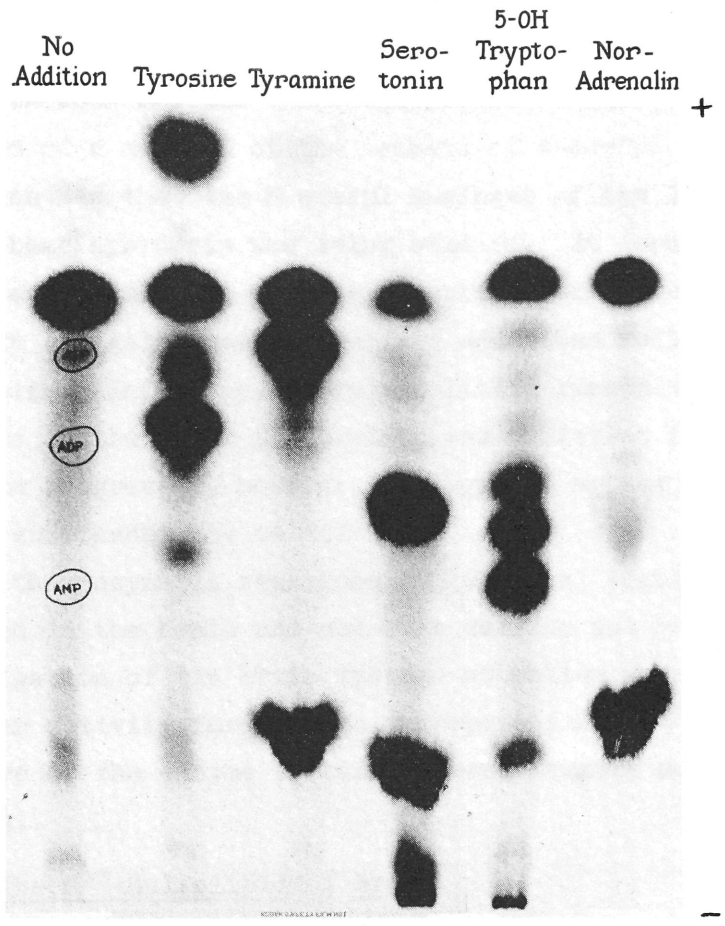


Fig. 36. See text for description.

The sulfurylation of various aminoalkyl phenols and related compounds.

DISCUSSION

At the time this work was initiated, the only sulfate-containing lipid to have been described was the cerebroside sulfate of Blix. One of the objects of this investigation was to delineate the sequence of sulfurylation in its biosynthesis. It soon became evident that cell-free enzyme preparations of rat brain and liver were able to transfer sulfate from PAPS into a lipid-soluble material. In both systems this transfer was stimulated by the addition to the incubation of a mixture of the isomers of N-acetyl sphingosine. A natural assumption was that the N-acetyl analogue of the Blix Compound was the substance whose synthesis was being studied. It seemed reasonable to suppose that the added ceramide was the precursor for the cerebroside sulfate-like material with the galactose and sulfate additions following acylation of the sphingosine. Initially, there was little reason to think that the sulfolipid formed in the liver preparation was different from that in the brain. As the work progressed, however, it began to appear more and more likely that the two substances may be different.

A detailed study of the enzymatic reactions and chemical identification of the sulfolipid produced in the brain has not been carried out because, after preliminary investigation of the brain system, attention was diverted to the liver; its greater activity for sulfate incorporation into lipid, as well as the soluble nature of the enzyme system rendered it more suitable for protein fractionation.

The S^{35}-Sulfolipid of Brain

Attempt at Characterization - Chemical identification of the brain S^{35}-sulfolipid has been limited to experiments on its acid degradation. At best, the quantity of labelled sulfolipid produced by this system is a fraction of a millimicromole, so that definitive chemical analysis is not feasible. Instead, indirect methods had to be resorted to. The results of the hydrochloric acid hydrolysis of the S^{35}-sulfolipid produced in the young rat brain homogenate are compatible with this substance being the N-acetyl analogue of the Blix Compound, but absolute proof is not yet available. The problem of identification is complicated by the fact that the ceramide, which is active in these preparations, is not one found in nature. Comparisons of physical properties of the natural cerebroside sulfate, which has a long-chained fatty acid, with the postulated N-acetyl analogue, therefore, are of limited value.

