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Alterations in Sulfolipid Metabolism in Malathion-Stressed Rats

Gail W. Disney
University of Tennessee, Knoxville

To the Graduate Council:

I am submitting herewith a dissertation written by Gail W. Disney entitled "Alterations in Sulfolipid Metabolism in Malathion-Stressed Rats." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Nutrition.

John T. Smith, Major Professor

We have read this dissertation and recommend its acceptance:

Mary Rose Gram, Frances A. Schofield, Ada Marie Campbell

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

November 17, 1972

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Vice Chancellor for
Graduate Studies and Research

ALTERATIONS IN SULFOLIPID METABOLISM IN MALATHION-STRESSED RATS

A Dissertation

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

Gail W. Disney

December 1972

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ABSTRACT

The effects of malathion stress on inorganic sulfur metabolism in female rats were investigated by extracting macromolecules containing ester sulfate from cellular lipoprotein. Sulfolipid, mucopolysaccharide, nucleotide, and sulfate transfer fraction were extracted from the lipoprotein prepared from five to seven pooled livers from rats fed diets containing 0.42, 0.10, and 0.0002 percent sulfate. Six groups of rats (two groups per diet) were fed the diets for 21 days. Rats were given either malathion in corn oil or only corn oil by stomach tube every 24 hours for three days and were killed on day 21. Lipoprotein was isolated from liver by exhaustive salt extraction. Determination of the specific activities of the macromolecules from lipoprotein by liquid scintillation counting revealed a statistically significant increase in $^{35}SO_4^=$ incorporation into sulfolipid of malathion-stressed rats as compared with controls fed the diet containing 0.10 percent sulfate. In general, malathion stress caused increases in specific activities of liver lipoprotein macromolecules. These increases were generally intensified by decreasing the level of dietary sulfate.

An investigation was initiated in order to identify the cellular component and the mechanism causing the increased $^{35}SO_4^=$ incorporation into sulfolipid observed in malathion-stressed rats. Total galactose, sulfate, and ^{35}S activity were determined in liver lipoprotein sulfolipid extracted from nuclei, mitochondria, and the residue from groups

of 16 and 20 pooled livers from malathion-stressed rats and controls, respectively. These rats were fed the diet containing 0.10 percent sulfate. Data indicated that nuclear sulfolipid was the component of lipoprotein most active in $^{35}SO_4^=$ incorporation and probably was responsible for the increased ^{35}S activity previously observed in cellular lipoprotein sulfolipid. The galactose:sulfate molar ratio in cellular lipoprotein sulfolipid from the cell particulates indicated that sulfate was being mobilized from sulfolipid.

Arylsulfatase activity was estimated in groups of three pooled livers from malathion-stressed rats and controls (five groups each) fed the diet containing 0.10 percent sulfate. A statistically significant increase in arylsulfatase activity per mg of nitrogen was found in liver homogenates of malathion-stressed rats as compared with controls. This finding provided further evidence for the mobilization of sulfate from sulfolipid from malathion-stressed rats.

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CHAPTER I

INTRODUCTION

The importance of inorganic sulfur to animals has been demonstrated in this laboratory (Nutrition Department, The University of Tennesee, Knoxville) by Button et al. (1) and by Michels and Smith (2). A study by Button et al. (1) showed that rats absorbed a major portion of the radioactive sulfate from dietary calcium or sodium ^{35}S-sulfate. The absorbed sulfate was utilized in sulfation of cartilage mucopolysaccharides. Michels and Smith (2) demonstrated that inorganic sulfur is needed in the diet of rats if the sulfur amino acid requirement is not to be increased.

Input into the natural sulfur cycle is being altered due to necessary efforts to decrease the amount of sulfur dioxide in the atmosphere. If this effort to rid the air of fossil fuel pollutants is carried to extremes, the natural sulfur cycle may be interrupted. Insecticides, another form of environmental stress, are directed primarily at insect pests but are also encountered by man and animals. Malathion is the most widely used organophosphorus insecticide for domestic application. Its classical effect is the inhibition of the enzyme acetylcholinesterase. Because malathion is one of the least toxic pesticides to animals, it is expected to replace the halogenated hydrocarbons such as DDT which are accumulated by the body. With increased use of malathion, there will be increased exposure and poisoning in man and animals. Proof that malathion, as a general esterase

inhibitor, interferes with normal sulfation in the rat has been shown in previous studies by Disney and Smith (3-5). Malathion-intoxicated rats had decreased $^{35}SO_4^=$ incorporation into rib cartilage mucopolysaccharide and increased mobilization of ^{35}S into urine and feces. Increased levels of dietary sulfate appeared to decrease the toxicity of malathion as indicated by decreases in the mobilization of ^{35}S into excreta. Increased exposure of man and animals to organophosphorus stress coupled with decreased sulfate intakes due to interruption of the natural sulfur cycle could result in a critical situation; therefore, the relationship of malathion stress to inorganic sulfur metabolism seemed to merit further study. Since previous investigations (3-5) have shown a relationship between malathion intoxication and dietary sulfate which could not be totally explained with data obtained from tissue studies, this investigation was undertaken to determine if the relationship between dietary sulfate and stress could be demonstrated with selected cellular macromolecules.

CHAPTER II

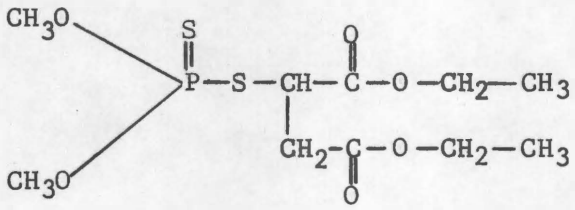
REVIEW OF THE LITERATURE

Preliminary investigations in this laboratory of liver lipoprotein and its fractions revealed that only the sulfolipid fraction is significantly affected by the stress of malathion intoxication. Therefore, a review of the literature to support this dissertation has been restricted to a discussion of malathion and sulfolipid metabolism.

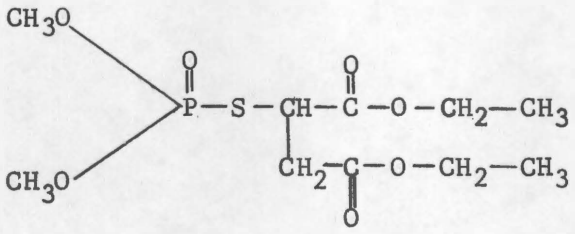
A. MALATHION

History

Malathion (Figure 1) is the least toxic member of a group of insecticides called the organophosphorus insecticides (6). Malathion was first used by the Germans in 1936 (7). The most firmly documented trait of organophosphorus insecticides is their ability to inhibit acetylcholinesterase (ACE), an enzyme essential for the proper functioning of the central nervous system. The use of ACE inhibitors as poisons is not a recent innovation. Calabar beans, seeds from the vine Physostigma venenosum Balfour, have been used for centuries as ordeal poisons in West African witchcraft trials. Feeding calabar beans generally served as a fair judge and jury because the guilty ate slowly, absorbed the poison and died while the innocent ate rapidly and regurgitated due to gastric irritation. Eserine (physostigmine), a cholinesterase inhibitor, was found to be the active ingredient in 1926, thus paving the way for



Malathion



Malaoxon

Figure 1. The structures of malathion and malaoxon.

the development of synthetic ACE inhibitors (7). Malathion was made available for experimental use by the American Cyanamid Corporation in 1950 (8). It is now one of the most widely used insecticides because of its unique ability to be of high toxicity to insects but of relatively low toxicity to mammals. In addition to species differences in toxicity, malathion intoxication also varies considerably with route and frequency of administration and with previous history of exposure to other chemicals. A review of the literature on intoxication variables has been previously prepared by Disney (4); therefore, this section of the review will deal primarily with topics under discussion in the literature since 1967.

Mechanism of Action

ACE is an enzyme essential for the proper functioning of the central nervous system. Acetylcholine is released at parasympathetic nerve endings and acts as a transmitter on the effector organ. Before another impulse can be transmitted, acetylcholine must be hydrolyzed by ACE to form acetic acid and choline (9). If acetylcholine is not hydrolyzed, there is continual transmission of the impulse resulting in muscle spasms, convulsion, and eventually death.

ACE has two adjacent binding sites for the substrate: one is anionic and normally binds the cationic quaternary nitrogen of choline; the other is an esteratic site consisting of a serine residue and another nucleophilic group (imidazole of a histidine residue) which binds the acetyl portion of the substrate. Choline is released leaving an

acetylated esteratic site. The acetyl group reacts with water to produce acetic acid and regenerate the enzyme (9).

During malathion intoxication, malaoxon (the oxygen analog of malathion), is thought to bind with the esteratic site of the enzyme (7). In an oxidation reaction occurring in liver microsomes, the double-bonded sulfur of malathion is replaced with oxygen to form malaoxon (10) (Figure 1, page 4). When malaoxon rather than malathion was administered to mice, the malaoxon was 20 times more toxic than malathion (11). Main and Braid (12) found that the oral LD_{50} of secondary standard malathion (96.8 percent pure) is 3.7 times as high as that of technical grade malathion (95.6 percent pure). They suggested that the technical grade may contain esterase-inhibiting malathion impurities from which the standard grades of malathion are free. This esterase-inhibiting impurity is believed to be malaoxon. Exposure to heat, air and ultraviolet light promotes the oxidation of malathion to malaoxon (13); therefore, the conditions under which the chemical is stored can greatly influence the LD_{50} level obtained.

There is some controversy as to whether the esteratic site on the enzyme is phosphorylated, alkylated, acylated or a combination of these. Main and Hastings (14) concluded that blockage of this site is primarily by phosphorylation. These investigators were studying the binding of analogues of malaoxon to cholinesterase by substituting methyl, n-propyl or n-butyl groups for the ethyl group of the diethylsuccinyl portion (acyl portion) of the molecule. They investigated the possibilities of phosphorylation and acylation. They did not consider alkylation (the

binding of the dimethoxy portion of the molecule). Consideration of binding energies suggested that one of the two acyl groups of malaoxon may bind to cholinesterase. This finding was supported by an investigation by Hassan and Dauterman (15) who found that the configuration of the diethylsuccinyl residue affects the affinity of the enzyme for the substrate. The d-isomer of malaoxon was a more potent inhibitor of ACE than was the l-isomer.

Although the diethylsuccinyl residue affects inhibition of acetylcholinesterase, there seems to be general agreement at this time that the dimethyl phosphoryl residue is most tightly bound to the enzyme (7, 16). In recent literature, the dimethyl phosphoryl residue is referred to as the "remaining group" and the diethylsuccinyl residue as the "leaving group" (15,16). The acyl residue is thought to be released when a stronger bond between the enzyme and the dimethyl phosphoryl residue is formed (7), but Main and Hastings (14) found that serum cholinesterase could not hydrolyze the carboxylic esters of malaoxon and that one of the two acyl groups binds to the active site; therefore, the portion released is not clearly understood.

The binding of organophosphorus insecticides such as malathion with ACE appears to be a combination of phosphorylation and alkylation with the phosphorus atom of malathion serving as the main link with the esteratic site of the enzyme (7,16). This linkage is in contrast with the dominant attraction of the sulfur atom of thioether carbamates for the anionic site of the enzyme (17). The carbamates and the organophosphorus insecticides are the two groups of pesticides which

act primarily by inhibiting ACE in the central nervous system (7). Zahavi et al. (18) have used analogues of the "remaining group" to study the esteratic site of organophosphorus sensitive and insensitive acetylcholinesterases of mites. These investigators concluded that the esteratic site of the sensitive enzyme is wide enough to accomodate the 0,0-dimethyl phosphoryl residue of malaoxon but not wide enough to accomodate the diethyl analogue. The esteratic site of the organophosphorus insensitive acetylcholinesterase was found to be too narrow to accomodate even the 0,0-dimethyl phosphoryl residue.

Parathion, another organophosphorus insecticide, is 20 to 30 times more toxic than malathion as judged from LD_{50} levels (19,20). Chiu et al. (16) determined affinity constants (Ka) and phosphorylation constants (kp) for a series of 0,0-dialkyl malaoxons and paraoxons in which the phosphorylalkoxy groups were varied from methyl to butyl. The Ka values were from two to ten times higher for the paraoxon series than for comparable members of the malaoxon series meaning that members of the paraoxon series bind to the enzyme more tightly than members of the malaoxon series. This finding explains the reason for the greater toxicity of paraoxon, the oxygen analogue of parathion.

Metabolism

Malathion is hydrolyzed by an enzyme found in rat liver and serum (20) and human liver (12). Cook and Yip (20) called the enzyme malathionase. They found that in vitro hydrolysis of malathion results in the formation of a monoacid derivative of malathion formed by the

removal of one ethyl group from the diethylsuccinyl residue of the molecule. Main and Braid (12) partially purified an enzyme from rat liver which hydrolyzed one of the carbethoxy groups of malathion. It was characterized as carboxylesterase or carboxylic-ester hydrolase (EC. 3.1.1.1). Data obtained by Main and Braid (12) suggest that carboxylesterase is capable of hydrolyzing the carboxyl esters of malaoxon as well as of malathion. They found the monoacid derivative to be non-toxic. Through the use of nuclear magnetic resonance and infrared spectroscopy, Chen et al. (21) concluded that only the α-monoacid is produced biologically.

Bourke et al. (22) isolated four urinary metabolites of malathion. Twenty-four hours after a dose of 125 mg/kg of malathion to rats, 83.44 percent was excreted in the urine. Analyses of body tissues revealed no unmetabolized malathion. When administered in corn oil, only 7.75 percent was unabsorbed at the time of death. Metabolites of malathion appeared in urine within the first two hours after administration; therefore, malathion must be degraded shortly after ingestion.

Organophosphorus insecticides are degraded predominantly by hydrolysis to less toxic products. Other reactions (primarily oxidative) occur, but hydrolysis is better understood (23). Recently, Miyata and Matsumura (24) detected four organophosphate degrading enzymes from the supernatant fraction of rat liver. Three of these enzymes were capable of hydrolyzing malathion: (a) the previously mentioned carboxylesterase, (b) a glutathione stimulated dealkylation enzyme, and (c) a desmethyl phosphatase not stimulated by glutathione.

The latter two enzymes explain the findings of metabolites other than monoacids in the urine of rats.

Sakai and Matsumura (25), by starch gel electrophoresis, isolated four esterases from mouse brain which had strong hydrolytic activity for the carboxylester site and five bands possessing the ability to degrade the malathion molecule at the methoxyphosphate site. These esterases would correspond respectively to types (a) and (c) detected by Miyata and Matsumura (24).

Malathion-esterases are inhibited by prior dosing with malathion itself or when malathion is administered simultaneously with several other pesticide compounds. The discovery of malathionase in liver by Cook et al. (26) came as a by-product of work initiated to search for an explanation of the potentiation of malathion intoxication by EPN (p-nitrophenyl thionobenzene phosphonate). Cook et al. (26) found that EPN inhibited the degradation of malathion by blocking the hydrolytic action of malathionase on malathion. Murphy (27) found that levels of malathion below those which inhibit brain or erythrocyte cholinesterase will inhibit hydrolysis of malathion. After single large doses of malathion, inhibition of malathion-esterase occurred sooner and persisted longer than cholinesterase inhibition. Results of several in vivo and in vitro experiments suggested that a metabolite of malathion (possibly malaoxon) was the actual inhibitor of malathion-esterase. Murphy and Cheever (28) found that dietary concentrations of malathion, which were less than those required to inhibit erythrocyte or brain cholinesterase, inhibited liver and plasma carboxylesterases which

degrade malathion, diethyl succinate or triacetin.

Results of a study by Cohen and Murphy (29) indicate that inhibition of binding of malaoxon to malathion-esterase leaves malaoxon free to inhibit ACE. They state that binding of malaoxon to noncritical tissue constituents can spare critical ACE and thus serve as a mechanism of protection against poisoning. This study suggests that malathion-esterases may act as noncritical binding sites for malaoxon inactivation. In in vitro studies, Hassan and Dauterman (15) found that malaoxon is the better inhibitor for malathion-esterase, but malathion is the better substrate for the enzyme. The inhibition of ACE and malathion-esterase by malaoxon probably overrides the detoxication of malathion in vivo. Hassan and Dauterman concluded that the most important feature governing the acute toxicity of malathion in mammals is the rate of inhibition of ACE and malathion-esterase by malaoxon. These rates are in turn dependent on the rate of oxidation of malathion to its P=O analogue, malaoxon.

