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Alterations of Sulfur Metabolism in Malathion-Treated Rats

Gail W. Disney
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To the Graduate Council:

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

John T. Smith, Major Professor

We have read this thesis and recommend its acceptance:

Mary Rose Gram, Anna Jean Treece

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 22, 1967

To the Graduate Council:

I am submitting herewith a thesis written by Gail W. Disney entitled "Alterations of Sulfur Metabolism in Malathion-Treated Rats." I recommend that it be accepted for nine quarter hours of credit in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

John T. Smith
Major Professor

We have read this thesis and recommend its acceptance:

Mary Rose Gram

Anna Jean Treece

Accepted for the Council:

Vice President for
Graduate Studies and Research

ALTERATIONS OF SULFUR METABOLISM IN MALATHION-TREATED RATS

A Thesis

Presented to

the Graduate Council of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

Gail W. Disney

December 1967

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TABLE OF CONTENTS

CHAPTER PAGE

CHAPTER PAGE

LIST OF TABLES

TABLE PAGE

CHAPTER I

INTRODUCTION

Malathion is an organophosphorus sulfur-containing insecticide and pesticide which was introduced by the American Cyanamid Corporation in 1950 for experimental use (1). Since that time there has been much work done on the properties and mode of action of malathion, but to this writer's knowledge, no investigation of the relationship between malathion and sulfur metabolism has been made. An addition to the already existing knowledge of this insecticide would seem imperative, especially since malathion itself contains sulfur.

In a study of the labilization of lysosomes as an aspect of the biochemical toxicology of anticholinesterases, Ntiforo and Stein (2) used the release of aryl-sulfatase from lysosomes as a measure of the lysosomal stability. They found that after a single intraperitoneal injection of malathion, the experimental lysosomes released aryl-sulfatase at a significantly higher rate than the controls. In this study, aryl-sulfatase release was used as a criterion for lysosomal stability because of its ease of measurement, but these data do suggest a relationship between malathion and sulfur metabolism not proposed by the investigators.

Malathion's effectiveness as an insecticide and pesticide is due to its ability to inhibit acetylcholine esterase. In addition to acetylcholine esterase, organophosphates have been shown to block a

number of other hydrolytic enzymes including pseudocholine esterase, lipase, other esterases, trypsin, and chymotrypsin (3). Bigley and Plapp (4), and Matsumura and Brown (5) found proof that malathion is a carboxyesterase inhibitor. Murphy (6) found that when malathion was given repeatedly in non-toxic doses, it inhibited its own further hydrolytic detoxication by inhibiting the malathion hydrolyzing esterase. Frazier (7) reported that inhibition of esterases other than acetylcholine esterase may be a significant factor in organophosphorus toxicity.

If malathion is a general esterase inhibitor as the above information suggests, it should have an inhibitory effect on the formation and fixation of ester sulfate in vivo. Since the fixation of sulfate has been suggested as a sufficiently sensitive technique for growth hormone assay (8), it is feasible that organophosphate toxicity could be detected by measuring the effects on sulfate fixation.

The utilization of organic and inorganic sulfur by the normal rat has been investigated by Michels and Smith (9). Since the effects of organophosphorus insecticide intoxication on sulfur metabolism may vary depending on the ratio of organic to inorganic sulfate in the diet, a comparison of the data obtained from the above study with data obtained when rats were fed these diets but were also given selected levels of malathion seems necessary.

The investigation reported in this thesis was designed to study the effects of malathion administration on sulfate fixation in rats and to determine whether or not these effects are related to the level of inorganic sulfate in the diet.

CHAPTER II

REVIEW OF THE LITERATURE

I. MALATHION

History

Malathion is the least toxic member of a group of insecticides called the organophosphorus insecticides (10). This group of insecticides resulted from the work of Gerhard Schrader of Germany in chemical warfare research prior to World War II. Little was known about organophosphorus insecticides until after the defeat of Germany (11). The first organophosphorus insecticides, parathion (O,O-diethyl-O-p-nitrophenyl-thiophosphate) and TEPP (tetraethylpyrophosphate) (12) are among the strongest ones in the group. Parathion, made available in 1946 (13), is one hundred times as toxic to mammals and three to four times as toxic to insects as malathion (14).

Properties

Malathion is the common name for S(1,2-dicarbethoxyethyl)-O,O-dimethyl dithiophosphate (Figure 1) (15). It was introduced for industrial use in the United States in 1952 (16) by the American Cyanamid Company (17). Malathion is a yellow-brown liquid with a high boiling point and a low vapor pressure. It is slightly soluble in water and miscible with many organic solvents.

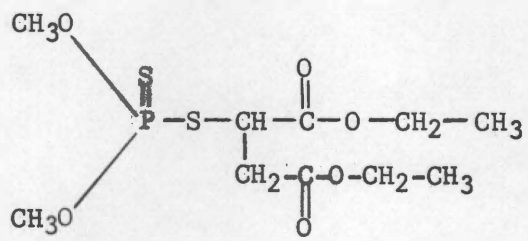

Figure 1. The structure of malathion.

Malathion also has an unpleasant, garlic-like odor which aids in its effectiveness as a pesticide. Majumder, Krishnakumari, and Krishna Rao (18), in testing twenty-seven pesticidal solutions, found that malathion had one of the highest degrees of repellency to the rat. In a high viscosity oil carrier, malathion sprayed on standard size jute bags at a concentration of 20 mg./square foot has a possible repellent action of 165 days.