Biosynthesis of the Cerebroside Sulfate - The stimulation in the brain homogenate of sulfate transfer from PAPS to lipid by N-acetyl sphingosine and not by psychosine is of interest, especially in view of the recent report of Cleland and Kennedy (207). Evidence that D-erythro-sphingosine* is a precursor for psychosine (galactosyl sphingosine) and subsequently for cerebroside synthesis by rat and guinea pig brain microsomes has been presented by these workers. With the microsomal preparation N-acetyl-threo-trans-sphingosine was inactive as an acceptor for galactose from UDP-Galactose, although the whole homogenate did have such activity; the erythro ceramide showed little activity in either system. Upon fractionation, the activity for the threo ceramide apparently disappeared while that for erythro-sphingosine was enhanced. It seems that the galactose-accepting ability of the threo ceramide was associated with some other subcellular fraction, perhaps the mitochondria. As mentioned by these authors, "it is possible that psychosine is an intermediate in cerebroside synthesis, and ceramides the intermediates in the syntheses of more complex glycolipids." Our finding of activity for the ceramide in a brain mitochondrial fraction is compatible with such a hypothesis, although the data is, as yet, in a preliminary form. Despite their findings, Cleland and Kennedy caution that the failure to find significant activity for the ceramides in their system "may be due to the lack of proper emulsification of the ceramides, and that ceramides synthesized within the microsomes may be more active than those added to the external medium." This point cannot be over-emphasized.

If sulfatide formation does proceed by way of the ceramide and the cerebroside, then one might expect the latter to accept sulfate from PAPS in the brain system. The fact that it did not does not rule out this mechanism. Tween 20 in concentrations which are not excessively inhibitory for the enzymatic reaction, did not eliminate the solubility problem encountered with the natural cerebroside. For this reason, it would be of interest to prepare and test the more easily emulsified N-acetyl analogue of the cerebroside in this system. In view of the very small amounts of sulfolipid produced, and the crude nature of the enzyme preparation, the

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* As noted in Table XVII, in the erythro configuration the secondary hydroxyl and amino groups are cis, whereas in the threo configuration they are trans.

lack of a clear-cut effect on addition of the uridine nucleotides is not unexpected and does not detract from the suggested mode of synthesis:

ceramide $\xrightarrow{\text{UDP-Gal}}$ cerebroside $\xrightarrow{\text{PAPS}}$ cerebroside sulfate (sulfatide)

The S^{35}-Sulfolipid of Liver

In the liver system both enzymatic and chemical data indicate that a direct sulfurylation of the primary hydroxyl function of N-acetyl sphingosine takes place. This reaction appears to account for at least a part of the sulfate incorporated into lipid, but because of poor over-all recoveries in the periodate experiments, it cannot be said with certainty that the primary hydroxyl-conjugate is the only product, although there is no evidence suggesting otherwise. N-acyl sphingosine sulfate has not been described previously. Its existence in any appreciable amount in nature and its physiological significance are unknown. The liver is the master organ for ethereal sulfate formation and its role in "detoxication" is well known (234). It is possible that the sulfated ceramide represents such a product.

The facts that the brain enzyme is particulate but the liver enzyme is soluble, and that brain is unable to sulfurylate chloramphenicol support the notion that the respective sulfolipids are different. The inability of the brain to sulfurylate the chloramphenicol is significant in this regard, provided, as seems likely, the chloramphenicol and N-acetyl sphingosine sulfokinase are one and the same enzyme. The striking structural similarities of these two compounds, the finding of sulfate attached to the primary hydroxyl group of both, the close association of enzymatic activities and requirements for both, and, most convincing, the inhibition of sulfurylation of the lipid by D-erythro-chloramphenicol support such a contention. Indeed, the ability of the erythro chloramphenicols to compete with N-acetyl sphingosine for sulfurylation warrants the examination for competition in other reactions where the ceramides take part, such as sphingomyelin synthesis *in vitro* and *in vivo* as in Niemann-Pick's Disease, reticular and histiocytic sphingomyelinosis.