In addition to the inhibition of malathion-esterases and ACE, malathion (probably as malaoxon) has been indicated as a general esterase inhibitor (30,31). Organophosphates have been shown to block a number of other hydrolytic enzymes including pseudocholine esterase, lipase, other esterases, trypsin, and chymotrypsin (32). All of these enzymes are capable of hydrolyzing carboxylic acid esters as does malathionase (carboxylesterase). They are ali- or B-esterases. Read and McKinley (33) state that there is ample evidence to show that most of the ali-esterases are inhibited by organophosphorus compounds.

After analyzing liver and kidney esterases in several mammals, they concluded that livers in general have greater ali-esterase content than kidney. Bull and human liver had the greatest ali-esterase content. Arylesterases (A-esterases) are not thought to be inhibited by organophosphates (34) but are known to hydrolyze organophosphates (35). Bergmann et al. (35) discovered a new type of esterase in hog kidney and called it C-esterase. C-esterase neither hydrolyzes organophosphates nor is inhibited by this group of compounds.

Effects Other than Inhibition of Acetylcholinesterase

Probably related to the ability of organophosphates to be multi-esterase inhibitors are effects other than ACE inhibition. One of these effects is called "delayed neurotoxicity" which is a limb paralysis occurring several days after exposure. Aldridge et al. (36) found that hens given malathion became weak but recovered within a month, while other organophosphates caused permanent limb paralysis. Prophylactic treatment with drugs known to relieve ACE inhibition did not prevent paralysis (37,38). Johnson (39) concluded that organophosphates phosphorylate a specific esterase in the nervous system soon after administration. The esterase was not specifically identified. The site for inhibition has been shown to be in the spinal cord where the clinical lesion occurs (37). Barnes and Denz (40) studied delayed neurotoxicity in rats and rabbits and concluded that the ability of organophosphates to cause demyelination is not solely or directly related to their ability to inhibit ACE.

Some of the organophosphates have produced teratogenic effects when fed to dams during the developmental period of the fetus (41). At levels tolerated by the dams, parathion, diazinon and tepa caused a great number of resorptions when administered intraperitoneally on the 11th day of pregnancy. At higher levels producing toxic symptoms in the dams, fetal malformations, resorptions and reduced birth weight occurred. Malathion at toxic dosages neither affected the weight of the fetuses nor produced malformations.

Ho and Gibson (42) found malformations in chicks when eggs were injected with 0.1 ml of 2 percent malathion in corn oil on the fifth day of incubation. The embryos had a generalized reduction in body size, delayed patterns of mineralization in certain endochondral bones and a reduction in the size of one or more limbs. Degenerative necrosis of the cartilage within both proximal and distal epiphyses was observed. The epiphyses, particularly the distal epiphysis, showed a progressive degeneration of the chondrocytes and a reduction in the amount of ground substance and in the degree of sulfation of the mucopolysaccharide component. Radioautographic studies by Gill and LaHam (43) showed that less ^{35}S was incorporated into chondroitin sulfate of the cartilage ground substance of malathion-treated embryos. Gill and LaHam observed that the mesenchyme tended not to differentiate into chondroblasts in these embryos, and if differentiation did occur, the chondroblasts were unable to synthesize a normal amount of ground substance. Thus, malathion produced a double effect: a partial block of differentiation and inability of those cells that appeared to

differentiate to carry out normal chondroitin sulfate synthesis. Upshall et al. (44) found that levels of acetylcholine in developing hen eggs do not appear to be related to the degree of teratogenesis, indicating that ACE and the cholinergic system do not play a major role in the differentiation processes involved in organophosphate-induced teratogenesis.

Prior to the finding of a relationship between malathion intoxication and chondroitin sulfate synthesis by Ho and Gibson (42) and Gill and LaHam (43), Disney and Smith (3-5) found that $^{35}SO_4^=$ incorporation into cartilage mucopolysaccharide was more depressed by malathion intoxication than was the activity of brain ACE. Disney (4) also found evidence that high levels of inorganic sulfur decrease the toxicity of malathion. Roe (45) has shown that inorganic sulfur in the diet of rats can be used in the detoxication of indoles. Indole-fed rats receiving sulfate supplements excreted more indican and gained more weight than the indole-fed controls. Boyd and Tanikella (46) and Boyd et al. (47) found that increased protein (casein) in the diet of rats increased the LD_{50} level of malathion. In relation to the studies of Disney and Smith (3-5), this finding is probably explained by the fact that increased protein would supply increased amounts of sulfur amino acids. Michels and Smith (2) found evidence for a relationship between dietary levels of organic and inorganic sulfur in the diet of rats. These investigations have shown that inorganic sulfur has a sparing effect on sulfur amino acids as evidenced by the fact that low inorganic sulfur

raised the methionine requirement of the rat.

Human Malathion Intoxication

Human malathion intoxication is not encountered as frequently as is intoxication by parathion or other more toxic pesticides, but the incidence of malathion poisoning is expected to increase as it replaces halogenated hydrocarbons such as DDT for general use. During the encephalitis epidemic in Corpus Christi, Texas, in the fall of 1966, malathion was applied by aerial spraying over the city and its outskirts to control the mosquito vector. In a group of 119 volunteers who received varying degrees of exposure, there was a 5 percent incidence of mild and transient symptoms such as headache, nausea and weakness. There was no correlation of symptom frequency or severity with ACE activity. The investigators, Gardner and Iverson (48), concluded that there is negligible risk to human health involved in aerial applications of malathion. The risk of poisoning is decreased by adequate covering of the skin. Wolfe et al. (49) found that there is greater potential for human malathion poisoning through dermal exposure than through respiratory exposure.

Alleviation of Intoxication

Drugs have been developed which will reactivate inhibited ACE. During inhibition, ACE acts as a nucleophile, and the hydroxyl group of serine in the esteratic site is phosphorylated forming the enzyme-substrate complex (50,51). The "leaving group" is released and, in the case of organophosphates, a dialkylphosphate group remains. The

necessary criterion for a compound to overcome the inhibition is a strongly cationic group at a significant intramolecular distance from a nucleophilic group (50). The oximes were developed to fit this description; of these, 2-pyridine aldoxime methiodide (PAM) is the most effective (Figure 2). At low concentrations, PAM rapidly removes the dialkylphosphate group from the esteratic site and restores enzymic activity (8,50). Spontaneous loss of one alkyl group from the bound dialkylphosphate makes reactivation much more difficult (6). This event is called "aging" of the enzyme-substrate complex (52). Atropine is often given simultaneously with PAM, but atropine is not specific for organophosphorus poisoning as is PAM. Atropine helps to relieve the symptoms of poisoning by acting as a respiratory stimulant and a muscle relaxant (53).

Drugs can be given prophylactically to prevent poisoning. Quinby (54) administered one gram of PAM three to four times per week for up to 22 weeks to 13 men working in a formulating plant in Washington. There were no signs of intoleration to PAM, and erythrocyte ACE levels were higher during the treatment period.

Detection of Organophosphorus Poisoning

The classical method of detecting organophosphorus poisoning is by monitoring ACE activity, but other methods are being devised. Shafik and Enos (55) have measured the level of alkylphosphates excreted in the urine of humans as an estimate of organophosphorus exposure. Mattson and Sedlak (56) have measured urinary phosphorus metabolites

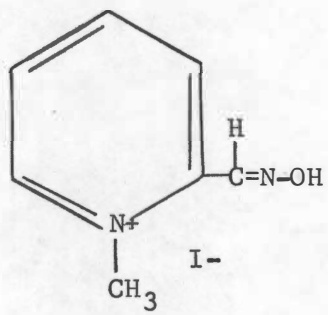


Figure 2. The structure of 2-pyridine aldoxime methiodide.

extractable with organic solvents in persons exposed to malathion. Durham et al. (57) found that the measurement of urinary p-nitrophenol is a valid procedure for determining human exposure to parathion. Bunyan et al. (58,59) have identified the type of organophosphorus poisoning in pheasants through the development of esterase electrophoregrams. The pesticide residue is extracted from tissue and added to a standard tissue extract with subsequent demonstration of typical inhibition in the resulting starch gel electrophoregram.

B. SULFOLIPID

Structure

Sulfolipid (sulfatide) is now thought to be composed of ceramide linked to a sulfated mono- or dihexose molecule (Figure 3) (60,61). Galactose was once thought to be the only hexose found in sulfolipid (62). Svennerholm (63) found this to be the case in human fetal brain, and Mårtensson (64) has found a sulfate-dihexose consisting of glucose and sulfated galactose in human kidney. Mårtensson (65) found two sulfatide fractions in human kidney: monohexose(galactose)-sulfatide with the same structure as brain sulfatide, and dihexose-sulfatide. On the basis of hydrolysis and periodate oxidation studies, dihexose-sulfatide is proposed to be galactosyl-glucosyl-ceramide esterified with sulfuric acid in the galactose moiety. Monohexose-sulfatides were isolated from kidneys in approximately three times the concentration of dihexose-sulfatides. There may possibly be other types of sulfated-galactolipids in tissues since Mårtensson (66) revealed the presence of the following

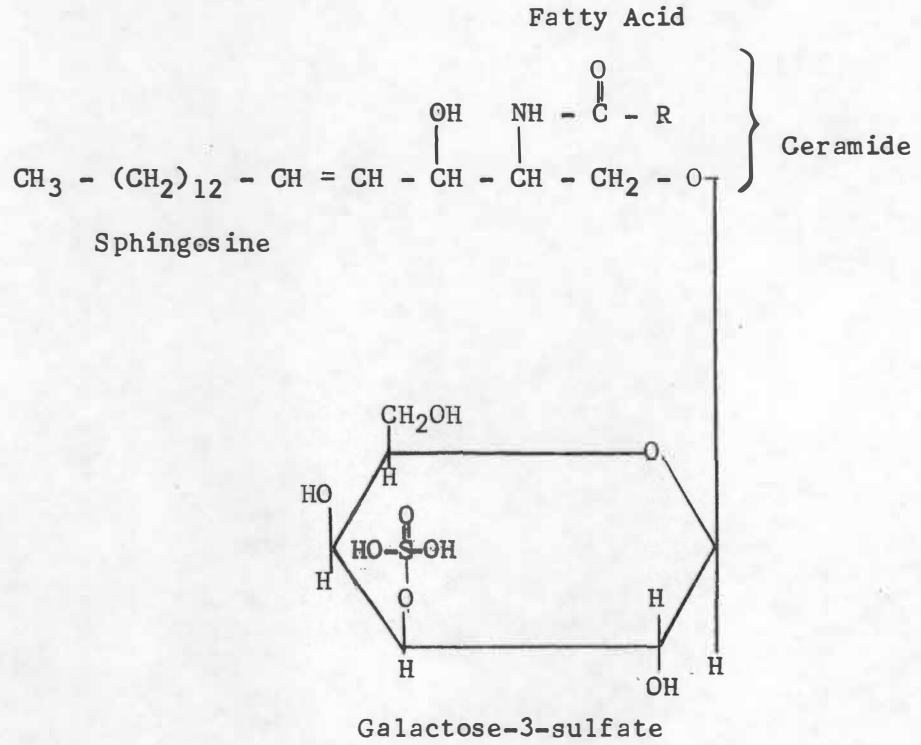


Figure 3. The presently accepted structure of monohexose-sulfatide.

glycolipids in human kidney (listed in decreasing order of concentration): aminoglycolipids, ceramide-trihexosides, ceramide-dihexosides and ceramide-monohexosides. He confirmed that the ceramide-dihexosides are a mixture of ceramide-lactosides and ceramide-digalactosides, and the ceramide-monohexosides a mixture of almost equal amounts of gluco- and galactocerebrosides.

These two studies by Mårtensson (65,66) demonstrate that the relative concentrations of glycolipids in tissues do not necessarily parallel the concentrations of the corresponding sulfolipids. In kidney, the monohexose sulfolipids were found in the greatest amount; conversely, the monohexose glycolipids were found in lowest concentration of the four glycolipids isolated. In brain, the galactocerebrosides are essentially the only neutral glycolipids and galactosulfolipids are the primary sulfolipids; therefore, the distributions of glycolipids and sulfolipids appear to be specific for different organs (66).

A consistent finding in all tissues is the location of the sulfate on the galactose moiety of the sulfolipid (62,65). Sulfolipids were once thought to be sulfated on carbon six of the galactose moiety (67). Yamakawa et al. (61) concluded that the sulfate was on the third hydroxyl of galactose based on methylation studies and infrared absorption spectra. Stoffyn and Stoffyn (68) confirmed this finding also using infrared spectra.

The fatty acid residue of sulfolipids is a long-chain fatty acid usually 18 to 24 carbons in length. Kishimoto and Radin (69) found

18:0 and 24:0 fatty acids in brain sulfatides. Mårtensson (65) found 22:0, 24:0 and 24:1 fatty acids predominating in human kidney. Pakkala et al. (70) studied very long chain fatty acids in human brain. They found similar amounts of fatty acids over 26 carbons in length in ceramides and sulfatides.

Biosynthesis

In 1960, Jatzkewitz (71) proposed that sulfatides are direct precursors of cerebrosides. Conversely, in 1964, Hauser (72) concluded from the relative pool size of cerebrosides and sulfatides in rat brain, that cerebrosides must be precursors of sulfatides. At the present time, there seems to be no doubt that sulfatides are synthesized by sulfation of a cerebroside, but there is still controversy concerning the pathway for cerebroside synthesis. Cerebroside formation may occur via two alternate pathways:

I. galactosylation of sphingosine, followed by N-acylation
   A. sphingosine + UDP-galactose → psychosine + UDP
   B. psychosine + acyl-CoA → cerebroside + CoA

II. acylation of sphingosine followed by galactosylation
   A. sphingosine + acyl-CoA → ceramide + CoA
   B. ceramide + UDP-galactose → cerebroside + UDP

Cleland and Kennedy (73) found an enzyme for pathway I, galactosyl-sphingosine transferase, in microsomes from the brains of guinea pigs and young rats. Since they could find no enzyme for pathway II, they concluded that cerebroside synthesis must proceed via psychosine.

Brady (74) followed cerebroside synthesis from psychosine and stearyl-1-^{14}C-CoA. Virtually all the radioactivity was present in the fatty acid portion of cerebroside. Sphingosine had some ability to replace psychosine. This ability was increased by the addition of UDP-glucose. Brady concluded that the psychosine pathway is the major route for cerebroside synthesis.

Morell and Radin (75) found proof for the existence of the second pathway for cerebroside synthesis. They found that a crude microsomal fraction from the brains of young mice catalyzed the formation of galactosylceramide from ceramide and UDP-galactose. The enzyme system exhibited high specificity: ceramide-containing nonhydroxy fatty acids would not stimulate galactose incorporation and only UDP-galactose or a UDP-galactose generating system acted as a sugar donor. Morell et al. (76) when testing the first pathway, could not observe psychosine acylation, but could demonstrate galactosylation of sphingosine.

Once cerebroside is formed, there seems to be no doubt concerning the mechanism of sulfation. Goldberg and Delbrück (77) were the first to demonstrate that 3'-phosphoadenosine-5'-phosphosulfate (PAPS) acts as the sulfate donor in forming sulfolipid. Goldberg and Delbrück observed the incorporation of $^{35}SO_4^=$ from ^{35}S-PAPS into sulfolipid of young rat brain and liver homogenates. Balasurbamanian and Bachhawat (78) found that cerebroside could be sulfated only when bound to a protein, presumably an enzyme. In 1965, McKhann et al. (79) found an enzyme in rat brain microsomes necessary for sulfatide formation. Addition of exogenous cerebroside resulted in a three-fold increase in

^{35}S-sulfate incorporation into sulfatide. They called the sulfate-transferring enzyme galactocerebroside sulfokinase. Farrell and McKhann (80) found that this enzyme would also catalyze the transfer of sulfate to lacto-cerebroside and psychosine, further indicating the possible existence of two biosynthetic routes for cerebroside.