Hazelton (19) of Hazelton Laboratories, one of the first investigators of malathion, found that malathion was quite stable when stored at room temperature in nonreactive containers. Lipparini (20), in a more recent publication, says that technical grade malathion (95 per cent pure) was practically unchanged in four months storage at 5°C., regardless of the container, but at 40°C., the decomposition was 15 to 20 per cent in four months. It is readily hydrolyzed at a pH above 7.0 or below 5.0, but is stable in aqueous solutions buffered at pH 5.26. Malathion is synthesized by the addition of O,O-dimethyldithiophosphoric acid to diethyl maleate (Figure 1). The presence of hydroquinone is necessary to suppress the polymerization of the unsaturated ester (1).

Mechanism of Action

Malathion's effectiveness as a pesticide and an insecticide is due to its ability to inhibit the enzyme acetylcholine esterase. This enzyme is 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 may be transmitted, the acetylcholine must be hydrolyzed by acetylcholine esterase to form acetic acid and choline (21). If acetylcholine is not hydrolyzed, there is a continual transmission of the impulse resulting in spastic movements, convulsion, and eventually death.

Effect of the Route of Administration on Toxicity

The route of administration of malathion has an effect on the rate and degree of intoxication. Natoff (22) found a difference in mice in the LD_{50} values obtained for intraperitoneal and oral routes (hepatic routes) and subcutaneous and intravenous routes (peripheral routes). He found that larger amounts of the organophosphorus insecticides were necessary for the LD_{50} dose when administered by the hepatic routes than by the peripheral routes. This suggests that the availability of the compounds for metabolism by the liver is a major factor in their toxicity. Compounds administered by the peripheral routes would enter the peripheral venous circulation directly and only about 27.5 per cent would traverse the liver during the first passage through the body. Administration of compounds by the hepatic routes results in their access to the peripheral venous circulation predominately by way of the hepatic portal system.

There is also a difference in the absorption of malathion when given by various routes. Of the four routes previously discussed, the best absorption is by the oral route. Vegetable oil solutions of malathion appear to facilitate the gastrointestinal absorption (23). Malathion is most poorly absorbed through the subcutaneous route. Stolman

and Stewart (24) gave human males 100 mg. of malathion as a 1 per cent talc dust. Less than 10 per cent of the malathion was absorbed.

Metabolism

Although the degree of toxicity varies with the route of administration, the basic chemistry of the metabolism of malathion is thought to be the same. Malathion is both activated and detoxified in the liver. In liver microsomes malathion is oxidized to its more toxic analog, malaoxon (25). In this reaction the double-bonded sulfur of malathion is replaced with oxygen. Nicotinamide adenine dinucleotide (NAD) and magnesium ions are required for this conversion in vitro.

Once malathion has been converted to the oxygen analog, several reactions can take place (26): (1) inhibition of acetylcholine esterase enzyme in blood and various organs, (2) hydrolysis to an inactive compound, and (3) inhibition of aliesterase enzymes in blood and various organs. Malathion inhibits acetylcholine esterase by phosphorylating the enzyme's serine hydroxyl group forming a covalent bond (3). This reaction deactivates acetylcholine esterase and prevents its normal action of hydrolyzing acetylcholine so that this compound accumulates at nerve synapses.

The second reaction, hydrolysis of malaoxon, detoxifies the compound. Casida (27) found that this detoxication results from hydrolysis by aliphatic esterases at each of the ester sites on the molecule to give at least thirteen theoretical metabolites, most of which have been isolated. Cook and Yip (28) and Casida agree that the most significant

site of hydrolysis is at the carboxyester group forming the non-toxic monocarboxylic acid analog. Stolman (23) also found that malathion monoacid was the principal urinary metabolite. He found that the principal degradation products in the feces were dimethylphosphate and O,O-dimethylphosphorothioate.

Species Difference in Intoxication

Malathion represents the only true success in developing an insecticide that is of low toxicity to mammals but highly toxic to insects. Detoxication, the second reaction, affords malathion this ability. Mammals are thought to be able to detoxify malathion to a much greater degree, much faster than insects (29). O'Brien (30) has proposed the idea that the species difference in susceptibility is due to the distance between the anionic and esteratic sites in the choline esterase of the two species. He proposed that the choline esterase of insects is better suited for reaction with malathion than the choline esterase of mammals. Metcalf (31) obtained data suggesting that the reduced mammalian toxicity may result from poor absorption.

Sex Difference in Intoxication

A sex difference in susceptibility to malathion poisoning and organophosphorus poisoning in general also exists. Taylor, Kalow, and Sellers (32) found that in rats up to 30 days of age, sensitivity to parathion is similar in males and females, but in adult rats, females form the toxic oxygen analog much more rapidly than males. Differences can be abolished by the administration of testosterone to females or

diethyl stilbestrol to males (27). In a study by Murphy and Dubois (33), activity of the livers of male rats was about four times as great as that of females as evidenced by the detoxication of 4 µg. of malaoxon per mg. of male rat liver and 0.99 µg. per mg. by the livers of female rats.

Response of Different Insect Strains

In addition to sex and species differences in response to malathion, there is also a difference in response by certain strains of insects, especially houseflies, mites, mosquitoes, and roaches. In contrast to the susceptibility of most insects, these strains of insects have developed a resistance to malathion. Most investigators believe that a high carboxyesterase activity is responsible for the resistance phenomena (34). Bigley and Plapp (4) found that the malathion resistant strain of the mosquito, Culex tarsalis, exhibited three times more carboxyesterase activity than a normal strain. Vass, Dauterman and Matsumura (35) found that the malathion resistant two-spotted spider mite has a superior ability to degrade malathion at the carboxyester as well as at the phosphoester bond. Matsumura and Dauterman (36) found that the insects had become resistant to the normal malathion molecule. Substitution of methyl groups at either end of the malathion molecule quickly diminished the degree of resistance. Oppenoorth (37) found that a certain type of gene called oligogene was responsible for an important part of the resistance in insects.