Relation of Stereochemistry and Solubility to Enzymatic Activity

There is one point that must be resolved. The erythro-chloramphenicols are much better sulfate acceptors than the threo isomers. The lipid studies, however, show the mixture of N-acetyl sphingosine isomers to be most active,

but of the two purified ceramides, the threo is much more active. The stereochemical requirements for enzymatic reactions involving sphingolipid substances in general - especially where the isomers differ greatly in solubility properties - is, at present, quite confusing. Sribney and Kennedy (184,188) have found the threo ceramides to be precursors in sphingomyelin synthesis by chicken liver particles, and have evidence that sphingomyelin thus formed is of the threo configuration, although only the erythro form is found in tissues. Whether or not the threo requirement for in vitro sphingomyelin synthesis is an artifact of the experimental conditions necessary for testing the enzyme is not known. The threo ceramides are more easily emulsified than the erythro compounds and may be detected as the active species simply because they have access to the enzyme surface. In an effort to get around this possibility, Sribney and Kennedy (184,188) found that with the acetylenic ceramides, which are more easily emulsified, the threo remains the active form. Nonetheless, a ready explanation for the synthesis of the unnatural threo sphingomyelin is not obvious. In our experiments, neither acetylenic compound is active. As already mentioned, there is another example of the superior activity of the threo compound, i.e. that it is the active species in accepting galactose from UDP-Galactose in brain homogenates (207).

In the sulfolipid experiments described in this paper, a simple explanation for the contradictory stereochemistry is not apparent. The different degrees of solubility and emulsifiability, even with the use of Tween 20, are so obvious to the naked eye that one is not at all surprised to find the order of activity paralleling the order of solubility within the ceramide group. From the chloramphenicol experiments it is seen that although the erythro compounds are much more active, the threo isomers have definite sulfate acceptor activity, provided that this low order of activity is not due to trace contamination with the erythro compound; this seems unlikely inasmuch as the D-threo is obtained from natural sources. Similarly, it is possible that there is a sulfurylating activity for both threo and erythro ceramides, but the former shows up as the more active of the two pure compounds because of its greater solubility. The greater activity of the crude lipid preparation than either of the purified isomers may be because of its increased solubility due to undefined "impurities". It is even possible that these "impurities" have contributed to the solubilization of the erythro

ceramide present in the isomer mixture, so that this "more natural" acceptor becomes the more active one.

The fact that the N-acetyl ceramide is active in this system, whereas the ceramides with longer-chain fatty acids are not, emphasizes the importance of solubility factors. The explanation, however, may be more complex. Since the N-acetyl analogue may not be found in nature, its reactivity in the liver system may have no counterpart among the natural ceramides. On the other hand, the much greater activity of the erythro than the threo chloramphenicols - where one is dealing with water-soluble reactants - may indicate that the erythro ceramide is the in vivo acceptor of sulfate from PAPS.

Metabolism of the Chloramphenicols

The sulfate ester of chloramphenicol has not been described previously. When natural chloramphenicol (D-threo) is administered by mouth to human subjects, only 5-10% of the administered dose is excreted unchanged in the urine. The bulk of the remainder is accounted for in the 24-hour urinary excretion as aromatic nitro compounds (223). Glazko et al (226) found the principal nitro compounds in the urine of the rat, dog, and man to be: 1) unchanged chloramphenicol, 2) the chloramphenicol base, and 3) the glucuronate conjugate of chloramphenicol. The glucuronic acid was found to be attached to the primary hydroxyl group of chloramphenicol. Although no other metabolites were identified, mention was made of the finding of a "faint spot" (as tested for the nitro group) that was close to the glucuronate band on paper chromatography of rat and dog urine; this might be the sulfate conjugate.