Tissue Distribution

There is an integral relationship between sulfatide and myelin synthesis. McKhann and Ho (81) found that the in vivo incorporation of ^{35}S into sulfatide is maximal in rats 15 to 20 days after birth. The onset of ^{35}S incorporation on the tenth day correlates with the histological appearance of myelin. Sulfatide is a component of membranous structures of the central nervous system other than myelin, but 70 to 80 percent of brain sulfatide is in this tissue. Peak galactolipid sulphotransferase activity corresponded to the peaks for myelin and sulfatide formation. All three factors began to increase on the tenth day after birth of the rat and peaked on the 20th day. In an earlier study, Davison and Gregson (82) also found maximum uptake of ^{35}S into lipid from 15 to 20 days after birth in rats. In humans, Davison and Gregson (82) found no significant differences in brain sulfatide content of either cerebral grey or white matter after age 30. They found that approximately 50 percent of total brain sulfatide is found in myelin. Their results suggest that sulfatide is a characteristic membrane lipid. These results have been confirmed by Galli and ReCecconi (83) and Suzuki et al. (84).

Myelin and sulfolipid are so integrally related that a study of myelination can be conducted simply by isolating sulfatide. Chase et al. (85) have found that the synthesis of sulfatide can be used as a biochemical marker for myelin formation. McKhann et al. (79) have also suggested that galactocerebroside sulfokinase may be used as a biochemical marker for myelination. Pritchard (86) studied in vivo labeling of sulfatides by ^{35}S in rat brain during early growth. As a result of studying four areas of the brain, he concluded that the myelin sulfatides of each area contained the major portion of the ^{35}S label.

Herschkowitz et al. (87,88) concluded that sulfatide is synthesized in microsomes and then transported to myelin membranes via a water soluble sulfatide-protein complex. Suzuki et al. (84) found that from 10 to 30 days after birth of the rat, there is a 55 percent increase in brain weight, a 200 percent increase in total brain lipid, and a 2100 percent increase in brain sulfatide. These increases seem to be due to an increase in total cell numbers rather than cell size or content. Mandel and Bieth (89) have shown that, in the rat, DNA increases after birth until the 15th day. In a study of rat brain neurons and astrocytes, Norton and Poduslo (90) found that lipids increase rapidly with age in whole brain but remain low in isolated cells, suggesting that the increase in lipid is due to an increased number of cells.

Myelin is found in brain white matter where it constitutes 40 percent of the dry weight of the tissue (91). The dry weight of rat (92) and human (93) brain myelin is approximately 80 percent lipid. This lipid is a very important constituent of brain since Cuzner et al.

(94) have found that, based on dry weights, myelin constitutes 36 percent of the total brain weight. McIlwain (95) states that white matter of rats is composed of 1.2 percent sulfatide while gray matter is 0.2 percent sulfatide. Norton and Autilio (96) found that bovine myelin lipid consists of about 3.0 percent sulfatide. Cuzner et al. (94) have shown that the concentration of sulfatide is greater in myelin than in whole brain when values are expressed as μmoles per mg dried lipid. Relating specific data from studies concerning total and component brain lipids is difficult since values are rarely stated on the same basis, but comparisons do give a concept of the location and concentration of sulfolipid in the brain.

Sulfolipid has been by far most frequently studied in brain, but other tissues have received some attention for sulfolipid studies. Mårtensson (65,66) isolated and identified monohexose- and dihexose-sulfatides in human kidney. Green and Robinson (97) studied the distribution of sulfolipid in brain, kidney and liver of the rat. Sulfolipid was found in greatest concentration in the mitochondria of brain [the fraction from which purest myelin is obtained (91)], in the microsomes of kidney, and in the soluble fraction of liver. Goldberg (98) found that enzymatic sulfation activity was greatest in a particulate, probably mitochondrial, fraction in young rat brain, whereas, in liver, a high speed supernatant fraction of the homogenate was active. The location of the enzyme corresponds with the location of sulfolipid found by Green and Robinson (97). In descending order of amount, Green and Robinson (97) found sulfolipid in rat liver in supernatant fluid, nuclei, microsomes and mitochondria. Spiro and McKibbin (99) found the

greatest amount of sulfolipid in the microsomes of rat liver. Getz et al. (100) found rat liver to contain approximately twice as much total lipid in microsomes as in mitochondria. Eichberg et al. (101) found more sulfolipid in microsomes of guinea-pig brain than in mitochondria; therefore, the distribution of sulfolipid does differ for different organs, but there is not enough agreement in the literature to establish a definite pattern for each organ at this time.

Turnover

Turnover of brain sulfolipid is believed to be very slow. By administration of ^{14}C-galactose and ^{35}S-sulfate to rats, Radin et al. (102) found no breakdown in sulfolipid in rat brain while cerebrosides underwent slow turnover. Heald and Robinson (67) concluded that brain sulfatide is metabolically inert. Green and Robinson (97) found that sulfatide turned over rapidly in all organs analyzed except brain. In brain, the maximum incorporation of an intraperitoneal injection of ^{35}S was at 48 hours. The level of brain radioactivity then remained constant until 16 days after injection. On day 34, three-fourths of the maximal uptake remained. In liver, spleen and heart, maximal labeling occurred at 12 hours. Only a small fraction of the ^{35}S incorporated into these tissues was present at 48 hours, and after 4 days, the radioactivity had virtually disappeared. In kidney, ^{35}S incorporation was maximal at 24 hours, and measurable amounts remained 32 days after injection. Green and Robinson (97) suggested that brain sulfatide ^{35}S incorporation is only by de novo synthesis, while in other tissues such

as liver, there is rapid sulfate exchange. Davison and Gregson (103) found the half-life for rat brain mitochondria sulfatide to be 38.6 days while this fraction in liver had a half-life of 10.3 days. Cuzner et al. (104) found that the half-life of rat brain sulfatide is over 100 days.

Several investigators have suggested that brain sulfatide is composed of two metabolic compartments: (a) a small and rapidly exchanging compartment and (b) a large and slowly exchanging compartment (105). Davison and Gregson (103) have estimated the fast pool to be equal to about 0.2 percent of the total myelin sulfatide. Fleischer and Rouser (105) suggested that myelin is the chief representative of the metabolically stable pool while mitochondria primarily compose the labile pool. Myelin was found to be associated with a large amount of cholesterol as compared with mitochondria. Eng and Smith (106) found that slowly metabolized lipids (ethanolamine phosphatide, sulfatide, cerebroside, and sphingomyelin) exist in myelin as a cholesterol complex while the more labile lipids (inositol phosphatide, lecithin, and serine) are found in an uncomplexed form. Autilio et al. (91) and Norton and Autilio (96) found that myelin lipids have more cholesterol and less phospholipid than whole white matter lipid. Eng and Smith (106) suggest that association with cholesterol may be responsible for increased stability of myelin.

Malnutrition and Sulfolipid Synthesis

Chase et al. (85) found that myelin sulfatide synthesis was markedly decreased in malnourished rats. If rats are malnourished

until after the onset of myelination, there is no significant increase in the rate of sulfatide synthesis with ad libitum feeding. During vitamin A deficiency, Clausen (107) observed a statistically significant decrease in sulfatide synthesis accompanied by a heavily depressed formation of PAPS, but Kean (108) found no effect of vitamin A deficiency on sulfatide formation. This controversy is very similar to the controversy of the effect of vitamin A deficiency on another group of ester sulfate compounds, the mucopolysaccharides (109,110).

Arylsulfatases

Arylsulfatases were first studied by Roy (111) who found ox liver sulfatase to have two distinct sulfatase fractions. Roy (112, 113) later named these fractions sulfatase A and B. Dodgson et al. (114) found evidence for at least three arylsulfatases in rat and ox liver, thereby discovering sulfatase C. The function of arylsulfatases B and C in mammals is not clear. A naturally occurring substrate for arylsulfatase A was not known until 1968 when Mehl and Jatzkewitz (115) isolated cerebroside sulfatase from pig kidney cortex and found it to consist of arylsulfatase A and a heat-stable polymer. In the presence of this complementary fraction, arylsulfatase A degraded sulfatide. Farooqui and Bachhawat (116) observed that purified chicken brain arylsulfatase A had sulfatide-degrading activity in the absence of a heat-stable polymer. These studies grew from the finding by Austin et al. (117) that accumulation of sulfatide in metachromatic leucodystrophy was accompanied by a low level of arylsulfatase.

Dodgson and Spencer (118) have divided the arylsulfatases into two types: those which are not inhibited by sulfate (type I) and those which are (type II). Very little seems to be known about type I arylsulfatase. Dodgson et al. (114) found it to be located predominantly in microsomes. Type I arylsulfatases are inhibited by cyanide but are hardly affected by phosphate or sulfate.

Type II arylsulfatases are arylsulfatases A and B. Arylsulfatases A and B follow quite different kinetics. Arylsulfatase B follows simple kinetics, but Roy (111) found arylsulfatase A to have anomalous kinetics. Roy (112) suggested that the failure of sulfatase A to exhibit direct proportionality between enzyme concentration and reaction velocity is due to polymerization of the enzyme molecules to give complexes which are more active enzymatically than are the unpolymerized molecules. Baum et al. (119) have proposed the anomalous kinetics to be due to the accumulation of a catalytically inactive form of the enzyme which can be reactivated by sulfate ions, one of the reaction products. In a subsequent study, Baum and Dodgson (120) found proof that the observed anomalies result from the slow exposure of a second substrate-binding site as the enzymic reaction proceeds. This second site can bind substrate to give a form of enzyme which is virtually inactive, or it can bind sulfate or phosphate ions and 4-nitrocathecol to give a form of the enzyme which is still active. The final rate of the reaction is believed by Baum and Dodgson (120) to be governed by the relative affinity of the new site for substrate or reaction products.

Sulfatases A and B have some characteristics in common, but also, some properties which prove that they are two different enzymes. The pH optimums for A and B are 4.7 (in acetate buffer) and 5.7, respectively (111). Sulfatase A is activated by sulfate ions on the alkaline side of pH 4.7 because sulfate ions cause a shift in the pH optimum. Sulfatase B is activated by chloride ions on the acid side of pH 5.7. Magnesium ions are without action on sulfatase B, but activate sulfatase A to a small extent (111). In general, both sulfatases are inhibited by sulfate. Roy found the inhibition of sulfatase B by sulfate to be noncompetitive (113) while there is a competitive inhibition of sulfatase A by sulfate (112,121).

Arylsulfatases A and B have wide distribution in mammalian tissues. Percy et al. (122) studied arylsulfatase A activity in rat kidney, liver and brain and in human fibroblasts and leukocytes. In rat tissues, kidney possessed the highest sulfatase A activity, followed by liver and brain. These findings are consistent with the ability of the human kidney to excrete high levels of sulfatide during metachromatic leukodystrophy (123) and with the greater half life of sulfatide in brain than in liver (103,104). Roy (111) found 70 percent of the arylsulfatase A activity in the mitochondrial fraction of ox liver. In a later study of ox liver arylsulfatases Roy (124) concluded that arylsulfatase A and B are lysosomal enzymes. The misconception that these enzymes are of microsomal or mitochondrial origin was due to lysosomal contamination of cellular particulates. Thyberg (125) found localization of arylsulfatases in lysosomes of epiphyseal cartilage of guinea pigs.

Arylsulfatase A and B seem to be sensitive to exogenous influences such as toxicants and dietary changes. Pokrovskii et al. (126) studied the effects of aflatoxin, a potent hepatocarcinogen, and mytomycin C, an antibiotic, on lysosomal enzyme activity. Aflatoxin caused a 276 percent increase in DNAase activity and a 50 percent increase in arylsulfatase A and B activity; conversely, mytomycin C effected a marked decrease in DNAase and arylsulfatase A and B activities. Aflatoxin-induced enzyme activation was accompanied by the release of enzymes into the supernatant fluid, which indicated an increase in permeability of the lysosomal membrane.

Pokrovskii and Tutel'yan (127) have measured the levels of liver protein, and the activities of DNAase and arylsulfatase A and B in rats fed diets containing 18.5 and 4.0 percent protein. There were increases in the activities of DNAase (20 percent) and arylsulfatase A and B (100 percent) on 30 days of feeding the diet containing 4.0 percent protein which were not reversed by transferring the experimental animals to the diet containing 18.5 percent protein. These investigators suggest that under the conditions of their experiment, lysosomal enzymes increase in activity in order to mobilize and redistribute cellular protein. In this study, the level of lysosomal enzyme activity in the supernatant fluid was not affected, indicating the absence of marked changes in the permeability of lysosomal membranes.

CHAPTER III

EXPERIMENTAL PROCEDURE

A. GENERAL PLAN

Previous work in this laboratory (3-5) has shown that malathion intoxication interferes with normal inorganic sulfur metabolism; therefore, malathion intoxication can be used as a tool in conducting model experiments to determine the effect of poisoning on mammalian inorganic sulfur metabolism. Maintaining normal inorganic sulfur metabolism is important for the well-being of mammals. Michels and Smith (2) have shown that a low dietary level of inorganic sulfur increases the requirement for sulfur amino acids. Wellers et al. (128) have also shown that inorganic sulfur can supply one-third of the total sulfur requirement and all of the sulfur required beyond that of amino acids. Interference in inorganic sulfur metabolism would be especially detrimental to populations of developing countries where pesticides are freely used and sources of sulfur amino acids are lacking.

Evidence of an interrelationship between malathion intoxication and inorganic sulfur metabolism has been shown by a depression in the ^{35}S incorporation into rib cartilage mucopolysaccharide and a mobilization of previously incorporated tissue ^{35}S into urine and feces of rats given three consecutive oral doses of malathion at 24 hour intervals (3-5). High (0.42 percent) dietary levels of inorganic sulfur decreased the apparent tissue mobilization and altered the normal labeling pattern

of rib cartilage mucopolysaccharides. A search for specific tissue sources of the mobilized ^{35}S was inconclusive but gave strong reason to suspect ester sulfate compounds (4). These studies pointed to the need for a more specific investigation of the effects of poisoning on inorganic sulfur metabolism.

In previous studies in this laboratory (3-5), the specific effects of malathion seemed to be masked when the entire tissue was examined, as in the case of the liver; therefore, the investigation recorded here was initiated for the purpose of studying the effects of poisoning on inorganic sulfur metabolism at the cellular level. The liver was chosen as the site for study since it is the center for the metabolism of malathion and other pesticides (129). Investigation of $^{35}SO_4^=$ incorporation into liver lipoprotein fractions has been used successfully by Fulton and Smith (138) to elucidate the effects of another type of stress, avitaminosis E, on inorganic sulfur metabolism; therefore, this investigation began with the isolation and fractionation of liver lipoprotein of malathion-fed rats and controls fed diets containing 0.0002, 0.10, and 0.42 percent inorganic sulfur.

Inbred Long Evans-Wistar cross adult rats weighing from 250 to 350 g served as the experimental animals throughout this study. Only female rats were chosen since this investigator and others (3-5, 129-131) have shown that female rats are more sensitive to malathion intoxication, based on the depression of acetylcholine esterase activity, than are males. The rats were housed in groups of five to eight in

wire-mesh cages and were given freshly prepared diets and distilled water ad libitum. The composition of the diets, as shown in Table 1, is a variation of the diets of Young and Dinning (132) as modified by Caputto et al. (133). The salt mixture of Hubbell et al. (134) was modified to obtain a low sulfate salt mixture by replacing the sulfate salts with chlorides and acetates. In order to obtain low (0.0002 percent), intermediate (0.10 percent) and high (0.42 percent) levels of dietary inorganic sulfur, the amounts of inorganic and organic sulfur were varied inversely so that the total percent sulfur as sulfate remained constant as shown in Table 2.