Synergists

Results from work of Plapp et al. (38) and Henneberry and Smith (39) provided evidence that with certain synergists, resistance to organophosphorus insecticides could be overcome by inhibition of degradation mechanisms. Potentiated toxicity or synergism between two compounds is assumed to be present if non-toxic doses of each when combined, produce a greater mortality than did twice these doses of either compound alone. EPN (p-nitrophenyl thionobenzene phosphonate), chlorothion, phostex (40) and TOTP (triorthotolyl phosphate) (41) have been found to potentiate malathion intoxication. EPN and malathion showed the greatest potentiation. When the two compounds were given orally to rats in an approximately equitoxic ratio of 25 parts of malathion to one part of EPN, the LD_{50} was 167 mg./kg. for malathion and 6.6 mg. for EPN, which represented a 10-fold increase in the toxicity of each agent. If the two compounds had exerted strictly additive toxicity the LD_{50} of this particular combination should have been 700 mg./kg. of malathion plus 32.5 of EPN (one-half of the LD_{50} of each compound) (42). A single dose of EPN inhibits the ability of rat liver to detoxify malaoxon for as long as 72 hours. In order for potentiation to occur, it appears that one or both compounds must inhibit inactivation of the other (40).

Fawley (26) showed that an effective in vivo dose of EPN depressed plasma choline esterase more than red cell choline esterase and that an effective dose of malathion depressed red cell choline esterase with little effect on plasma choline esterase. The above reactions occured because acetylcholine esterase, found in red blood cells, is

specifically inhibited by malathion while butyrocholine esterase, or pseudo-choline esterase, found in plasma, is specifically inhibited by EPN. Given together, these insecticides gave plasma-red-cell choline esterase depression similar to that produced by malathion alone but from a much lower level of administration. This could be interpreted to mean that EPN helped to make malathion available to the blood stream choline esterase enzymes.

Malathion has also been shown to potentiate itself. Murphy (6) found that guinea pigs and rats pretreated with doses of malathion which did not produce inhibition of choline esterase, but which did inhibit malathion-esterase (detoxifying enzyme), were more susceptible to poisoning by a second dose of a quantity of malathion which was not sufficient to cause choline esterase inhibition in animals not pretreated. The results of this investigation indicate that an early biochemical event leading to poisoning by malathion is the inhibition of its own further hydrolytic detoxication by a metabolite of the parent insecticide. Murphy proposed that this metabolite was malaoxon. In this study Murphy also found that technical grade malathion (95 per cent pure) was more toxic than primary grade (99.6 per cent pure), probably due to malaoxon present in the 95 per cent pure sample as an impurity.

Determination of Intoxication in Man

Since malathion is a specific inhibitor of acetylcholine esterase, the depression of this enzyme in red blood cells is measured in order

to determine the degree of malathion intoxication. Frequent determination of acetylcholine esterase depression is especially important for those persons who are in frequent contact with malathion or other organophosphorus insecticides since depression occurs before other symptoms of poisoning are manifested. There are several ways of measuring acetylcholine esterase activity in red blood cells (44): (1) measurement of the amount of acetic acid produced, (2) measurement of the amount of thiocholine developed due to enzyme hydrolysis of acetylthiocholine, (3) Warburg manometric technique, and (4) measurement of metabolites in the urine. Kits for quick, easy, and fairly accurate estimation of acetylcholine esterase depression have been manufactured for use in factories and field work. The use of these kits is based on a change in pH as a result of liberated acetic acid.

Symptoms of Intoxication

In humans, the symptoms of malathion poisoning (45) are anorexia, nausea, sweating, muscular fasciculations, abdominal cramps, vomiting, diarrhea, salivation, involuntary defecation and urination, weakness, drowsiness, and slurring of words. Death usually results from respiratory arrest. In man, 150 mg./kg. will produce moderate symptoms, while 600 mg./kg. is the estimated lethal dose for the oral route as compared with 1400 mg./kg. and 1000 mg./kg. for male and female rats respectively (46).

Residues on Food

Very few human deaths have been attributed to malathion poisoning as compared to other organophosphorus poisons, and most of these were suicides or poisoning in very young children. To this writer's knowledge, no deaths have occurred from malathion residues on foods. The application of malathion is recommended no later than 21 days before harvest. Malathion residue levels in the soil and on plants drop rapidly (47). Koivistoinen (48) found that the rate of disappearance depended on the amount applied; the less applied, the more rapid the disappearance. Malathion residues on lettuce were found in significant quantities for only 2-4 days after spraying (49). There was no off-flavor in meat of malathion-treated hogs or cattle (50), and no detectable malathion in the milk or meat of cows fed 200 parts per million (p.p.m.) of malathion based on the total food intake for 41-44 days (51). The residue tolerance according to the Miller Amendment to Public Law 518 is 8 p.p.m. actual malathion in or on raw agricultural commodities (14).

Aleviation of Malathion Intoxication

Extensive research has been done in order to find chemicals which will reverse the effects of malathion poisoning. These effects can be reversed if the malathion-enzyme complex has not undergone "ageing" (52). Ageing of the complex occurs when an alkyl group is removed from the malathion residue joined to the acetylcholine esterase so that the chemicals now used in treatment of malathion poisoning are

not effective in freeing the enzyme. Hobbiger (53) proposed that the conversion leads to additional bonding so that the malathion residue is more tightly bound to the enzyme.