In the lower mammals, such as the rat and guinea pig, lesser amounts are excreted in the urine and more in the gastrointestinal tract by way of the bile. The experiments of Glazko et al (235) on the biliary excretion of the optical isomers of chloramphenicol in the rat indicate that the metabolism of the erythro compound differs from that of the threo. They found a large amount of glucuronate conjugate in the bile with all the chloramphenicol isomers, but in addition, with D-erythro chloramphenicol there was a second component with a mobility on paper chromatography corresponding to that of the free base of chloramphenicol. This has appparently not been further characterized. No mention was made of a sulfate-conjugate of the D-erythro compound.

Molecular Requirements for Sulfurylation of Chloramphenicol and Related Compounds

It is of interest that, with the important exception of the erythro-threo relationship, many of the molecular requirements for the sulfurylation of the chloramphenicols and related compounds are those that are important for the biological activity of the natural compound. The latter are summarized by Hahn et al (236):

1. Over 100 chemical variations of the acylamide side chain have been reported. It appears that one fact that determines the biological activity is the electronegativity of the acyl substituent. The less electronegative acetyl group produces a substance with only 14% of the natural biological activity, and the free base has only 1.8% of this activity (192). As noted in our studies, sulfurylation was also significantly lower in these cases.

2. Comparison of a group of differently para-substituted compounds reveals a proportionality between the electronegativity of the para-substituent and the antibiotic activity; Woolley (237) found only 6% of the original biological activity when hydrogen replaced the nitro group, and Smith and Worrel (238) found the amino analogue to be inactive. Similarly, in our studies a positive ρ has been found for the para-substituted benzyl alcohols.

3. The relation between the aromatic character of the ring system and biological activity was studied. No activity was found when the phenyl group was replaced by isopropyl, cyclohexyl or hydrogen (239), and 1.6% activity when replaced by 4-pyridyl (240). In the series of aliphatic alcohols with aromatic side group, it was likewise noted that the pyridyl derivatives had little to no activity for sulfurylation,and compounds containing no aromatic nucleus or unsaturation in the chain were inactive.

The sulfokinase for the chloramphenicols and for N-acetyl sphingosine does not appear to be the same enzyme as that for phenols or for dehydroepiandrosterone, for the reasons listed under Experimental. But it still may be that the sulfokinase studied here has some other substrate as its natural one, such as a steroid alcohol. The 21-hydroxy steroids (e.g. desoxycorticosterone) which are, in effect, primary aliphatic alcohols and have been shown to be sulfurylated, although rather poorly (54), have not been tested as substrates for this enzyme system.

In the system described in this paper, the erythro-chloramphenicols are much better sulfate acceptors than N-acetyl sphingosine. Although it

is not surprising to find an unnatural substrate such as the chloramphenicols to be more active than the "natural" N-acetyl analogue, it may be that within the environment of the cell the ceramide is the most active sulfate acceptor with this enzyme. Emulsifiability or water-solubility is undoubtedly an important factor in the different activites in the test tube.

Sulfurylation of other primary alcohols - Until very recently, reports of sulfurylation of primary alcohols have been limited to those of the polysaccharides - where carbon-6 of a hexose is conjugated with sulfate - choline, and the steroids mentioned above. In preliminary experiments, Boström and Vestermark (241) have found that the administration of aliphatic primary alcohols to rats leads to the excretion of the corresponding sulfate-conjugate in the urine. These workers have also reported evidence for the sulfurylation of these alcohols by rat liver supernate from ATP and inorganic $S^{35}O_4$ (242). Until the details of these experiments, especially the concentrations of the alcohols used, have been presented, these studies remain incomplete. In a more recent investigation, these same workers (243) have described the enzymatic sulfurylation of aliphatic polyols, such as the glycols and other diols in which the hydroxyl groups are separated by several carbons, e.g., 1,6-hexanediol. The concentrations of alcohol in the medium required for maximal sulfurylation vary from 0.1% to 5%. The very high concentrations used raise the question of non-enzymatic reactions; appropriate control experiments have not been reported. It may be of interest that the alcohol that was sulfurylated at the lowest concentration (0.01% for the appearance of a weak new spot) was 1,6-hexanediol, which, in the partially purified system at the concentration (1.0 μmole per ml. of incubation mixture) used in our experiments, was probably not sulfurylated at all. In any case, the chloramphenicol-ceramide system appears to be different from that described by the Swedish workers.