Six groups of five to seven rats were fed the three diets shown in Table 1 with two groups fed each diet. The rats were "prepped" for 17 days by feeding a diet containing the appropriate level of inorganic sulfur. On day 18 of the diet-feeding schedule, 250 mg malathion per kg of rat with corn oil as a carrier (100 mg malathion/ml corn oil) were administered to the three experimental groups of rats by stomach tube. The three corresponding control groups were given equivalent amounts of corn oil by stomach tube. Three consecutive tube feedings were given at 24 hour intervals. Malathion is well absorbed by the oral route and disperses evenly in corn oil, a carrier which facilitates its absorption (135). The level of malathion chosen was 250 mg per kg of rat since levels below 250 mg per kg were found to be of little value in this type of study (4,5) and illness or death occurred frequently at higher levels. Stavinoha et al. (136) have shown that symptoms of organophosphorus poisoning become maximal at about the third

TABLE 1

Composition of diets

Component	Diets A 0.42% $SO_4^=$	B 0.10% $SO_4^=$	C 0.0002% $SO_4^=$
		g/100 g	
Cornstarch	32.00	32.00	32.00
Sucrose	30.00	30.00	30.00
Casein	15.00	15.00	15.00
Non-nutritive bulk[1]	10.00	9.89	9.72
Fat	6.00	6.00	6.00
Cod liver oil	2.00	2.00	2.00
Vitamin mixture[2]	2.00	2.00	2.00
Basic salt mixture[3]	1.34	1.34	1.34
$CaCO_3$	0.91	1.24	1.34
$CaSO_4 \cdot 2H_2O$	0.75	0.18	0.00
L-cysteine	0.00	0.35	0.60

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio 44128.

[2]Nutritional Biochemicals Corporation, Cleveland, Ohio 44128. Vitamin diet fortification mixture formulated to supply the following amounts of vitamins (g/kg vitamin premix): thiamine hydrochloride 1.0, riboflavin 1.0, niacin 4.5, p-aminobenzoic acid 5.0, calcium pantothenate 3.0, pyridoxine hydrochloride 1.0, ascorbic acid 45.0, inositol 5.0, choline chloride 75.0, menadione 2.25, biotin 0.020, folic acid 0.090, vitamin B-12 0.00135, γ-tocopherol 5.0, vitamin A 2 x 10^8 units, vitamin D 4 x 10^8 units, and sufficient glucose to make 1 kg.

[3]1.34 g equals in mg: 92.8 $MgCO_3$, 207.0 NaCl, 336.0 KCl, 636.0 KH_2PO_4, 61.5 $FePO_4 \cdot 2H_2O$, 0.2 KI, 0.3 NaF, 1.8 $AlK(SO_4)_2 \cdot 12H_2O$, 2.1 $Cu(C_2H_3O_2)_2 \cdot H_2O$, 1.2 $MnCl_2 \cdot 6H_2O$.

TABLE 2

Calculated levels of dietary sulfur

Diet	Percent sulfur as sulfate		
	Inorganic	Organic	Total
A	0.42	0.25	0.67
B	0.10	0.57	0.67
C	0.00	0.67	0.67

day of administration. The malathion used throughout this study was technical grade (95.6 percent pure) and was kindly donated by the American Cyanamid Company, Princeton, New Jersey.

Rats were killed by decapitation 32 hours after the third tube feeding of malathion. Since Fulton (137) and Fulton and Smith (138) have shown that liver lipoprotein is maximally labeled 8 hours after a subcutaneous injection of $Na_2{}^{35}SO_4$, rats were injected with approximately 10 μCi of carrier free $Na_2{}^{35}SO_4$ in 0.5 ml of isotonic saline 8 hours before sacrifice. Livers from five to seven rats from each of the six groups were pooled as a group and were stored at -20°C until lipoprotein isolation began.

The cellular lipoprotein was prepared from pooled livers of each group by the salt extraction method of Smith et al. (139) as modified by Levin and Thomas (140). Determination of the specific activity of the unfractionated lipoprotein was followed by isolation and determination of the specific activity of each of the following fractions in this order: sulfolipid, mucopolysaccharide, nucleotide, sulfate transfer fraction and the remaining insoluble fraction. The lipid extraction method used extracts cerebroside as well as sulfolipid (141). Ribose was determined in the nucleotide fraction.

After the effects of malathion intoxication were observed in fractions of total cellular lipoprotein of liver, the effect of malathion intoxication on nuclear, mitochondrial and residual liver lipoprotein was determined. Only diet B (0.10 percent inorganic sulfur) was fed during this investigation since statistically significant effects of malathion

intoxication were found only when the specific activities of the sulfolipid fractions of malathion-treated rats fed diet B were compared with those of control rats. Two groups of 21 rats each were fed diet B and were given malathion or corn oil, injected subcutaneously with $Na_2{}^{35}SO_4$, and sacrificed as described previously. On the day of sacrifice, 16 rats remained in the malathion group and 20 in the control group. The livers from each group were pooled and nuclei and mitochondria were isolated. Cellular lipoprotein was extracted from the nuclear, mitochondrial, and residual particulates. Sulfolipid was then extracted from the lipoprotein from each of these particulates. In order to determine whether the effect of malathion intoxication on liver lipoprotein is due to alterations in the amount of sulfolipid synthesized or whether it is due to altered turnover rates, galactose, sulfate, and the specific activity of the lipoprotein sulfolipid from each particulate were determined. The ratio of sulfate to galactose (mmoles $SO_4^=$ per mmole galactose), the counts per minute per mmole sulfate and the counts per minute per mg of galactose were calculated.

The activity of liver arylsulfatase was determined in another group of rats in order to determine if the activity of this sulfolipid-degrading enzyme was affected in malathion-intoxicated rats and to correlate the enzyme activity with data obtained in the previously described experiments. Two groups of rats were fed diet B during the 17 day prep period: 24 in the malathion group and 18 in the control group. The rats were then given three tube feedings of malathion or corn oil. The rats were injected subcutaneously with $Na_2{}^{35}SO_4$ and killed as

described previously on day 21 of the feeding period. On the day of sacrifice, 19 rats remained in the malathion group and 16 in the control group. Arylsulfatase activity was determined in sets of three pooled livers in the malathion and control groups. Deoxyribonucleic acid and nitrogen determinations were performed on whole liver homogenates and nitrogen and arylsulfatase in dialyzed homogenates. The weight of each set of pooled livers was determined before analyses. Nitrogen was determined by the micro-Kjehdahl procedure as standardized by Willets and Ogg (142).

B. METHODS

Determination of ^{35}S Specific Activity in Fractions of Cellular Lipoprotein

Unfractionated cellular lipoprotein and each of the fractions obtained were evaluated for their specific activities according to the method of Mahin and Lofberg (143). Approximately 10 mg of the unfractionated lipoprotein and the mucopolysaccharide, sulfate transfer, and residue fractions were transferred to counting vials and weighed to 1×10^{-4} decimal places. Two ml of the sulfolipid and the nucleotide fractions were evaporated to dryness at 50° in counting vials. All samples were analyzed in duplicate. To each vial was added 0.2 ml water, 0.2 ml 70 percent perchloric acid and 0.4 ml hydrogen peroxide. The samples were heated in a water bath at 70° to 80° for approximately 40 minutes or until clear and colorless. The samples were then allowed to cool at room temperature and 6.0 ml of ethyl cellosolve and 10.0 ml

of PPO (2,5-diphenyloxazol) in toluene (12.0 g PPO per liter of toluene) were added. Samples were capped, mixed, and radioactivity was evaluated by liquid scintillation counting using a Pickernuclear/Liquimat 220 set for less than 2 percent statistical error. There was no appreciable quenching as indicated by channels ratio data.

Determination of Total Sulfur as Sulfate in Sulfolipid

The chloroform-methanol extracts of liver sulfolipid were diluted to 100 ml with 2:1 chloroform-methanol. Four ml of the sulfolipid dilutions were analyzed in duplicate for each sample. The chloroform-methanol extract was pipetted into a 50 ml centrifuge tube and the extract evaporated to dryness at 50°. The centrifuge tubes containing the dried sulfolipid extract were then held upright in beakers and the extracts heated gently with a 50 percent nitric acid solution until the yellow-brown fumes subsided. Five ml of a combustion mixture composed of 600 ml concentrated nitric acid, 300 ml 70 percent perchloric acid, and 9.0 g cupric nitrate were pipetted into each sample and the sample heated until a blue residue remained as the sample was evaporated to dryness.

After the above combustion procedure was completed, samples were prepared for sulfate analyses by atomic absorption spectrophotometry by the method of Roe et al. (144). Dry combusted samples were dissolved in 5.0 ml of distilled water, and 1.0 ml of a 5 percent lanthanum chloride and 2.0 ml of a 15 percent barium chloride solution were pipetted into each sample to precipitate the sulfate as barium

sulfate. Samples were mixed and then centrifuged at 715 x g in an International Model SBV centrifuge. The resulting precipitate was washed twice with 5.0 ml of distilled water to remove the lanthanum chloride and excess barium. As the supernatant fluid was removed after the last washing, the tube was left inverted to drain and the upper portion of the tube was dried with paper tissue. The precipitate was dissolved in 10 ml of an alkaline disodium ethylenediamine tetra-acetate (EDTA) solution prepared by dissolving 10 g EDTA in 500 ml of distilled water, adding 20 g of sodium hydroxide, and diluting to 2000 ml. Duplicate working standards which contained 0.00 and 0.10 ml of a stock sulfate solution were prepared concurrently with each set of samples. The stock sulfate solution was made by dissolving 1.479 g of sodium sulfate in 500 ml of distilled water to yield a concentration of 2000 ppm of sulfate.

Samples and standards were aspirated into the flame of a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer prepared for the determination of barium. A barium hollow cathode tube was operated at 20 milliamperes. The following settings were made: monochromator, 276 nanometers (nm), visible; gain, 5 to 6; slit, 3; flow rate of nitrous oxide (the oxidizer), 8.5; flow rate of the acetylene (the fuel), slightly above 15. After the nitrous oxide burner was ignited, the fuel flow was adjusted to give the characteristic pink cone. Percent absorption, an exponential function, was converted to absorbance, a linear function, so that the mmoles sulfate per ml of sulfolipid extract could be calculated according to the following equations:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \text{concentration of standard (ppm)} \times \text{ml aspirated} \times \frac{\text{volume (ml) or weight (mg) of sample analyzed}}{\text{total sample volume (ml) or weight (mg)}} = \mu gS \text{ as } SO_4^{=} \text{ per ml or mg of sample}$$

$$\frac{\mu gS \text{ as } SO_4}{96{,}000} = \text{mmole } SO_4^{=} \text{ in sample}$$

Isolation of Liver Nuclei, Mitochondria, and Residue

Liver nuclei and mitochondria were isolated by the method of Hogeboom (145), utilizing the homogenizing solutions of Keller and Zamecnik (146). Livers were excised and placed in chilled beakers containing cold medium A which consisted of 0.35 M sucrose, 0.035 M potassium bicarbonate, 0.004 M magnesium chloride·$6H_2O$, and 0.025 M potassium chloride. Livers were homogenized in 3.0 ml of medium A per g of tissue using a motor-driven Potter-Elvehjem homogenizer with a teflon pestle. The homogenate was centrifuged in plastic tubes at 700 x g for 10 minutes in a Lourdes refrigerated centrifuge. The supernatant fluid was decanted and saved for isolation of mitochondria. Nuclei were re-homogenized with medium A and centrifuged as described above.

Nuclei were purified by homogenization in 10.0 ml of medium B per tube. Medium B consisted of 0.9 M sucrose, 0.004 M magnesium chloride·$6H_2O$ and 0.025 M potassium chloride. The homogenate was centrifuged at 700 x g for 10 minutes. Homogenization and centrifugation were repeated twice to obtain purified nuclei. The supernatant fluids were combined with their corresponding fluids from other centrifugations.

The combined supernatant fluids for each sample were adjusted to contain 0.8 M sucrose so that the mitochondrial fraction would not be contaminated with myelin since myelin floats on 0.8 M sucrose (147). The supernatant fluids were centrifuged at 15,000 x g for 10 minutes in metal tubes. The supernatant fluid was carefully decanted from each sample, leaving the isolated mitochondria.

The residual fraction was isolated from the supernatant fluids by centrifuging at 15,000 x g for 60 minutes after the supernatant fluids were adjusted to contain 0.1 M sucrose. The molarity of the supernatant fluids was adjusted in order to simulate the conditions of lipoprotein extraction from liver. The residual fraction should contain any cell components other than nuclei and mitochondria which are found in lipoprotein.

Lipoprotein was extracted from each fraction by the method of Smith et al. (139) as modified by Levin and Thomas (140). Total lipid was extracted from the lipoprotein of each fraction according to the method of Smith et al. (139). Lipoprotein and sulfolipid extraction have been described in detail by Fulton (137) and will not be included in this manuscript. The specific activity and sulfate content of the sulfolipid fraction were determined as described previously and the counts per minute per mmole of sulfate calculated. Galactose was determined as described in the following section and the counts per minute per mg of galactose and the mmoles sulfate per mmole of galactose calculated.

Determination of Galactose in Sulfolipid

Galactose in sulfolipid samples was determined according to the method of Fisher et al. (148). Duplicate 4.0 ml portions of the chloroform-methanol sulfolipid extract were evaporated to dryness in a 50° oven in hydrolysis tubes. Samples were then hydrolyzed in sealed tubes for 2 hours at 100° with 3.0 N hydrochloric acid according to the method of Radin et al. (149). The release of galactose at 30 minutes and 1, 2, and 3 hours was determined. Maximum amounts of galactose were found at 2 hours of hydrolysis in agreement with Radin et al. (149). Hydrolyzed samples were then filtered into test tubes. The filter paper was washed with a small amount of water. The sample was evaporated to dryness in a 50° oven. The tubes were placed in an ice bath, and 2.0 ml of cold 3.0 N sulfuric acid and 2.0 ml of a cold orcinol solution were pipetted into each test tube. Care was taken to keep samples cold so that premature and uneven color development would be avoided. The orcinol solution was prepared by dissolving 2.0 g of orcinol in 50 ml of water and adding a cooled solution made by mixing 20 ml of concentrated sulfuric acid with 30 ml of water. Standard glucose and galactose solutions containing 0.1 mg per ml in 3.0 N sulfuric acid were prepared. A blank and samples of both the standards containing 0.1 and 0.2 mg were treated in the same way as the sulfolipid samples. Sufficient 3.0 N sulfuric acid was added to the blank and standards to bring the volume to 2.0 ml before the orcinol solution was added. Six ml of a 92 percent sulfuric acid solution were rapidly added to each sample and mixing was completed by forcing a stream of

nitrogen through a small glass tube placed in each sample. The glass tube was rinsed in 3.0 N sulfuric acid after each sample. Samples were then heated exactly 4 minutes in a water bath maintained at 67-68° with good circulation. After 5 minutes cooling in an ice bath, color density was estimated in a Beckman B spectrophotometer at 470 and 560 nm.

This method is based upon the difference in absorption of the orcinol complex of glucose and galactose at 470 and 560 nm so that true estimations of both glucose and galactose can be obtained. The glucose- and galactose-orcinol complexes have maximal absorbance at 470 and 560 nm, respectively. Since both complexes absorb at each wavelength, values were calculated from the quadratic equations derived by Fisher et al. (146). These equations are

$$\hat{u} = 1.05x + 2.57y + 0.98x^2 + 1.32xy + 0.32y^2$$

$$\hat{v} = 1.27x + 5.08y + 0.21x^2 - 0.06y - 2.27y^2$$

where x and y equal mg of glucose and galactose in the sample analyzed and $\hat{u}$ and $\hat{v}$ are the estimated absorbances at 470 and 560 nm. Standard glucose and galactose solutions were run simultaneously with the unknowns for comparison with the values Fisher et al. (148) used in deriving their equations. The equations were solved for x and y by these equations:

$$\frac{du}{dx} = A = 1.05 + 1.96x + 1.32y$$

$$\frac{du}{dy} = B = 2.57 + 1.32x + 0.64y$$

$$\frac{dv}{dx} = C = 1.27 + 0.42x - 0.06y$$

$$\frac{dv}{dy} = D = 5.08 - 0.06x - 4.54y$$

Corrections to the true values of x and y were made using the following equations:

$$AD - BC = 1/M$$

$$\Delta x = M(D\Delta u - B\Delta v)$$

$$\Delta y = M(A\Delta v - C\Delta u)$$

$$\Delta u = u - \hat{u}$$

$$\Delta v = v - \hat{v}$$

The mgs total galactose were divided by 180 to convert mgs to mmoles.