The most successful treatment of malathion poisoning has resulted from the use of a combination of three chemicals (54). This so-called AMP therapy includes the use of atropine, metaraminol, and an oxime, pyridine aldoxime methanesulfonate (P_2S). When malathion or organophosphorus poisoning occurs, atropine (33) should be given every ten minutes in 5 mg. doses until signs of atropinization appear (dry skin, dilated pupils, tachycardia, etc.). Atropine will competitively block the action of acetylcholine that is accumulating at muscarinic receptor sites but it will not free acetylcholine esterase from its inhibitor.

Metaraminol, 10 mg., can be given with atropine. It has been shown to reduce the side effects of atropine and to enhance the antagonistic action of atropine against acetylcholine accumulation (54).

The third member of the AMP therapy team, pyridine aldoxime methanesulfonate (P_2S), will reverse the phosphorylation of acetylcholine esterase (37). This reversal involves a competition between the hydroxyl groups of serine and the oxime for the phosphoryl (malathion) group. The covalent bond with serine is broken and a new covalent bond is formed with the hydroxylamine freeing the enzyme, acetylcholine esterase (3, 55). It has been shown in animal studies that oximes in combination with atropine can decrease the toxicity of organophosphorus compounds more than a hundred fold (32).

Acute episodes of organophosphorus poisoning seldom last longer than 48 hours; most deaths occur within the first 24 hours. If a patient can survive for two days, he will usually recover completely (32), but cases of organophosphorus poisoning have been reported in which patients developed paralysis of the legs, arms, and hands after the usual symptoms of poisoning had subsided (56). This paralysis is thought to be due to demyelinating lesions in the spinal cord caused by the accumulation of unhydrolyzed acetylcholine at cholinergic synapses. TOCP (tri-ortho-cresylphosphate) (7), DFP (di-isopropyl-fluorophosphonate), and mipafox (bis-monoisopropyl-amino fluorophosphine oxide) have caused this delayed paralysis. Malathion has caused muscular weakness, but not paralysis (56).

Malathion Prophylaxis

In addition to uses as pesticides and insecticides, malathion is also used to treat myasthenia gravis and glaucoma. Myasthenia gravis (57) is a chronic disease characterized by weakness and abnormal fatigability of skeletal muscles. Since deficient release of acetylcholine is believed to be responsible for this disease, malathion can be administered to inhibit acetylcholine esterase and cause an accumulation of acetylcholine.

Glaucoma (58) is a disease of the eye evidenced by an increase in intraocular pressure. Failure to control the increase in intraocular pressure results in irreparable damage to the eyes and eventual blindness. One of the reasons for the increase in intraocular pressure

is thought to be obstruction of outflow channels, causing a decreased flow of aqueous humor. Malathion therapy possibly lowers the intraocular pressure by decreasing the flow of aqueous humor into the eye and/or by increasing the permeability of the blood-aqueous humor barrier.

In addition to increasing the permeability of the blood-aqueous humor barrier in glaucoma, malathion has been shown to increase the permeability of lysosomes, resulting in the release of aryl-sulfatases (2). Because of this and other relationships between malathion and sulfur metabolism proposed in Chapter I, a discussion of sulfur metabolism would seem appropriate to include in this review. The oxidation of sulfur has been reviewed in detail by Rutledge[1] and will not be reviewed in this paper. This portion of the review will be limited to a discussion of active sulfate, 3'-phosphoadenosine 5'-phosphosulfate (PAPS), and its role in ester formation.

II. 3'-PHOSPHOADENOSINE 5'-PHOSPHOSULFATE (PAPS)

Formation of PAPS

Sulfate is found, mostly in the form of esters, in a great number of compounds widely distributed in living organisms. It is now known that PAPS is responsible for the formation of these esters. Demeio began the study of sulfate activation in 1952 when he found that ATP (adenosine triphosphate) could furnish the energy for sulfate activation (59). Bernstein and McGilvery (60) further clarified the mechanism of

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[1]Rutledge, H. R. 1966 Inhibition of cysteine oxidation in vitamin E deficiency. Unpublished Ph. D. Thesis. The University of Tennesee.

sulfate activation when they found proof that two steps were involved in the activation, each having a specific enzyme. In 1955, Hilz and Lipmann (61) found that active sulfate was a mixed anhydride between sulfate and phosphate, the phosphate being most likely linked to adenosine. Soon after this discovery Robbins and Lipmann (62, 63) isolated PAPS and revealed its structure. Baddiley, Buchanan, and Letters (64) strengthened the findings of Robbins and Lipmann by synthesizing PAPS. After the isolation of PAPS, Bandurski and coworkers (65) and Robbins and Lipmann (63) identified the mechanism and enzymes involved in the formation of active sulfate (Figure 2). In the first reaction, catalyzed by ATP-sulfurylase, ATP reacts with inorganic sulfate to yield adenosine 5'-phosphosulfate and pyrophosphate. Since the reverse reaction is thermodynamically favored, the forward reaction as well as the over-all synthesis of PAPS is considerably enhanced by the removal of pyrophosphate by pyrophosphatase. Adenosine 5'-phosphosulfate (APS) kinase catalyzes the second reaction in which APS plus ATP irreversibly form active sulfate, 3'-phosphoadenosine 5'-phosphosulfate, and ADP. Both enzymes require as cofactors magnesium or other divalent cations. Bandurski and Wilson (65) assigned to magnesium the role of enabling sulfurylase to bind sulfate so that sulfurylase-magnesium-sulfate is the reagent which attacks ATP. Peck (66) proposed an alternate second reaction occurring in certain microorganisms in which instead of the formation of PAPS, the enzyme APS-reductase catalyzed the reaction of APS with two electrons to form AMP (adenosine monophosphate) and sulfite.