Possible Need for Additional Factors in Sulfate Transfer

There are several findings in the rat liver system that are not readily explained. 1) The stimulation by ATP of incorporation of $S^{35}O_4$ from $PAPS^{35}$ into lipid with all enzyme preparations except that obtained by DEAE-cellulose chromatography was discussed in the Experimental Section. Analogous experiments were not performed with the chloramphenicols or other sulfate acceptors. 2) The stimulatory effect of the second ethanol fraction for

sulfurylation of D-erythro-chloramphenicol and dehydroepiandrosterone (and perhaps to a lesser extent for p-nitrophenol) as well as for the lipid remains to be explained. In the lipid experiments an unsuccessful effort was made to detect the accumulation of intermediates by pre-incubation with this ethanol fraction. Whether this fraction takes a direct part in the sulfurylation sequence or acts simply by providing a more favorable environment for sulfurylation of the compound in question by the removal or modification of some competing or inhibitory substance is not known. These results bring to mind the experiments of Bernstein and McGilvery (25), and of Brunngraber (42) detailed in the Historical Section, which indicated the possibility of additional co-factors or enzymes being involved in phenol sulfurylation. The findings of Roy (82) that arylamine sulfurylation is stimulated by 17-oxosteroids in rat liver may indicate similar reactions with other sulfokinases, although he was unable to get this effect with the sulfurylation of phenol.

Sulfate Activation and Transfer in Marine Plants and Animals

Activation and Transfer to Polysaccharides - The generality of PAPS formation and its role as the carrier of active sulfate have been extended to cell-free extracts of the marine algae and the mucous gland of Busycon. In both systems evidence has been found for the incorporation of sulfate from PAPS into mucopolysaccharide. The characteristics of the enzymatic system in the mucous gland extract are such as to make it most unlikely that the mucopolysaccharide sulfurylation is due to the action of a sulfatase. That PAPS and not APS is the sulfate donor is indicated by the experiments in Busycon. If endogenous sulfate activation was required and phosphate was not included in the incubation, APS accumulated, presumably due to enzymatic dephosphorylation, and no sulfurylation of mucopolysaccharide occurred. As has been the case in other systems, with the snail gland extract no evidence could be found for the existence of sulfurylated intermediates in mucopolysaccharide synthesis.

Nature of the Endogenous Sulfate Acceptor in Busycon - The identity of the endogenous sulfate acceptor in Busycon gland remains to be elucidated. Available data strongly suggest that the compound and its sulfated product contain a free amino group. It may be that the sulfurylation occurs on a

phenolic hydroxyl. The presence of rather large amounts of pharmacologically active amines such as serotonin, tyramine, and octopamine (p-hydroxyphenylethanolamine) in the hypobranchial glands of the molluscs has been previously reported (244). Evidence is presented in this thesis that the endogenous compound in the Busycon gland is not serotonin, tyramine, or the adrenalines, but is a similar compound. Whether this compound occurs naturally in the Busycon gland, or is released in large quantities only on autolysis has not been determined.

Sulfurylation of Aminoalkyl Phenols - A number of aminoalkyl phenols or their metabolites could be sulfurylated in the snail preparation as well as in rat liver supernate where the pattern of sulfate conjugates appears, in general, to be more complex. The formation of tyrosine-o-sulfate could not be shown in either system when tyrosine was included in the incubation. Each system appeared to form different sulfurylated products on addition of this phenolic amino acid. With the snail preparation, only one sulfate-conjugate was produced; its behavior on paper electrophoresis at acid and alkaline pH's resembled that of tyrosine-o-sulfate, but the latter had a slightly faster mobility. In the snail, tyramine appeared to be sulfurylated on the phenolic hydroxyl, and in the rat liver two sulfurylated compounds appeared, one having a much faster mobility than the other. No further attempt has been made to characterize these compounds, but it does seem clear from this work that enzymatic sulfurylation, at least in vitro, is possible at several levels of degradation of these natural amines.