Determination of Sulfatase Activity in Liver

The liver homogenate for sulfatase activity was prepared by the method of Farooqui and Bachhawat (116). Sets of three livers were homogenized in 2 ml of 0.2 M sodium acetate buffer (pH 5.0) per g of tissue using a motor-driven Potter-Elvehjem homogenizer with a teflon pestle. The 0.2 M sodium acetate buffer solution was prepared by mixing together 133 ml of 1.0 N sodium hydroxide, 200 ml of 1.0 N acetic acid, and 500 ml distilled water; adjusting to pH 5.0 with glacial acetic acid; and adding distilled water to equal 1 liter. The samples were dialyzed for 8 hours against 500 volumes of 0.02 M tris-HCl buffer, pH 7.4 at 4°. The tris-HCl buffer was prepared by dissolving 2.42 g of Sigma 121 in approximately 800 ml of distilled water, adjusting the pH of the solution to 7.4 with concentrated hydrochloric acid with the use of a pH meter, and making the final volume 1 liter.

Arylsulfatase activity was determined according to the method of Roy (111). The dialyzed sample was diluted with 4 volumes of cold distilled water, mixed, and 0.2 ml portions transferred in duplicate to test tubes. In preparation for enzyme analysis, 0.6 ml of the buffer-substrate solution was adjusted to pH 5.0 with 0.1 N hydrochloric acid. The buffer-substrate solution was composed of 0.2 ml of 0.5 M acetate buffer, pH 5.0, and 0.4 ml of 6 mM p-nitrocatechol sulfate (0.0934 g per 50 ml) for each sample analyzed. A pH of 5.0 was chosen for the acetate buffer since this is the pH chosen by Farooqui and Bachhawat (116) for determining arylsulfatase A activity. The 0.5 M sodium acetate buffer solution was prepared by adding 500 ml of 1.0 N sodium hydroxide to 300 ml of 1.0 N acetic acid, adjusting to pH 5.0 with glacial acetic acid, and adding enough distilled water to equal 1 liter.

The buffer substrate solution and the enzyme preparation in the tubes were equilibrated at 37° in a shaking water bath. After equilibration, 0.6 ml of buffer-substrate solution was added to each enzyme sample. The samples were incubated for 1 hour with shaking at 37°. A blank containing 0.2 ml of the enzyme preparation was prepared for each sample. The buffer-substrate solution was added to the blanks at the end of the 1 hour incubation period. A standard containing 40 µg 4-nitrocatechol (0.8 ml of stock solution) was treated in the same way as samples except that no buffer-substrate solution was added. The stock 4-nitrocatechol solution was prepared by dissolving 2.5 mg in 50 ml of water to equal a concentration of 50 µg per ml.

At the end of the incubation period, 3.0 ml of 2 percent phosphotungstic acid in 0.1 N hydrochloric acid were added to each tube and the contents were mixed. Samples were centrifuged at 715 x g for 10 minutes to remove protein. Three ml of the clear supernatant fluid was pipetted into 5.0 ml of alkaline quinol made by adding 5.0 ml of 4 percent hydroquinone in 0.1 N hydrochloric acid (made up weekly) to 100 ml of 2.5 N sodium hydroxide: 5 percent sodium sulfite·$7H_2O$. Samples were mixed and optical densities of samples estimated in a Beckman B spectrophotometer at a wavelength of 520 nm. Standard samples were read against a mixture of 3.0 ml of distilled water and 5.0 ml alkaline quinol. Samples were read against their corresponding blanks. A standard curve was prepared and demonstrated that the Beer-Lambert Law was followed under the conditions of this experiment. The following equation describes the procedure for calculating arylsulfatase activity expressed as µg 4-nitrocatechol present at the end of the reaction period:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \text{concentration of standard (µg 4-nitrocatechol)} \times \frac{\text{volume of homogenate}}{\text{volume of homogenate analyzed}} = \text{µg 4-nitrocatechol in total homogenate.}$$

Determination of Deoxyribonucleic Acid in Liver Homogenates

DNA was extracted from 5.0 ml samples of undialyzed liver homogenates by the method of Schneider (150). Samples were pipetted into 15 ml centrifuge tubes. Five ml of 20 percent trichloroacetic acid (TCA) were added to each tube. Samples were mixed, centrifuged at

715 x g for 10 minutes, supernatant fluid was discarded, and the procedure repeated. The residue was then resuspended in 6.0 ml of 5 percent TCA and heated in a 90° water bath for 15 minutes. Samples were cooled and centrifuged as above. The supernatant fluids were decanted, the residue resuspended in 3.0 ml of 5 percent TCA, and the samples centrifuged a third time. Supernatant fluids were combined and diluted to 10 ml with 5 percent TCA.

The amount of DNA in extracts was determined by the method of Seibert (151). Duplicate 2.0 ml samples were pipetted into test tubes. Four ml of diphenylamine reagent were added to each tube. Samples were covered and immersed in a boiling water bath for exactly 10 minutes. Samples were removed from the water bath and immersed in cold water. Color density was estimated in a Beckman B spectrophotometer at a wavelength of 650 nm.

The diphenylamine reagent was prepared by dissolving 1 g of purified diphenylamine in 100 ml of glacial acetic acid and 2.75 ml of concentrated sulfuric acid. Diphenylamine was purified by dissolving approximately 5 g in 20 ml of 95 percent ethanol by heating and cooling to crystallize. The procedure was repeated using the resulting crystals. The crystals were washed with cold distilled water and dried in a 50° oven.

Deoxyribose was used for making the DNA standard solution. DNA was calculated to be 40 percent deoxyribose. The stock solution was made by dissolving 40 mg of deoxyribose in 250 ml of distilled water. A standard curve demonstrated that the Beer-Lambert Law was followed

under the conditions of this experiment. The color density of a standard equivalent to 0.8 mg DNA was determined simultaneously with the samples. The total mgs of DNA in the DNA extract were calculated according to the following equation:

$$\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \text{concentration of standard} \times \frac{\text{volume of DNA extract}}{\text{volume of extract analyzed}} = \text{total mg DNA in extract.}$$

C. STATISTICAL METHODS AND COMPUTATIONS

Data were analyzed by paired or unpaired comparisons or by Duncan's multiple range test for equal or unequal replications as described by Steel and Torrie (152). The Olivetti-Underwood Programma 101 was programmed to facilitate calculation and computation of data.

CHAPTER IV

RESULTS

Data obtained from analyses of cellular lipoprotein fractions from livers of malathion-treated and untreated rats are shown in Tables 3 through 10. These data were normalized to decrease the variation due to the specific activity of the $Na_2{}^{35}SO_4$ administered. The variation was not due primarily to decay of ^{35}S but to the necessity of using several different dilutions of $Na_2{}^{35}SO_4$ during the period in which this study was conducted. Levin and Thomas (140) and Fulton and Smith (138) have shown that the procedure used in this study for extraction of cellular lipoprotein is quite reproducible; therefore, the specific activities of the fractions of cellular lipoprotein were normalized based on the differences in specific activities of the unfractionated lipoprotein. Specific activities of liver lipoprotein obtained from the control group fed a diet containing an intermediate level (0.10 percent) of inorganic sulfur were chosen as the basis for normalizing the data since these conditions most nearly represent normal conditions. A ratio was established between the unfractionated lipoprotein value obtained from control rats in the diet B group having the highest specific activity and each of the other unfractionated lipoprotein control values. These ratios were used to equalize the specific activities of lipoprotein and were applied to data obtained from fractionation of lipoprotein since differences among replications

TABLE 3

Incorporation of $^{35}SO_4^{=}$ into cellular lipoprotein of livers from control and malathion-treated rats fed diets containing three levels of inorganic sulfur[a]

Diet	Percent dietary sulfate	Mg malathion/kg of rat	
		0	250
		cpm/mg lipoprotein	
A	0.42	224 ± 14[b]	199 ± 15[be]
B	0.10	202 ± 00[cd]	260 ± 20[cde]
C	0.0002	215 ± 27[c]	261 ± 32[c]

[a]Lipoprotein was extracted from pooled livers of five to seven rats.

[b]Mean of eight replications ± SE.

[c]Mean of seven replications ± SE.

[d]Difference statistically significant ($P<0.05$) by the method of paired comparisons (152).

[e]Difference is statistically significant ($P<0.05$) by the method of unpaired comparisons (152).

TABLE 4

The concentration of sulfur as sulfate in sulfolipid extracted from liver lipoprotein from control and malathion-treated rats[a]

Diet	Percent dietary sulfate	Mg malathion/kg rat	
		0	250
		mmoles $SO_4^{=}$/mg lipoprotein x 10^6	
A	0.42	11.7 ± 1.9[b]	9.2 ± 2.4[b]
B	0.10	7.7 ± 1.4[c]	7.6 ± 1.2[c]
C	0.0002	8.4 ± 1.4[c]	7.3 ± 2.1[c]

[a]Each sulfolipid sample was extracted from the lipoprotein of five to seven rats.

[b]Mean of six replications ± SE.

[c]Mean of five replications ± SE.

TABLE 5

Incorporation of $^{35}SO_4^=$ into the sulfolipid fraction of liver lipoprotein from control and malathion-treated rats[a]

Diet	Percent dietary sulfate	Mg malathion/kg of rat	
		0	250
		cpm/mg lipoprotein	
A	0.42	59 ± 6[b]	58 ± 5[b]
B	0.10	60 ± 12[cd]	95 ± 23[cd]
C	0.0002	58 ± 9[c]	82 ± 18[c]

[a]Each sulfolipid sample was extracted from the lipoprotein of five to seven rats.

[b]Mean of six replications ± SE.

[c]Mean of five replications ± SE.

[d]Difference is statistically significant ($P<0.05$) by the method of paired comparisons (152).

TABLE 6

Specific activity of sulfolipid sulfur as sulfate extracted from liver lipoprotein from control and malathion-treated rats[a]

Diet	Percent dietary sulfate	Mg malathion/kg of rat	
		0	250
		cpm/mmole $SO_4^=$ x 10^{-5}	
A	0.42	64 ± 16[b]	88 ± 22[bd]
B	0.10	89 ± 21[c]	155 ± 39[c]
C	0.0002	90 ± 18[c]	220 ± 99[cd]

[a]Each sulfolipid sample was extracted from lipoprotein from the livers of five to seven rats.

[b]Mean of six replications ± SE.

[c]Mean of five replications ± SE.

[d]Statistically significant ($P<0.01$) by the method of unpaired comparisons (152).

TABLE 7

Specific activity of mucopolysaccharide extracted from liver lipoprotein from control and malathion-treated rats[a]

Diet	Percent dietary sulfate	Mg malathion/kg of rat	
		0	250
		cpm/mg	
A	0.42	504 ± 126[b]	420 ± 92
B	0.10	481 ± 187	531 ± 169
C	0.0002	424 ± 147	796 ± 313

[a]Each sample was extracted from pooled livers of five to seven rats.

[b]Means of five replications ± SE.

TABLE 8

Specific activity of nucleotide extracted from liver lipoprotein from control and malathion-treated rats[a]

Diet	Percent dietary sulfate	Mg malathion/kg of rat	
		0	250
		cpm/mg ribose x 10^{-5}	
A	0.42	185 ± 38[b]	197 ± 38[b]
B	0.10	181 ± 25[c]	176 ± 29[c]
C	0.0002	218 ± 42[c]	281 ± 63[c]

[a]Each sample was extracted from lipoprotein from pooled livers from five to seven rats.

[b]Mean of six replications ± SE.

[c]Mean of five replications ± SE.

TABLE 9

Incorporation of $^{35}SO_4^{=}$ into sulfate transfer fraction of lipoprotein extracted from the livers from control and malathion-treated rats[a]

Diet	Percent dietary sulfate	Mg malathion/kg of rat	
		0	250
		cpm/mg	
A	0.42	516 ± 185[b]	378 ± 116[b]
B	0.10	389 ± 85[c]	723 ± 336[c]
C	0.0002	345 ± 67[c]	419 ± 140[c]

[a]Each fraction was isolated from pooled livers of five to seven rats.

[b]Mean of eight replications ± SE.

[c]Mean of seven replications ± SE.

TABLE 10

Specific activity of the insoluble fraction of cellular lipoprotein extracted from the livers from control and malathion-treated rats[a]

Diet	Percent dietary sulfate	Mg malathion/kg of rat	
		0	250
		cpm/mg	
A	0.42	217 ± 89[b]	181 ± 65[b]
B	0.10	119 ± 39[c]	107 ± 27[c]
C	0.0002	211 ± 124[c]	271 ± 184[c]

[a]Insoluble fraction was obtained from lipoprotein from pooled livers of five to seven rats.

[b]Mean of eight replications ± SE.

[c]Mean of seven replications ± SE.

for each fraction should be proportional to differences among replications in specific activity of lipoprotein. There is no standard error for the specific activity of lipoprotein obtained from rats fed diet B as shown in Table 3 because of the method of normalizing the specific activities as explained above.

Malathion intoxication caused increases in the specific activities of lipoprotein from rats fed diets containing intermediate (0.10 percent) and low (0.0002 percent) levels of inorganic sulfur, but did not evoke an increase when rats were fed the diet containing a high level (0.42 percent) of inorganic sulfur (Table 3). The increase in lipoprotein specific activity is statistically significant by paired comparisons (152) when the values for the control and the experimental group fed diet B are compared ($P < 0.05$). This statistical significance is not solely due to the lack of variation in the control group caused by normalizing. Statistical analyses of the original data for all three diets demonstrated the same level of significance.

Decreasing the level of dietary sulfate below 0.42 percent caused decreases in the specific activities of unfractionated lipoprotein from the control group. The reverse is true in the malathion-treated group; decreasing the level of dietary sulfate below 0.42 percent caused increases in specific activities. These changes were not magnified when the level of sulfate was decreased below 0.10 percent. Specific activities of unfractionated liver lipoprotein from rats in the experimental group fed diets A and B are significantly different ($P < 0.05$) by the method of unpaired comparisons (152).

A comparison of the data obtained from analyses of the sulfolipid fraction of liver lipoprotein from control and experimental groups is shown in Tables 4, 5, and 6, pages 53-55. There is a trend toward a lower sulfate content in the sulfolipid fraction of malathion-treated rats as compared with controls as shown in Table 4, but these decreases are not statistically significant by paired comparisons (152). Decreasing the level of dietary sulfate below 0.42 percent caused decreases in the sulfate concentration of lipoprotein sulfolipid when values for rats fed diets B and C are compared with values for rats fed diet A in both the control and the malathion-treated groups.

As shown in Table 5, there is an increase in the incorporation of $^{35}SO_4^=$ into lipoprotein sulfolipid when controls are compared with malathion-treated rats fed diets B and C. This increase in $^{35}SO_4^=$ incorporation is not found on examination of the values obtained from the lipoprotein sulfolipid of rats fed the diet containing a high level of inorganic sulfur (0.42 percent). As was the case with the data in Table 3, page 52, for the specific activity of lipoprotein, the only statistically significant difference is found when the values for the sulfolipid specific activity of diet B rats are compared ($P < 0.05$). Values within the malathion-intoxicated group of rats show increases in the incorporation of $^{35}SO_4^=$ into lipoprotein sulfolipid of rats fed diets containing the two lower levels of dietary sulfate as compared with values for rats fed diet A with the greatest increase occurring when rats were fed diet B. The $^{35}SO_4^=$ incorporation into lipoprotein sulfolipid of control rats remained essentially constant within the range of dietary sulfate fed.