1. ATP + S $\xleftrightarrow[\text{Sulfurylase}]{\text{ATP-}}$ APS + PP ΔF°, + 11,000

PP $\xrightarrow{\text{PP-ase}}$ 2P ΔF°, - 5,000

2. APS + ATP $\xrightarrow[\text{kinase}]{\text{APS-}}$ PAPS + ADP ΔF°, - 6,000

Over-all reaction

2ATP + S $\longrightarrow$ PAPS + 2P + ADP ΔF°, 0

Figure 2. Formation of PAPS.

Factors other than the presence of ATP and magnesium have been found to affect the formation and utilization of PAPS. Hypertension (67) and progesterone (68) increase the utilization of PAPS while UTP (uridine triphosphate) (69) and estradiol (70) inhibit the uptake of sulfate from PAPS. There is much controversy concerning the effect of vitamin A on sulfation by PAPS. Some investigators[2] (71, 72) say a deficiency or excess of vitamin A causes decreased incorporation of ^{35}S while others say (73, 74) this is not the case. There is also disagreement regarding the reactivation of PAPS by vitamin A. Subba Rao and Ganguly (75) helped to solve this controversy when they found that the success of vitamin A in reactivating PAPS depends on the degree of vitamin A deficiency. In mild or moderate vitamin A deficiency, addition of vitamin A in some form will reactivate PAPS, while in severe deficiency, vitamin A has no effect on restoring PAPS activity. Several investigators (76, 77, 78) have found that vitamin A deficiency and toxicity damage cell membranes. It has been proposed that this effect is the cause of altered sulfation especially since there are reports of the release of aryl-sulfatases in both vitamin A deficiency and toxicity (74). Sundaresan and Wolf[3] found that the first step in PAPS formation was the one depressed in vitamin A deficiency.

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[2]Sundaresan, P. R., R. M. Elford and G. Wolf 1964 The relation of vitamin A to sulfate activation and steroid hydroxylation. Federation Proc., 23: 479 (abstract).

[3]Sundaresan, P. R. and G. Wolf 1963 Evidence for the participation of a vitamin A derivative in ATP-sulfurylase action. Federation Proc., 22: 293 (abstract).

The transfer of sulfate from PAPS to various acceptors is catalyzed by a group of enzymes called sulfokinases. Each type of compound may require a different sulfokinase. The compounds known to accept sulfate from PAPS through the action of sulfokinases can be grouped into three classifications (79): (1) aromatic hydroxyls, (2) aliphatic hydroxyls, and (3) aromatic or aliphatic amines.

Sulfate Acceptors

Aromatic hydroxyls. The transfer of sulfate from PAPS to aromatic hydroxyls was first demonstrated by Gregory and Lipmann (80) in the sulfation of phenolic compounds. Sulfurylation of phenols is a two-step reaction: first, the activation of sulfate and second, the transfer of sulfate from PAPS to phenols catalyzed by phenol sulfokinase (71). PAPS acts as a coenzyme in the transfer of sulfate to phenols. This reaction was at first thought to occur only in the liver where it serves to detoxify phenolic compounds. In 1961, Wortman (81) found that phenol sulfotransferase was active in beef cornea in the transfer of sulfate from p-nitrophenyl sulfate via PAPS to corneal mucopolysaccharides. The transfer of sulfate from p-nitrophenyl sulfate to phenol is a sensitive assay for the PAP or phenol sulfokinase (81). Methods for the assay and identification of PAPS are its almost complete hydrolysis by 0.1 N. HCl at 37°C. in 30 minutes, its absorption maximum at 260 mμ, and its hydrolysis by 3'-nucleosidase which cleaves the 3'-phosphate group (82).

Phenol sulfokinase is now thought to be a relatively unspecific enzyme (82). In addition to beef cornea and liver, it has also been found in kidney, intestinal mucosa and granulation tissues, although its function in each of these tissues has not been elucidated (83).

Adams (83) found evidence that several rather specific steroid sulfokinases exist. Enzymes capable of conjugating steroids to form sulfate esters had been found only in liver when Adams began her investigation. Unfractionated liver preparations had been found to sulfate a wide range of steroids. Nose and Lipmann (84) were able to separate an enzyme fraction which sulfated estrone from one which sulfated dehydroisoandrosterone. Adams found that extracts of chick embryonic cartilage were found capable of sulfating estrogenic steroids but not a variety of other steroids tested. She concluded that in mammalian cartilage extracts, two separate enzymes are responsible for sulfating the simple phenols and the phenolic steroids.