The possible importance of the sulfurylation reaction in regulating the metabolism of these physiologically active substances has not been explored. It is of interest in this regard that Roche et al (72) have suggested that the physiological role of the sulfuric acid ester of triiodothyronine may differ from that of the glucuronate-conjugate. They have discussed the possibility that the sulfate-conjugate might be a form in which the hormone is transported to the receptors or a reserve form of the hormone. At present, these are still speculations. The estrogen sulfates have recently been implicated as competitors of pyridoxal phosphate. Mason and Gullekson (245,246) have found the inhibition of pyridoxal phosphate-dependent enzymes (kynurenine transaminase, kyrureninase, and muscle phosphorylase) by rather low concentrations of the sulfate esters of several estrogens. Suggestive evidence for the possible physiological significance of the

inhibitory reaction has been obtained. Similarly, Christensen et al (247) have recently reported that the estrogen sulfates are able to stimulate the amino acid concentration by Ehrlich ascites tumor cells - a pyridoxal phosphate stimulated process.

In Vivo Sulfurylation in Porphyra

The experiments reported here on the in vivo assimilation of inorganic sulfate by photosynthesizing Porphyra umbilicalis are of a preliminary nature, but raise some interesting points for further study. The incorporation of $S^{35}O_4$ into the polysaccharide of the marine alga is light-dependent for at least the first 15 minutes. That this effect is not due to transport has not been proved but seems most likely. The incorporation of significant radioactivity into the sulfated mucopolysaccharide after two hours in the dark may be due to the energy provided for sulfate activation by substrate level phosphorylation. Because of the very small total quantity of $S^{35}O_4$ added in these experiments, relatively little energy would be required.

The formation of several alcohol-soluble sulfurylated compounds has been found in vivo. It is not known whether these are related in any way to the sulfated polysaccharides, either as intermediates for synthesis or as degradation products. Choline sulfate has been described in the red marine algae (85), but none of the radioactive substances produced in these experiments could be identified with this compound. The radioactive spot on the electrophoretogram which probably has no net charge and is acid-resistent, must represent a more reduced form of sulfur than sulfate. Taurine and the sulfur-containing amino acids are possibilities. In fact, Lindberg (85) has isolated taurine and N-methyl taurine from Porphyra umbilicalis. If the compound described by Benson et al (175) were formed in vivo by Porphyra, then one would expect to find on the electrophoretogram an anionic radioactive material, presumably galactose-sulfonate, that appeared during the course of the acid hydrolysis and persisted throughout the hydrolysis. Such was not found.

The sulfur and sulfate metabolism of the marine algae represents a rich and practically unexplored area in biology. Much of the research on the marine algae has been concerned with chemical analyses of the complex polysaccharides (132). Only very recently have studies been initiated on the biosynthesis of algal components from the enzymological point of view (248,249).

It is to be expected that in the future, much valuable information on the reactions of sulfurylation and sulfate reduction in these organisms will be forthcoming.

CONCLUSION

The study of the biochemistry of sulfate has only just begun. The unexpected has often been more common than the expected, and such will probably continue to be the case until the importance and diversity of sulfate in biology is more fully understood. There are two areas of sulfate metabolism in particular from which we may expect new and significant discoveries: the sulfolipids, and the sulfate-containing proteins. Research on these problems has barely begun. Although the sulfate moiety itself is an example of simplicity, its study is made difficult and interesting because of the complex nature of the compounds with which it is covalently linked. Much of the progress in this field will depend on the rate with which information becomes available on the chemistry and biochemistry of the remainder of the molecule. With the use of radioactive tracers we are able to find substances which are formed only in minute amounts in nature, and which might otherwise go undetected. Their exact chemical constitution and physiological significance may follow some time after the initial discovery.

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