As shown in Table 6, page 55, the cpm/mmole $SO_4^=$ increased within the control group as the level of dietary inorganic sulfur decreased. This increase was magnified by malathion intoxication; there was a 41 percent overall increase in the normal group as compared with a 150 percent overall increase in the malathion-treated group. The latter percentage increase is statistically significant by the method of unpaired comparisons ($P < 0.01$) (152). When the malathion-treated groups were compared with their controls for each of the three diets, malathion intoxication appeared to cause an increase in $^{35}SO_4^=$ incorporation which became greater when diets containing increasingly lower levels of inorganic sulfur were fed. A comparison of the values for cpm/mmole $SO_4^=$ for control and experimental groups results in percent increases of 38, 74 and 144 for values from rats fed diets containing 0.42, 0.10, and 0.0002 percent inorganic sulfur, respectively. These increases in cpm/mmole $SO_4^=$ are not statistically significant, although a characteristic pattern is established.

Malathion intoxication did not evoke statistically significant effects on $^{35}SO_4^=$ incorporation into mucopolysaccharide, but an interesting reversal in the pattern of labeling in the control group as compared with the experimental group can be seen in Table 7, page 56, as the level of dietary sulfate decreases. There is a decrease in $^{35}SO_4^=$ incorporation into mucopolysaccharide with decreases in dietary sulfate in the control group while the opposite pattern is obtained in malathion-treated rats. This altered pattern was also observed previously by Disney (4) in malathion-treated rats fed these diets. When

comparing the specific activities of liver lipoprotein mucopolysaccharides extracted from malathion-treated rats with those of controls, the same pattern is found which was observed in unfractionated lipoprotein and in the incorporation of $^{35}SO_4^=$ into lipoprotein sulfolipid; i.e., there is a decrease (16 percent) in the specific activity of the fraction from malathion-treated rats fed diet A, but increases (10 and 88 percent, respectively) when rats were fed diets B and C which contain increasingly lower levels of inorganic sulfur.

An irregular pattern of $^{35}SO_4^=$ activity in the nucleotide fraction is observed in Table 8, page 57, although a greater percentage increase is obtained when data for diet C rats are compared (29 percent increase) than when data for diet A rats are compared (7 percent increase). Statistical analyses of values in this table showed no significant differences by paired comparisons (152). Examination of within group changes also revealed no statistically significant differences by the method of unpaired comparisons (152), but there are increases in cpm/mg ribose when values for both the control and the malathion-treated groups fed diet A are compared with values from rats fed diet C. The increase is greater in the malathion-treated group (43 percent as compared with 18 percent).

The incorporation of $^{35}SO_4^=$ into the sulfate transfer fraction (STF) of rat liver lipoprotein showed the same pattern for between group comparisons as the data for specific activity of unfractionated lipoprotein, the incorporation of $^{35}SO_4^=$ into lipoprotein sulfolipid, and the specific activity of mucopolysaccharide material. As shown

in Table 9, page 58, malathion intoxication caused a 27 percent decrease in $^{35}SO_4^=$ activity of lipoprotein STF when rats were fed diet A, but caused 86 and 21 percent increases, respectively, when diets B and C were fed. The differences between these values are not statistically significant by paired comparisons (152). Within group comparisons of $^{35}SO_4^=$ incorporation into STF show the same general trend found in the mucopolysaccharide fraction; a decrease in specific activity with decreased dietary sulfate in the control group but an increase in the malathion-treated group when rats fed the two lower levels of dietary sulfate are compared with rats fed diet A.

The specific activity of the fraction of liver lipoprotein remaining after the mucopolysaccharide material, sulfolipid, nucleotide and STF were extracted is shown in Table 10, page 59. There was a 17 percent decrease in ^{35}S activity of the insoluble fraction from malathion-treated rats fed diet A as compared with their controls. This decrease was lessened when rats were fed a lower level of inorganic sulfur; there was a 10 percent decrease in the ^{35}S activity of malathion-treated rats fed diet B containing 0.10 percent inorganic sulfur. When rats were fed the diet low in inorganic sulfur (0.0002 percent), there was a 28 percent increase in the specific activity of the insoluble fraction.

Fractionation of cellular lipoprotein from malathion-treated and untreated rats fed diets containing three levels of inorganic sulfur identified a statistically significant effect of malathion only on the sulfolipid fraction when expressed as cpm/mg of lipoprotein. Although

not statistically significant, the data from other fractions revealed interesting ^{35}S labeling patterns. Unfractionated lipoprotein and three of five of its fractions had the same labeling pattern when malathion-treated rats were compared with controls: a decrease in specific activity in malathion-treated rats fed the diet high in inorganic sulfur, but increases when the diets containing lower levels of inorganic sulfur were fed. In unfractionated lipoprotein and the three fractions which showed a common labeling pattern (sulfolipid when expressed as cpm/mg lipoprotein, mucopolysaccharide, and STF), the diet B groups showed a greater increase in ^{35}S activity in every case except in mucopolysaccharide. Also the two ester sulfate fractions isolated, sulfolipid and mucopolysaccharide, showed the same patterns for specific activity within the malathion-treated group: an increase in activity with decreasing dietary inorganic sulfur (see Tables 6 and 7, page 55 and 56). The normal labeling patterns for these two fractions were in direct opposition.

Since the fractionation of liver cellular lipoprotein identified sulfolipid obtained from rats fed diet B as the only fraction significantly affected by malathion intoxication, cellular particulates were isolated from control and malathion-treated rats fed diet B (0.10 percent inorganic sulfur). The purposes of this study were to identify particulates responsible for the effects of malathion intoxication observed previously and to determine if the increase in $^{35}SO_4^=$ incorporation into lipoprotein sulfolipid from livers of malathion-treated rats is due to increased synthesis or a combination of degradation of the

sulfate ester bond and replacement of the lost sulfate with sulfate of a higher specific activity. Table 11 shows that there were fewer mmoles of sulfate in the sulfolipid extract from particulate lipoprotein from malathion-treated rats as compared with controls. Nuclear lipoprotein sulfolipid appeared to be more active in $^{35}SO_4^=$ incorporation than mitochondrial or residual particulates. Malathion intoxication caused an increase in the specific activity of sulfate (cpm/mmole) in the lipoprotein sulfolipid from all three particulates. The percentage increases for the malathion-treated as compared with the control groups for the three particulates are 279, 70, and 131 for nuclei, mitochondria and residue, respectively. As shown in Table 12, there was less galactose in the nuclear and mitochondrial lipoprotein sulfolipid of malathion-treated rats as compared with controls, but more in the lipoprotein sulfolipid from the residue. The cpm/mg of galactose in the nuclear lipoprotein sulfolipid were greater for malathion-treated rats than for controls, but the reverse was true in the mitochondrial and residual lipoprotein sulfolipid. In all three particulates, there was a lower molar ratio of sulfate to galactose in the lipoprotein sulfolipid from malathion-treated rats than from the untreated group.

Arylsulfatase activity was measured in liver homogenates of malathion-treated and untreated rats in order to determine if the decreased amounts of sulfolipid sulfate and the decreased sulfate to galactose molar ratios in malathion-treated rats were due to increased arylsulfatase activity. As shown in Table 13, the amount of the substrate, p-nitrocatechol sulfate, hydrolyzed by arylsulfatase was

TABLE 11

Total sulfate, ^{35}S activity, and specific activity of sulfolipid sulfate from liver lipoprotein extracted from cellular particulates of control and malathion-treated rats[a]

Cellular particulates	Mg malathion/ kg of rat	mmoles $SO_4^=$ x 10^4	cpm x 10^{-2}	cpm/mmole $SO_4^=$ x 10^{-4}
Nuclei	0	235	145	62
	250	86	202	235
Mitochondria	0	180	184	107
	250	47	86	182
Residue	0	170	67	51
	250	55	64	118

[a]Cellular particulates were extracted from 16 pooled livers from control rats and 20 pooled livers from malathion-treated rats fed a diet containing an intermediate level of sulfate (0.10 percent).

TABLE 12

Specific activity, mmole ratio and total galactose in the sulfolipid fraction of lipoprotein prepared from the cellular particulates of livers from control and malathion-treated rats[a]

Cellular particulates	Mg malathion/ kg of rat	Galactose		
		mg	cpm/mg x 10^{-2}	mmoles $SO_4^=$/ mmole
Nuclei	0	2.18	67	1.94
	250	1.67	121	0.92
Mitochondria	0	2.52	73	1.29
	250	2.04	42	0.42
Residue	0	1.84	36	1.66
	250	2.29	28	0.43

[a]Cellular particulates were extracted from 16 pooled livers from control rats and 20 pooled livers from malathion-treated rats fed a diet containing an intermediate level of sulfate (0.10 percent).

TABLE 13

The effect of malathion intoxication on liver arylsulfatase activity[a]

Mg malathion/kg of rat	µg NC[b]/g liver x 10^{-2}	µg NC/mg DNA x 10^{-2}	µg NC/mg N
0	113 ± 13[c]	116 ± 13	263 ± 17[d]
250	118 ± 7	123 ± 6	326 ± 20[d]

[a]Arylsulfatase activity was estimated in homogenates of three pooled livers of rats fed a diet containing an intermediate level of sulfate (0.10 percent).

[b]4-nitrocatechol (NC).

[c]Mean of five replications ± SE.

[d]Difference is statistically significant ($P<0.05$) by the method of unpaired comparisons (152).

increased in malathion intoxication when based on liver weight, mg DNA and mg nitrogen. The amount of the product, 4-nitrocatechol, was significantly increased when controls were compared with the experimental group only when based on mg nitrogen ($P < 0.01$). The nitrogen content based on mg DNA as shown in Table 14 was not significantly different in malathion-treated rats as compared with controls.

TABLE 14

Nitrogen content of livers of control and malathion-treated rats[a]

Mg malathion/kg of rat	mg N/mg DNA
0	32.15 ± 1.91[b]
250	32.26 ± 1.38

[a]Nitrogen was estimated in homogenates of three pooled livers of rats fed a diet containing an intermediate level of sulfate (0.10 percent).

[b]Mean of five replications ± SE.

CHAPTER V

DISCUSSION

The effect of malathion intoxication on cellular lipoprotein and its fractions from livers of rats fed diets containing three levels of inorganic sulfur resulted in a general ^{35}S labeling pattern as follows: a decrease in specific activity when rats were fed diet A but increases in specific activity when rats were fed diets B and C which contain lower levels of inorganic sulfur. $Na_2{}^{35}SO_4$ was administered to rats on the third day of malathion treatment; therefore, the diet-related differences in the specific activities of lipoprotein and its fractions probably result from attempts to repair effects of malathion intoxication. As shown in previous studies of the relationship of malathion intoxication to inorganic sulfur metabolism (4), less $^{35}SO_4{}^{=}$ was mobilized from the tissues of malathion-treated rats fed diet A (0.42 percent inorganic sulfur), indicating that rats fed the diet containing a high level of inorganic sulfur were not stressed as greatly as rats fed diets containing the lower levels of inorganic sulfur. The increased specific activity of lipoprotein and its fractions from malathion-stressed rats fed diets intermediate and low with respect to sulfate in this study also may reflect a protective effect of a high level of dietary sulfate. The increased specific activities of lipoprotein fractions from malathion-stressed rats as compared with controls fed diets B and C could be envisioned to be due to replacement

of tissue sulfate mobilized during malathion intoxication. In the previous study in which mobilization of ^{35}S was observed, the rats were fed a ^{35}S labeled diet before malathion was administered; therefore, when $^{35}SO_4^=$ is administered after intoxication, as in the study presently under discussion, replacement of mobilized unlabeled sulfate with $^{35}SO_4^=$ would result in increased tissue specific activities. Rats fed diet A were stressed to a lesser degree and probably did not mobilize as much tissue sulfate during malathion intoxication as rats fed the diets containing lower levels of sulfate; therefore, these rats did not incorporate as much $^{35}SO_4^=$ into tissue-sulfate compounds as the malathion-stressed rats in the other two groups. PAPS (3'-phosphoadenosine 5'-phosphosulfate) and its metabolites are the primary sulfated compounds isolated in the nucleotide fraction from cellular lipoprotein.[1] As a result of malathion-stress, a 7 percent increase in the specific activity of the nucleotide fraction was obtained when rats were fed diet A compared with a 29 percent increase in values from rats fed diet C. The comparative increase in specific activity of the nucleotide fraction of malathion-treated rats fed diet C could be interpreted as indicating the presence of more PAPS and therefore more sulfate incorporating ability in malathion-stressed rats fed the low sulfate diet. Increased PAPS synthesis, in this case, would be induced by malathion intoxication.

Malathion-stress caused a reversal in the normal labeling patterns of liver lipoprotein fractions. Values for specific activities

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[1] Button, G. M., unpublished observation.

of lipoprotein fractions in general decreased within the control group as the level of dietary sulfate decreased. This trend has also been previously observed in the specific activity of cartilage mucopolysaccharide by Disney (4) and Michels and Smith (2). The trend for values for specific activity within the malathion group was a reversal of the control values or an increase in specific activity with decreased dietary inorganic sulfate (Table 7, page 56). The reason for the altered pattern of lipoprotein fractions within the malathion-stressed group is due to attempts to recover from the trauma of malathion intoxication. This trauma was increased as the level of dietary sulfate decreased.

Although consistent trends are observed in the data from lipoprotein fractionation, the only statistically significant difference between groups ($P < 0.05$) was the increase in incorporation of $^{35}SO_4^=$ into the sulfolipid fraction. Davison and Gregson (82) concluded that sulfolipids are true membrane lipids. Organophosphorus insecticides have been shown to have a specific effect on membranes, i.e., demyelination (40); therefore, the reason for a specific effect of malathion on the sulfolipid fraction from lipoprotein may be due to a specific effect on cell membrane sulfolipid. Feland and Smith (153) found that malathion intoxication caused a loss in mitochondrial integrity as demonstrated by decreased mitochondrial swelling. They found the loss in mitochondrial integrity to be accompanied by decreased mitochondrial

hexosamine content. Mitochondria were not analyzed for sulfolipid content in the study by Feland and Smith (153).

In order to examine the effect of malathion stress on sulfolipid from cellular lipoprotein particulates, nuclei, mitochondria and the residual portion (which would normally be in lipoprotein) were isolated from rat liver. Galactose, sulfate, and ^{35}S activity were determined in the lipoprotein prepared from these three particulates in an attempt to elucidate the reason for increased $^{35}SO_4^=$ incorporation in the sulfolipid fraction of malathion-stressed rats (see Tables 11 and 12, pages 67 and 68). The question asked of this study was: is the increased $^{35}SO_4^=$ incorporation due to (a) de novo synthesis or to (b) exchange of labeled for unlabeled $SO_4^=$?

The mmoles $SO_4^=$ in lipoprotein sulfolipid were greatly decreased in all three particulates. Nuclei were the only cellular components showing an increase in total ^{35}S activity, but when data were expressed as cpm/mmole $SO_4^=$, an increase in specific activity was observed in all three particulates. The greatest increase in specific activity of lipoprotein sulfolipid was observed in the nuclei; therefore, lipoprotein sulfolipid from nuclei appeared to be more active than lipoprotein sulfolipid from mitochondria or the residue in incorporating $^{35}SO_4^=$.

Examination of the ratio of $^{35}SO_4^=$ incorporated into sulfolipid to the mgs of galactose indicates the relative difference in ^{35}S labeling of the sulfolipid molecule (see Table 12, page 68) in control and malathion-stressed rats. As seen in Table 11, page 67, the lipoprotein sulfolipid from nuclei of malathion-stressed rats contained the lowest

amount of sulfate and the most ^{35}S activity. When ^{35}S activity is expressed as cpm/mg galactose, the only increase in the specific activity of galactose was found in the nuclear sulfolipid; therefore, nuclear sulfolipid appeared to be most active in replacing the mobilized sulfate with labeled sulfate.

Cellular lipoprotein prepared by exhaustive salt extraction as in this study consists primarily of membranes. Hogeboom et al. (154) found 26.8×10^{-6} nuclei and 10.7×10^{-9} mitochondria per 100 mg of fresh liver homogenate; therefore, nuclear membranes represent a large percentage of the composition of lipoprotein. The increased specific activity of lipoprotein was probably to a great extent a reflection of the increased ^{35}S activity in nuclear lipoprotein from malathion-stressed rats. The decreased integrity of the mitochondrial membrane found in the study by Feland and Smith (153) was probably due to decreased membrane sulfolipid as well as decreased hexosamine.