L-tyrosine O-sulfate has been identified as a component of mammalian urines, mammalian fibrinogens, and gastrin (isolated from hog mucosa), but its synthesis in vivo is not clear. Evidence suggests that the carboxyl group of L-tyrosine must be blocked before the phenolic hydroxyl group of the amino acid can undergo sulfation in the rat liver phenol-sulfotransferase system (79). Jones and coworkers (85) found that L-tyrosylglycine can act as a sulfate acceptor in the rat liver while glycyl-L-tyrosine cannot. One of the products of the sulfation of L-tyrosylglycine was identified as L-tyrosylglycine-O-sulfate which may account in part for its presence in urine. The

sulfurylation from PAPS to amino alkyl phenols such as tyramine, noradrenalin, and serotonin has been observed in extracts of snail gland and of rat liver.[4]

Aliphatic hydroxyls. The most important ester compounds in the aliphatic hydroxyl group are the carbohydrates. D'Abramo and Lipmann (86) demonstrated that PAPS was responsible for the sulfation of chondroitin sulfate in vitro when the extract was incubated with adenosine triphosphate and magnesium chloride. PAPS is the precursor of chondroitin sulfate in cornea and the molecule is polymerized before sulfation. Suzuki and Strominger (87) found that chondroitin sulfates A and B and chondroitin sulfate C isolated from shark cartilage were sulfated on the fourth and sixth carbon respectively. These investigators (88) also found that an enzyme isolated from the isthmus of hen oviduct catalyzed the transfer of sulfate from PAPS to monosaccharides and oligosaccharides containing acetylgalactosamine.

There is evidence for the existence of sulfokinases specific for the various chondroitin sulfates.[5] An enzyme specific for chondroitin sulfate in embryonic calf cartilage has been isolated. A human

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[4]Goldberg, I. H., and A. Delbrück 1959 Transfer of sulfate from 3'-phospho-adenosine-5'-phospho-sulfate to lipids, mucopolysaccharides, and amino alkyl phenols. Federation Proc., 18: 235 (abstract).

[5]Haseqawa, E., A. Delbrück and F. Lipmann 1951 Sulfate transfer specificity for chondroitin sulfates in tissue preparations. Federation Proc., 20: 86 (abstract).

chondro-sarcoma extract has shown specificity for chondroitin sulfate C. In calf embryo skin extract, a differentiation between the sulfokinases for chondroitin sulfate A and C and for B has been found. Balasubramanian and Bachhawat (89) demonstrated the presence in rat brain of an enzyme specific for heparitin sulfate and chondroitin sulfate B.

McKhann, Levy, and Ho (90), in working with another aliphatic hydroxyl, found that galactocerebrosides were sulfated by a soluble enzyme obtained from the microsomal fraction of rat brain. Hauser (91) has suggested that galactocerebrosides are the precursors of sulfatides. McKhann concluded that galactocerebrosides are sulfated by PAPS to form sulfatides and that the sulfate-transferring enzyme is galactocerebroside sulfokinase.

Although no information was found in the literature concerning the sulfation of choline in mammals, this reaction has been studied in fungi. The isolation of the choline ester of sulfuric acid from a natural source was first done in 1937 by Woolby and Peterson (92) from Aspergillus sydowi. Since that time choline sulfate synthesis in vitro has been observed in a number of extracts of fungi in the presence of ATP and magnesium ions. The synthesis process is catalyzed by choline sulfokinase (93). In all fungi tested choline sulfate was necessary for the transfer of sulfate from PAPS to choline. Choline sulfate in fungal mycelia is envisaged as a source of easily assimilated sulfate existing in an activated state. The pathway of utilization is thought to involve transfer of sulfate from choline sulfate to PAP to form PAPS

and the subsequent reduction of PAPS to give sulfite and eventually cysteine (94). This reaction was also discussed by Peck (66).

Amines. Less information is found in the literature about the third group of ester sulfates, the amines. Spolter and Marx (95) have shown that labeled inorganic sulfate was incorporated into heparin by a mouse mast-cell tumor homogenate thus proving that PAPS is involved in sulfation of heparin.

After reviewing sulfation research, findings indicate that much is still unknown. However, there appears to be universal agreement that inorganic sulfur is coupled to the formation of ester sulfates by energy from ATP. The enzymes which catalyze these coupled reactions are specialized esterases; therefore, since malathion was shown to be an esterase inhibitor, a relationship between sulfate fixation and malathion intoxication seems to be a reasonable assumption.

CHAPTER III

EXPERIMENTAL

I. GENERAL PLAN

The main objective of this investigation as originally planned was to study the effects of malathion administration on sulfate fixation in rats. Later an investigation was made to determine whether or not these effects are related to the level of inorganic sulfate in the diet. Both of these problems were investigated in addition to others which grew from the initial investigation. This study was divided into seven experiments which are described in chronological order. Methods common to many experiments are discussed in the latter part of this chapter.

Experiment 1

This part of the study was designed to investigate the effects of malathion administration on brain acetylcholine esterase activity and on ^{35}S-sulfate uptake by rib cartilage mucopolysaccharides. To begin the study, a survey was conducted using several levels of malathion ranging from 100 to 1000 mg./kg. of rat. Certain levels were selected for more intensive investigation as a result of this survey. Both male and female rats were used in order to determine whether or not there is a sex difference in response to malathion as has been reported by other investigators (27, 32, 33). Rats weighing between

200 g. and 250 g. were used for the survey and all of the other experiments. The rats were fed lab chow[1] and received food and distilled water ad libitum. They were housed individually in wire mesh cages. The female rats were Wistar rats from the Nutrition Department colony as were those used in all subsequent experiments except experiment 3. The male rats used in this experiment and the females used in experiment 3 were Sprague Dawley rats obtained from the Animal Husbandry colony.

The malathion[2] was administered by stomach tube with corn oil as a carrier. Malathion is well absorbed by the oral route and disperses evenly in corn oil, a carrier which facilitates its absorption (23). There were three rats in each group for each replication: one receiving malathion in oil, one receiving a sham tube feeding of oil, and one receiving no treatment. The rat given corn oil was used as the control since Baron (6) has shown that oral administration of 5 to 10 ml. of corn oil per kg. produces a significant inhibition of the activity of certain esterases. The concentration of malathion in the corn oil solution was 100 mg./ml. and 200 mg./ml., the concentration used depending on the level of malathion to be administered. As a result of using these concentrations, all rats on the study received less than a total of 2.0 ml. of corn oil during the 3 day treatment period regardless of the level of malathion given.

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[1] Purina Laboratory Chow, Ralston Purina Company, St. Louis.

[2] Donated by the American Cyanamid Company, Princeton, N. J.

The rats were given malathion and oil every 24 hours for 3 days. Stavinoha et al. (96) has shown that symptoms of organophosphorus poisoning become maximal at about the third day of administration. He observed that the acetylcholine content of the brain tissue rises to the highest level on the third day, indicating that acetylcholine esterase is most inhibited during this period. On the third day, the rats were injected subcutaneously with approximately 10 µc. $Na_2{}^{35}SO_4$ as carrier free $Na_2{}^{35}SO_4$ in 0.5 ml. of isotonic saline. They were killed by decapitation 24 hours after this injection. Bostrom (97) has shown that maximum incorporation of ^{35}S into chondroitin sulfates of rib cartilage occurs in 24 hours. Decapitation was chosen as the method for killing since Crossland (98) has shown that anaesthetization of animals before killing causes the acetylcholine content of brain to increase by 40 per cent. Immediately after decapitation, the brain, liver, and rib cartilage were excised from each rat. The liver and rib cartilage were stored at -20°C. until assays for ^{35}S could be made. The brain was placed in cold Ringer's buffer solution and stored at 4°C. The acetylcholine esterase activity of each brain was determined.

Experiment 2

In addition to giving malathion by stomach tube, this pesticide was also fed in the diet of rats. The composition of the diet used is a modification of the diet of Cuputto et al. (99) and is the same as that shown for diets B and C in Table 1 except that corn oil was substituted for lard. The salt mixture used in this study is a

TABLE 1

COMPOSITION OF THE DIETS

Component	Quantity Per 100 Grams		
	A 0.42% SO_4	B and C 0.10% SO_4	D 0.0002% SO_4
	g.	g.	g.
Casein	15.00	15.00	15.00
L-cysteine	00.00	00.35	00.60
Sucrose	30.00	29.65	29.40
Cornstarch	32.00	32.00	32.00
Non-nutritive bulk[1]	10.00	10.00	10.00
Lard	6.00	6.00	6.00
Cod liver oil	2.00	2.00	2.00
Vitamin mixture[1]	2.00	2.00	2.00
Salt mixtures:			
A--14.00% SO_4	3.00	--	--
B--3.34% SO_4	--	3.00	--
C--0.00% SO_4	--	--	3.00

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

modification of the salt mixture of Hubbell et al. (100). Its composition is shown under salt mixture B on Table 2. Sufficient $Ca^{35}SO_4$ was added as part of the $CaSO_4$ in the salt mix to provide approximately 1500 counts per minute/gram of diet. The diet used was 0.10 per cent inorganic sulfur as sulfate and 0.57 per cent neutral sulfur as sulfate. This ratio of inorganic to organic sulfur as sulfate in the diet is the usual ratio.

Five and 10 mg. malathion/g. of diet were fed by dissolving the required amount of the insecticide in corn oil and mixing it with the diet. Adult rats of the weights used in this experiment (200-250 g.) are known to eat 10-12 g. of diet/day; therefore, as a result of feeding the above concentrations of malathion in the diet, the rats ate approximately 250 and 500 mg. of malathion/day. Six female rats were fed diets containing 5 mg./g. diet, and five female rats were fed diets containing 10 mg./g. diet. Each rat fed malathion had a control which was fed the same diet but without malathion. The animals were housed separately in wire mesh cages and received food and water ad libitum. At the end of the feeding period, the rats were killed by decapitation and the brain, liver, and ribs of each animal removed. The ribs and livers were stored at -20°C. until analyzed for ^{35}S-sulfate incorporation. The brains were refrigerated in Ringer's buffer solution.

Experiment 3

Four adult female Sprague Dawley rats were fed malathion-^{35}S[3] in corn oil by stomach tube every 24 hours for 3 days. Two of the

[3]Obtained from The Radiochemical Centre, Amersham, England.

TABLE 2

COMPOSITION OF SALT MIXTURES

Component	Quantity Per 100 Grams		
	A 14.00% SO_4	B 3.34% SO_4	C 0.007% SO_4
	g.	g.	g.
$CaCO_3$	30.346	41.250	44.750
$CaSO_4 \cdot 2H_2O$	25.097	6.000	0.000
Cornstarch	0.000	8.193	10.693
$MgCO_3$	3.060	3.060	3.060
NaCl	6.900	6.900	6.900
KCl	11.200	11.200	11.200
KH_2PO_4	21.200	21.200	21.200
$FePO_4 \cdot 2H_2O$	2.050	2.050	2.050
KI	0.008	0.008	0.008
NaF	0.010	0.010	0.010
$AlK(SO_4)$	0.017	0.017	0.017
$Cu(C_2H_3O_2)_2 \cdot H_2O$	0.072	0.072	0.072
$MnCl_2 \cdot 4H_2O$	0.040	0.040	0.040

rats were fed 250 mg. and two 500 mg. of malathion/kg. of rat. The rats were housed in metabolism cages[4] and fed lab chow and distilled water ad libitum. Urine and feces were collected daily. One ml. of 0.1 N. HCl was added to each urine vial to retard spoilage. On the fourth day, 24 hours after the last malathion feeding, the rats were killed by decapitation and their livers, brains, and ribs removed and stored as described previously for analysis.