Cellular lipoprotein without nuclei and mitochondria consists primarily of cell membranes and is equivalent to the residual lipoprotein prepared in this study. Residual lipoprotein had the greatest decrease in the molar ratio of sulfate to galactose, and therefore, may have been the most affected by malathion intoxication. However, there appeared to be little effort in this particulate to replace the mobilized sulfate since the cpm/mg of galactose were decreased when controls were compared with malathion-stressed rats as was the case in mitochondrial lipoprotein.

When a ratio was calculated for mmoles $SO_4^=$ to mmoles of galactose, the ratio was lower in lipoprotein sulfolipid from malathion-treated rats as compared with controls in all three particulates. The interrelationships among these data indicate that in all three particulates, malathion intoxication caused release of the sulfate esterified to the galactose moiety of sulfolipid.

If sulfate is released from sulfolipid during malathion intoxication as the data discussed above indicate, liver arylsulfatase activity should be increased in malathion-stressed rats. Determination of arylsulfatase activity in homogenates of livers from control and malathion-treated rats revealed an increase in arylsulfatase activity, thereby supplying additional evidence that sulfate was mobilized from sulfolipid of malathion-stressed rats. Pokrovskii and Tutel'yan (127) found increased arylsulfatase activity in the supernatant fluid of livers from rats stressed by another toxic substance, aflatoxin. The release of lysosomal arylsulfatases into liver supernatant fluid of aflatoxin-stressed rats was due to increased lysosomal permeability. The author proposes that malathion intoxication also causes increased lysosomal permeability. Feland and Smith (153) have shown that mitochondria from malathion-stressed rats exhibit increased permeability. It seems reasonable to assume that malathion intoxication would also affect the permeability of other cellular organelles since malathion was shown to have a specific effect on sulfolipid, a constituent of membranes.

During malathion intoxication, it is proposed that arylsulfatase in released into the cytoplasm and hydrolyzes the ester sulfate bond

of membrane sulfolipid to release sulfate. The purpose of the release of sulfate from sulfolipid is not clear. Roe (144) has shown that increased dietary sulfate decreased the toxicity of indole which is excreted in the urine as an ethereal sulfate. There is no evidence in the literature to support the presence of a sulfated excretory product of malathion. The sulfate released from sulfolipid may not be bound to the pesticide but may by indirectly involved in its detoxication. Fukami and Shiskido (155) have shown that a liver enzyme which cleaved parathion, an organophosphorus pesticide, required reduced glutathion for its activity in demethylating parathion. Consumption of inadequate levels of inorganic sulfur results in oxidation of neutral sulfur to supply the inorganic sulfur needs. Neutral sulfur could be lacking during periods of excessive glutathion synthesis, thereby depleting the reserve source of inorganic sulfur. Under conditions such as these if dietary sources of inorganic sulfur were not adequate, tissue sulfate would have to be mobilized to supply inorganic sulfur needs.

Mobilization of sulfate from such an essential ester sulfate as sulfolipid may seem unlikely; perhaps another class of ester sulfate compounds such as mucopolysaccharide would seem a more logical source for the mobilized sulfate. The ease of release of sulfate from the compound may be the reason for the selection of sulfolipid rather than mucopolysaccharide for the source of the mobilized sulfate. The ester bond of sulfolipid can be cleaved directly by arylsulfatase A (115) to release sulfate while mucopolysaccharide must first be hydrolyzed into

simpler fragments before it can be desulfated (118,156).

The data discussed in this dissertation indicate an increased need for dietary inorganic sulfur during organophosphorus stress. This study seems particularly relevant when the current problems of atmospheric pollution are considered. There seems to be reason for concern as the use of organophosphorus pesticides and other types of physiological stress increase while accompanied by efforts to rid the air of fossil fuel pollutants, a link in our natural sulfur cycle.

CHAPTER VI

SUMMARY

Macromolecules (sulfolipid, mucopolysaccharide, nucleotide, and sulfate transfer fraction) were extracted from cellular lipoprotein from the livers of control and malathion-treated rats fed diets containing 0.42, 0.10, and 0.0002 percent sulfate. A statistically significant increase was found in in vivo $^{35}SO_4^=$ incorporation into the sulfolipid fraction of malathion-stressed rats as compared with controls fed the diet containing 0.10 percent sulfate. In general, malathion stress caused increases in specific activities which were intensified by decreasing the level of dietary sulfate.

Determination of total galactose, sulfate, and ^{35}S activity in liver lipoprotein sulfolipid extracted from nuclei, mitochondria, and the residue of rats fed a diet containing 0.10 percent sulfate indicated that nuclear sulfolipid was the component most active in $^{35}SO_4^=$ incorporation and probably was responsible for the increased ^{35}S activity previously observed in cellular lipoprotein sulfolipid. The galactose: sulfate molar ratio in cellular lipoprotein sulfolipid from the cell particulates indicated that sulfate was being mobilized from sulfolipid. Further evidence for the mobilization of sulfate from sulfolipid was provided by the finding of a statistically significant increase in arylsulfatase activity per mg of nitrogen in liver homogenates of malathion-stressed rats as compared with controls.

LITERATURE CITED

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1. Button, G. M., R. G. Brown, F. G. Michels and J. T. Smith 1965 Utilization of calcium and sodium sulfate by the rat. J. Nutr. 87: 211.

2. Michels, F. G., and J. T. Smith 1965 A comparison of the utilization of organic and inorganic sulfur by the rat. J. Nutr. 87: 217.

3. Disney, G. W., and J. T. Smith 1967 Sulfur metabolism and malathion toxicity. Federation Proc. 26: 427.

4. Disney, G. W. 1967 Alterations in sulfur metabolism in malathion-treated rats. Unpublished Master's thesis, The University of Tennessee, Knoxville.

5. Disney, G. W., and J. T. Smith 1970 Malathion intoxication and sulfation of mucopolysaccharides. J. Agr. Food Chem. 18: 540.

6. Frear, D. E. H. 1955 Chemistry of the Pesticides. D. Van Nostrand Company, Inc., New York, p. 86.

7. Casida, J. E. 1964 Esterase inhibitors as pesticides. Science 146: 1011.

8. Martin, H. 1961 Guide to the Chemicals Used in Crop Protection, Pub. 1093, Canada Department of Agriculture, Queens' Printer and Controller of Stationery, Ottowa, p. 273.

9. White, A., P. Handler and E. L. Smith 1968 Principles of Biochemistry. McGraw-Hill Book Company, Inc., New York, p. 866.

10. Davison, A. N. 1955 The conversion of schradan (OMPA) and parathion into inhibitors of cholinesterase by mammalian liver. Biochem. J. 61: 230.

11. Namba, T. 1971 Cholinesterase inhibition by organophosphorus compounds and its clinical effects. Bull. World Health Org. 44: 289.

12. Main, R. A., and P. E. Braid 1962 Hydrolysis of malathion by ali-esterases in vitro and in vivo. Biochem. J. 84: 255.

13. Dauterman, W. C. 1971 Biological and nonbiological modifications of organophosphorus compounds. Bull. World Health Org. 44: 133.

14. Main, A. R., and F. L. Hastings 1966 A comparison of acylation, phosphorylation and binding in related substrates and inhibitors of serum cholinesterase. Biochem. J. 101: 584.

15. Hassan, A., and W. C. Dauterman 1968 Studies on the optically active isomers of 0,0-diethyl malathion and 0,0-diethyl malaoxon. Biochem. Pharmacol. 17: 1431.

16. Chiu, Y. C., A. R. Main and W. C. Dauterman 1969 Affinity and phosphorylation constants of a series of 0,0-dialkyl malaoxons and paraoxons with acetylcholinesterase. Biochem. Pharmacol. 18: 2171.

17. Mahfouz, A. M. M., R. L. Metcalf and T. R. Fukuto 1969 Influence of the sulfur atom on the anticholinesterase and insecticidal properties of thioether N-methylcarbamates. J. Agr. Food. Chem. 17: 917.

18. Zahavi, M., A. S. Tahori and F. Klimer 1971 Insensitivity of acetylcholinesterases to organophosphorus compounds as related to size of esteratic site. Mol. Pharmacol. 7: 611.

19. Dubois, K. P. 1971 The toxicity of organophosphorus compounds to mammals. Bull. World Health Org. 44: 233.

20. Cook, J. W., and G. Yip 1958 Malathionase II. Identity of a malathion metabolite. J. A. O. A. C. 41: 407.

21. Chen, P. R., W. P. Tucker and W. C. Dauterman 1969 Structure of biologically produced malathion monoacid. J. Agr. Food Chem. 17: 86.

22. Bourke, J. B., E. J. Broderick, L. R. Hackler and P. C. Lippold 1968 Comparative metabolism of malathion-C^{14} in plants and animals. J. Agr. Food Chem. 16: 585.

23. Menzer, R. E., and W. C. Dauterman 1970 Metabolism of some organophosphorus insecticides. J. Agr. Food Chem. 18: 1031.

24. Miyata, T., and F. Matsumura 1972 Organophosphate degrading enzymes in the crude supernatant fraction from the rat liver. J. Agr. Food Chem. 20: 30.

25. Sakai, K., and F. Matsumura 1968 Esterases of mouse brain active in hydrolyzing organophosphate and carbamate insecticides. J. Agr. Food Chem. 16: 803.

26. Cook, J. W., J. R. Blake, G. Yip and M. Williams 1958 Malathionase I. Activity and inhibition. J. A. O. A. C. 41: 399.

27. Murphy, S. D. 1967 Malathion inhibition of esterases as a determinant of malathion toxicity. J. Pharmacol. Exp. Ther. 156: 352.

28. Murphy, S. D., and K. L. Cheever 1968 Effect of feeding insecticides. Inhibition of carboxyesterase and cholinesterase activities in rats. Arch. Environ. Health 17: 749.

29. Cohen, S. D., and S. D. Murphy 1972 Inactivation of malaoxon by mouse liver. Proc. Soc. Exp. Biol. Med. 139: 1385.

30. Aldridge, W. N. 1953 Serum esterases 1. Two types of esterases (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination. Biochem. J. 53: 110.

31. Aldridge, W. N. 1953 The inhibition of erythrocyte cholinesterases by tri-esters of phosphoric acid. 3. The nature of the inhibitory process. Biochem. J. 54: 442.

32. Albert, A. 1965 Selective Toxicity. John Wiley and Sons, Inc., New York, p. 280.

33. Read, S. I., and W. P. McKinley 1963 A comparison of the relative amounts of tissue esterases in liver and kidney homogenates. J. A. O. A. C. 46: 863.

34. Villeneuve, D. C., and W. P. McKinley 1968 Inhibition of beef liver hydrolytic enzymes by organophosphorus pesticides. J. Agr. Food Chem. 16: 290.

35. Bergmann, F., R. Segal and S. Rimon 1957 A new type of esterase in hog-kidney extract. Biochem. J. 67: 481.

36. Aldridge, W. N., J. M. Barnes and M. K. Johnson 1969 Studies on delayed neurotoxicity produced by some organophosphorus compounds. Ann. N. Y. Acad. Sci. 160: 314.

37. Johnson, M. K. 1969 A phosphorylation site in brain and the delayed neurotoxic effect of some organophosphorus compounds. Biochem. J. 111: 487.

38. Johnson, M. K. 1969 Delayed neurotoxic action of some organophosphorus compounds. Brit. Med. Bull. 25: 231.

39. Johnson, M. K. 1969 The delayed neurotoxic effect of some organophosphorus compounds. Biochem. J. 114: 711.

40. Barnes, J. M., and F. A. Denz 1953 Experimental demyelination with organophosphorus compounds. J. Path. Bacteriol. 65: 597.

41. Kimbrough, R. D. 1968 Effect of organic phosphorus compounds and alkylating agents on the rat fetus. Arch. Environ. Health 16: 805.

42. Ho, M., and M. A. Gibson 1972 Histogenesis of the tibio tarsus in malathion-treated chick embryos. Can. J. Zool. 50: 771.

43. Gill, G. R., and Q. N. LaHam 1972 Histochemical and radiographic investigations of malathion-induced malformations in embryonic chick limbs. Can. J. Zool. 50: 349.

44. Upshall, D. G., Jean-Claude Roger and J. E. Casida 1968 Biochemical studies on the teratogenic action of bidrin and other neuroactive agents in developing hen eggs. Biochem. Pharmacol. 17: 1529.

45. Roe, D. A. 1971 Effects of methionine and inorganic sulfate on indole toxicity and indican excretion in rats. J. Nutr. 101: 645.

46. Boyd, E. M., and T. K. Tanikella 1969 The acute oral toxicity of malathion in relation to dietary protein. Arch. Toxikol. 24: 292.

47. Boyd, E. M., C. J. Krijnen and T. K. Tanikella 1970 The influence of a wide range of dietary protein concentration on the acute oral toxicity of malathion. Arch. Toxikol. 26: 125.

48. Gardner, A. L., and R. E. Iverson 1968 The effect of aerially applied malathion on an urban population. Arch. Environ. Health 16: 823.

49. Wolfe, H. R., J. F. Armstrong, D. C. Staiff and S. W. Comer 1972 Exposure of spraymen to pesticides. Arch. Environ. Health 25: 29.

50. Froede, H. C., and I. B. Wilson 1971 Acetylcholinesterase. In: The Enzymes, ed. P. D. Boyer, Vol. 5, Academic Press, New York, p. 87.

51. Wilson, I. B. 1951 Acetylcholinesterase XI. Reversibility of tetraethyl pyrophosphate inhibition. J. Biol. Chem. 190: 111.

52. Quinby, G. E. 1964 Further therapeutic experience with pralidoximes in organic phosphorus poisoning. J. Amer. Med. Ass. 187: 202.

53. Stecher, P. G., ed. 1960 The Merck Index of Chemicals and Drugs, Merck and Co., Rahway, New Jersey, p. 111.

54. Quinby, G. E. 1968 Feasibility of prophylaxis by oral pralidoxime. Arch. Environ. Health 16: 812.

55. Shafik, M. T., and H. F. Enos 1969 Determination of metabolic and hydrolytic products of organophosphorus pesticide chemicals in human blood and urine. J. Agr. Food Chem. 17: 1186.

56. Mattson, A. M., and V. A. Sedlak 1960 Ether-extractable urinary phosphates in man and rats derived from malathion and similar compounds. J. Agr. Food Chem. 8: 107.

57. Durham, W. F., H. R. Wolfe and J. W. Elliott 1972 Absorption and excretion of parathion by spraymen. Arch. Environ. Health 24: 381.

58. Bunyan, P. J., D. M. Jennings and A. Taylor 1968 Organophosphorus poisoning: Some properties of avian esterases. J. Agr. Food Chem. 16: 326.

59. Bunyan, P. J., D. M. Jennings and A. Taylor 1968 Organophosphorus poisoning: Diagnosis of poisoning in pheasants owing to a number of common pesticides. J. Agr. Food Chem. 16: 332.

60. Goldberg, I. H. 1961 The sulfolipids. J. Lipid Res. 2: 103.

61. Yamakawa, T., N. Kiso, S. Handa, A. Makita and S. Yokoyama 1962 On the structure of brain cerebroside sulfuric ester and ceramide dihexoside of erythrocytes. J. Biochem. 52: 226.

62. Chargaff, E., C. Levine and C. Green 1948 Techniques for the demonstration by chromatography of nitrogenous lipid constituents, sulfur-containing amino acids and reducing sugars. J. Biol. Chem. 175: 67.

63. Svennerholm, L. 1964 The distribution of lipids in the human nervous system I. Analytical procedure. Lipids of foetal and newborn brain. J. Neurochem. 11: 839.

64. Mårtensson, E. 1963 On the sulfate containing lipids of human kidney. Acta Chem. Scand. 17: 1174.

65. Mårtensson, E. 1966 Sulfatides of human kidney: Isolation, identification, and fatty acid composition. Biochim. Biophys. Acta 116: 521.

66. Mårtensson, E. 1966 Neutral glycolipids of human kidney: Isolation, identification, and fatty acid composition. Biochim. Biophys. Acta 116: 296.

67. Heald, P. J., and M. A. Robinson 1961 The metabolism of sulphatides in cerebral tissues. Biochem. J. 81: 157.

68. Stoffyn, P., and A. Stoffyn 1963 Structure of sulfatides. Biochim. Biophys. Acta 70: 218.

69. Kishimoto, Y., and N. S. Radin 1966 Metabolism of brain glycolipid fatty acids. Lipids 1: 47.

70. Pakkala, S. G., D. L. Fillerup and J. F. Mead 1966 The very long chain fatty acids of human brain sphingolipids. Lipids 1: 449.

71. Jatzkewitz, H. 1960 Die leukodystrophie, typ scholz als sphingolipoidose. Z. Physiol. Chem. 318: 265.

72. Hauser, G. 1964 Labeling of cerebrosides and sulfatides in rat brain. Biochim. Biophys. Acta 84: 212.

73. Cleland, W. W., and E. P. Kennedy 1960 The enzymatic synthesis of psychosine. J. Biol. Chem. 235: 45.

74. Brady, R. O. 1962 Studies on the total enzymatic synthesis of cerebrosides. J. Biol. Chem. 237: PC 2416.

75. Morell, P., and N. S. Radin 1969 Synthesis of cerebroside by brain from uridine diphosphate galactose and ceramide-containing hydroxyl fatty acids. Biochem. 8: 506.

76. Morell, P., E. Costantino-Ceccarine and N. S. Radin 1970 The biosynthesis by brain microsomes of cerebrosides containing non-hydroxy fatty acids. Arch. Biochem. Biophys. 141: 738.

77. Goldberg, I. H., and A. Delbrück 1959 Transfer of sulfate from 3'-phosphoadenosine-5'-phosphosulfate to lipids, mucopolysaccharides and aminoalkyl phenols. Federation Proc. 18: 235.

78. Balasurbamanian, A. S., and B. K. Bachhawat 1965 Formation of cerebroside sulphate from 3'-phosphoadenosine-5'-phosphosulphate in sheep brain. Biochim. Biophys. Acta 106: 218.

79. McKhann, G. M., R. Levy and W. Ho 1965 The effect of galactocerebrosides on the synthesis of sulfatides. Biochem. Biophys. Res. Comm. 20: 109.

80. Farrell, D. F., and G. M. McKhann 1971 Characterization of cerebroside sulfotransferase from rat brain. J. Biol. Chem. 246: 4694.

81. McKhann, G. M., and W. Ho 1967 The in vivo and in vitro synthesis of sulphatide during development. J. Neurochem. 14: 717.

82. Davison, A. N., and N. A. Gregson 1962 Physiological role of cerebronsulfuric acid (sulfatide) in the brain. Biochem. J. 85: 558.

83. Galli, C., and D. ReCecconi 1967 Lipid changes in rat brain during maturation. Lipids 2: 76.

84. Suzuki, K., S. E. Poduslo and W. T. Norton 1967 Gangliosides in the myelin fractions of developing rats. Biochim. Biophys. Acta 144: 375.

85. Chase, H. P., J. Dorsey and G. M. McKhann 1967 The effect of malnutrition on the synthesis of a myelin lipid. Pediatrics 40: 551.

86. Pritchard, E. T. 1966 In vivo labeling of sulfatides from sulfate-^{35}S in rat brain during early growth. J. Neurochem. 13: 13.

87. Herschkowitz, N., G. M. McKhann, S. Saxena and E. M. Shooter 1968 Characterization of sulfatide-containing lipoproteins in rat brain. J. Neurochem. 15: 1181.

88. Herschkowitz, N., G. M. McKhann, S. Saxena, E. M. Shooter and R. Herndon 1969 Synthesis of sulfatide-containing lipoproteins in rat brain. J. Neurochem. 16: 1049.

89. Mandel, P., and R. Bieth 1951 Fixation du terme de la croissance du cerveau chez le rat par l'elude de l'acide deoxy-pentose nucleique. Experientia 7: 343.

90. Norton, W. T., and S. E. Poduslo 1971 Neuronal perikarya and astroglia of rat brain: Chemical composition during myelination. J. Lipid Res. 12: 84.

91. Autilio, L. A., W. T. Norton and R. D. Terry 1964 The preparation and some properties of purified myelin from the central nervous system. J. Neurochem. 11: 17.

92. Fishman, M. A., P. Madyastha and A. L. Prensky 1971 The effect of undernutrition on the development of myelin in the rat central nervous system. Lipids 6: 458.

93. O'Brien, J. S., and E. L. Sampson 1965 Brain lipids. III. Lipid composition of the normal human brain; gray matter, white matter and myelin. J. Lipid Res. 6: 537.

94. Cuzner, M. L., A. N. Davison and N. A. Gregson 1965 The chemical composition of vertebrate myelin and microsomes. J. Neurochem. 12: 469.

95. McIlwain, H. 1955 Biochemistry and the central nervous system. J. and A. Churchill, Ltd., London, p. 148.

96. Norton, W. T., and L. A. Autilio 1965 The chemical composition of bovine CNS myelin. Ann. N. Y. Acad. Sci. 122: 77.

97. Green, J. P., and J. D. Robinson, Jr. 1960 Cerebroside sulfate (sulfatide A) in some organs of the rat and in mast cell tumor. J. Biol. Chem. 235: 1621.

98. Goldberg, I. H. 1960 Parallels between enzymatic sulfurylation of N-acetyl sphingosine and chloramphenicol. Federation Proc. 19: 220.

99. Spiro, M. J., and J. M. McKibbin 1956 The lipids of rat liver fractions. J. Biol. Chem. 219: 643.

100. Getz, G. S., W. Bartley, F. Stirpe, B. M. Notton and A. Renshaw 1962 The lipid composition of rat-liver mitochondria, fluffy layer and microsomes. Biochem. J. 83: 181.

101. Eichberg, J., V. P. Whittaker and R. M. C. Dawson 1964 Distribution of lipids in subcellular particles of guinea-pig brain. Biochem. J. 92: 91.

102. Radin, N. S., F. B. Martin and J. R. Brown 1957 Galactolipide metabolism. J. Biol. Chem. 224: 499.

103. Davison, A. N., and N. A. Gregson 1966 Metabolism of cellular membrane sulfolipids in the rat brain. Biochem. J. 98: 915.

104. Cuzner, M. L., A. N. Davison and N. A. Gregson 1965 Chemical and metabolic studies of rat myelin of the central nervous system. Ann. N. Y. Acad. Sci. 122: 86.

105. Fleischer, S., and G. Rouser 1965 Lipids of subcellular particles. J. A. O. A. C. 42: 588.

106. Eng, L. F., and M. E. Smith 1966 The cholesterol complex in the myelin membrane. Lipids 1: 296.

107. Clausen, J. 1969 Effect of vitamin A deficiency on myelination in the central nervous system of the rat. Eur. J. Biochem. 7: 575.

108. Kean, E. 1970 Vitamin A deficiency and glycolipid sulfation. J. Lipid Res. 11: 284.

109. Wolf, G. 1961 Vitamin A in adrenal hormone and mucopolysaccharide biosynthesis. Amer. J. Clin. Nutr. 9: 36.

110. Pasternak, C. A., and D. B. Thomas 1969 Metabolism of sulfated mucopolysaccharides in vitamin A deficiency. Amer. J. Clin. Nutr. 22: 986.

111. Roy, A. B. 1953 The sulphatase of ox liver. 1. The complex nature of the enzyme. Biochem. J. 53: 12.

112. Roy, A. B. 1953 The sulphatase of ox liver. 2. The purification and properties of sulphatase A. Biochem. J. 55: 653.

113. Roy, A. B. 1954 The intracellular distribution of sulphatase. Biochim. Biophys. Acta 14: 149.

114. Dodgson, K. S., B. Spencer and J. Thomas 1955 Studies on sulphatases. 9. The arylsulphatases of mammalian liver. Biochem. J. 59: 29.

115. Mehl, E., and H. Jatzkewitz 1968 Cerebroside 3-sulfate as a physiological substrate of arylsulfatase A. Biochim. Biophys. Acta 151: 619.

116. Farooqui, A. A., and B. K. Bachhawat 1972 Purification and properties of arylsulphatase A from chicken brain. Biochem. J. 126: 1025.

117. Austin, J., A. S. Balasubramanian, T. Pattabiramam, S. Saraswathi, D. K. Basu and B. K. Bachhawat 1963 A controlled study of enzymic activities in three human disorders of glycolipid metabolism. J. Neurochem. 10: 805.

118. Dodgson, K. S., and B. Spencer 1957 Assay of sulfatases. In: Methods of Biochemical Analysis, ed. D. Glick, Vol. IV, Interscience Publishers, Inc., New York, p. 211.

119. Baum, H., K. S. Dodgson and B. Spencer 1958 Studies on sulphatases. 21. The anomalous kinetics of arylsulphatase A of human tissues: The anomalies. Biochem. J. 69: 567.

120. Baum, H., and K. S. Dodgson 1958 Studies on sulphatases. 22. The anomalous kinetics of arylsulphatase A of human tissues: Interpretation of the anomalies. Biochem. J. 69: 573.

121. Roy, A. B. 1957 The kinetics of sulphatase A. Experientia 13: 32.

122. Percy, A. K., D. F. Farrell and M. M. Kaback 1972 Cerebroside sulfate (sulfatide) sulfohydrolase: Improved assay method. J. Neurochem. 19: 233.

123. Philippart, M., L. Sarbive, C. Meurant and L. Mechler 1971 Human urinary sulfatides in patients with sulfatidosis (metachromatic leukodystrophy). J. Lipid Res. 12: 434.

124. Roy, A. B. 1960 The sulphatase of ox liver. 7. The intracellular distribution of sulphatases A and B. Biochem. J. 77: 380.

125. Thyberg, J. 1972 Ultrastructural localization of aryl sulfatase activity in the epiphyseal plate. J. Ultrast. Res. 38: 332.

126. Pokrovskii, A. A., L. V. Kravchenko and V. A. Tutel'yan 1971 Study of the enzyme activity of lysosomes in the presence of aflatoxin and mytomycin C. Biokhimiya 36: 690 (translated in Biochemistry (Biokhimiia) 36: 579).

127. Pokrovskii, A. A., and V. A. Tutel'yan 1968 Changes in the enzymes of lysosomes during protein deficiency. Biokhimiya 33: 809 (translated in Biochemistry (Biokhimiia) 33: 662).

128. Wellers, G., G. Boelle and J. Chevan 1960 Studies of sulfur metabolism. 4. Part replacement of endogenous loss of sulfur by sodium sulfate in the adult rat. J. Physiol. (Paris) 52: 903.

129. Casida, J. E. 1963 Problems posed by plant and animal metabolism of pesticides. In: New Developments and Problems in the Use of Pesticides, Foods Protection Committee, Food and Nutrition Board, Pub. 1082, National Academy of Sciences-National Research Council, Washington, D. C., p. 39.

130. Murphy, S. D., and K. P. Dubois 1955 Quantitative measurement of inhibition of the enzymatic detoxification of malathion by EPN. Proc. Soc. Exp. Biol. Med. 96: 813.

131. Taylor, W. J., W. Kalow and E. A. Sellers 1965 Poisoning with organophosphorus insecticides. Can. Med. Ass. J. 93: 966.

132. Young, J. M., and J. S. Dinning 1951 A relationship of vitamin E to nucleic acid metabolism. J. Biol. Chem. 193: 743.

133. Caputto, R., P. B. McCay and M. P. Carpenter 1958 Requirements of Mn^{++} and Co^{++} for the synthesis of ascorbic acid by liver extracts of animals deprived of tocopherol. J. Biol. Chem. 233: 1025.

134. Hubbell, R. G., L. B. Mendel and A. J. Wakeman 1937 A new salt mixture for use in experimental diets. J. Nutr. 14: 273.

135. Stolman, A., and C. P. Stewart 1965 The absorption, distribution, and excretion of poisons and their metabolites. In: Progress in Chemical Toxicology, ed. A. Stolman, Academic Press, New York, p. 137.

136. Stavinoha, W. B., J. A. Rieger, Jr., L. C. Ryan and P. W. Smith 1966 Effects of chronic poisoning by an organophosphorus cholinesterase inhibitor on acetylcholine and norepinephrine content of the brain. Advan. Chem. Ser. 60: 79.

137. Fulton, S. F. 1966 The effect of avitaminosis E on the incorporation of ^{35}S-sulfate by rat tissue components. Unpublished Master's thesis, The University of Tennessee, Knoxville.

138. Fulton, S. F., and J. T. Smith 1970 Effect of avitaminosis E on the incorporation of ^{35}S-sulfate by rat tissue components. J. Nutr. 100: 525.

139. Smith, J. T., A. J. Funckes, A. J. Barak and L. E. Thomas 1957 Cellular lipoproteins. I. The insoluble lipoprotein of whole liver cells. Exp. Cell Res. 13: 96.

140. Levin, E., and L. E. Thomas 1961 Cellular lipoproteins. III. The insoluble lipoproteins of rat liver cell fractions. Exp. Cell Res. 22: 363.

141. Svennerholm, L. 1956 The quantitative estimation of cerebrosides in nervous tissue. J. Neurochem. 1: 42.

142. Willets, C. O., and C. L. Ogg 1950 Report on standardization of microchemical methods. Micro-Kjeldahl nitrogen determination. J. A. O. A. C. 33: 179.

143. Mahin, D. T., and P. T. Lofberg 1966 A simplified method of sample preparation for determination of tritium, carbon-14, or sulfur-35, in blood or tissue by liquid scintillation counting. Anal. Biochem. 16: 500.

144. Roe, D. A., D. S. Miller and L. Lutwak 1966 Estimation of sulfur in biological materials by atomic absorption spectrometry. Anal. Biochem. 15: 313.

145. Hogeboom, G. H. 1955 Fractionation of cell components of animal tissues. In: Methods in Enzymology, Vol. I, eds. S. P. Colowick and N. O. Kaplan, Academic Press, Inc., New York, p. 16.

146. Keller, E. B., and P. C. Zamecnik 1956 The effect of guanosine diphosphate and triphosphate on the incorporation of labeled amino acids into proteins. J. Biol. Chem. 221: 45.

147. Whittaker, V. P. 1965 The applications of subcellular fractionation techniques to the study of brain function. In: Progress in Biophysics and Molecular Biology, eds. J. A. V. Butler and H. E. Huxley, Vol. 15, Pergamon Press, New York, p. 80.

148. Fisher, H., R. G. Hansen and H. W. Norton 1955 Quantitative determination of glucose and galactose. Anal. Chem. 27: 857.

149. Radin, N. S., F. B. Lavin and J. R. Brown 1955 Determination of cerebrosides. J. Biol. Chem. 217: 789.

150. Schneider, W. C. 1945 Phosphorus compounds in animal tissue. 1. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. 161: 293.

151. Seibert, F. B. 1940 Removal of the impurities, nucleic acid and polysaccharide, from tuberculin protein. J. Biol. Chem. 133: 593.

152. Steel, R. G. D., and J. H. Torrie 1960 Principles and Procedures of Statistics, McGraw-Hill Book Company, Inc., New York.

153. Feland, B., and J. T. Smith 1972 Malathion intoxication and mitochondrial damage. J. Agr. Food Chem. In press.

154. Hogeboom, G. H., W. C. Schneider and M. J. Striebich 1952 Cytochemical studies. V. On the isolation and biochemical properties of liver cell nuclei. J. Biol. Chem. 196: 111.

155. Fukami, J., and T. Shiskido 1966 Nature of a soluble, glutathion-dependent enzyme system active in cleavage of methyl parathion to desmethyl parathion. J. Econ. Entomol. 59: 1338.

156. Tudball, N., and E. A. Davidson 1969 Isolation of a novel sulphatase from rat liver. Biochim. Biophys. Acta 171: 113.

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