Experiment 4

In order to compare the effects of malathion administration on different sulfur pools, rats were fed diets containing ^{35}S either as ^{35}S-cysteine (diet B) or $Ca^{35}SO_4$ (diet C) for a period of 10 days. There were 8 rats in each group (four on diet B and four on diet C): one receiving no corn oil, one receiving only corn oil, and two receiving malathion in oil. There were five groups of rats in which the two rats on each diet to be given malathion received 250 and 500 mg. of malathion/kg. In prior experiments 500 mg. of malathion/kg. was used successfully, but in this experiment 500 mg. seemed to be the LD_{50} level for the rats used. An increase in the toxicity of the malathion may be one explanation for this phenomenon. Because several of the rats receiving 500 mg. of malathion/kg. died, two groups of rats were given 250 and 400 mg./kg. Since a large majority of the rats that died were fed the cysteine-^{35}S diet, in one group of 4 rats,

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[4] Hoeltage, Inc.

both rats receiving malathion were given 500 mg. of malathion/kg. of rat. During the following 3 days the appropriate rats as described above received malathion in oil or oil alone by stomach tube every 24 hours. The rats were housed in metabolism cages. Urine and feces were collected daily and analyzed for ^{35}S activity. The rats were killed by decapitation and the livers, brains, and ribs excised.

Experiment 5

In order to determine whether or not the effects of malathion administration on sulfur metabolism were related to the level of inorganic sulfate in the diet, four diets were fed to four groups of rats, with five rats on each diet. The compositions of the diets and salt mixtures are shown in Tables 1 and 2, pages 28 and 30, respectively. The diets of low (0.00 per cent), normal (0.10 per cent), and high (0.42 per cent) levels of inorganic sulfur as sulfate were obtained by varying the amount of $CaSO_4$ used in the salt mixtures (Table 2). Button et al. (101) has shown that these salt modifications have no ill effects on the rat. The ratio of inorganic to neutral sulfur was varied inversely so that the total per cent sulfur as sulfate remained constant (Table 3).

The diets were fed for a total of 21 days. For the first 7 days, the rats were fed non-radioactive diets. During the next 10 days, the sulfur pools of the rats were labeled by feeding the previously described diets containing either ^{35}S-cysteine as the sulfur amino acid or $Ca^{35}SO_4$ in the salt mixture as shown in Table 3. During

TABLE 3

CALCULATED LEVELS OF DIETARY SULFUR AND DISTRIBUTION OF RADIOACTIVITY

Diet	Per Cent Sulfur as Sulfate		
	Inorganic	Neutral	Total
A	0.42 ($Ca^{35}SO_4 \cdot 2H_2O$)	0.25	0.67
B	0.10	0.57 (^{35}S-cysteine)	0.67
C	0.10 ($Ca^{35}SO_4 \cdot 2H_2O$)	0.57	0.67
D	0.00	0.67 (^{35}S-cysteine)	0.67

the last 4 days, the non-radioactive diets were again fed. Feed intakes were recorded daily for each rat. During the last 3 days, malathion in corn oil was given by stomach tube to all the rats each 24 hours at the level of 250 mg./kg. of rat.

The rats were maintained in metabolism cages during the entire 21 day period, but urine and feces were not collected until the eighth day when feeding of the radioactive diets was begun. At this time, the funnels were attached to the cages and total urine and feces were collected for each rat during the next 10 days. The urine and feces were collected daily during the time of malathion administration. The rats were killed by decapitation 24 hours after the last tube feeding. The ribs were excised and stored at -20°C.

Experiment 6

Exactly the same procedure was used for experiment 6 as for experiment 5 except that the rats were given 400 mg. of malathion instead of 250 mg. of malathion/kg. as above. In this study, excreta were collected only during the period of malathion administration.

Experiment 7

Efforts were made to try to identify the radioactive compounds being excreted in the urine and feces of rats given ^{35}S and malathion. The urine and fecal samples collected from rats in experiments 5 and 6 were combined according to the diet consumed. A 3.0 g. sample of the combined feces from rats fed each diet was extracted with hot distilled water and filtered through Whatman No. 42 filter paper until the

filtrate ran through clear. The specific activity of each original fecal sample and of each filtrate was determined in order to ascertain whether or not the radioactive compound in the feces was water soluble.

The urine samples were acidified with 0.5 N. HCl and 100 ml. portions of each of the four combined urines were shaken with ether. The activity of the original urine samples and the activity of the urine layer remaining after shaking with ether was determined in order to find whether the radioactive compound in urine obtained from rats given malathion and ^{35}S is water or ether soluble.

The fecal filtrates and the urine samples were put through a Dowex 1X8, 200-400 mesh, formate column. After each sample passed through the column, the column was washed with distilled water. The activity of the filtrate and of the water collected from the column were determined. The column was eluted with 200 ml. of the following solutions: 0.25 M. sodium formate, 0.25 M. sodium formate adjusted to pH 4.7 with 0.25 M. formic acid, 0.25 M. formic acid, 1.0 M. sodium formate, and finally 1.0 M. formic acid. The eluate from each solution was collected automatically in 5 ml. fractions.

In order to locate the tubes which contained the radioactive compound or compounds, every fifth sample collected was analyzed for ^{35}S activity. The four tubes of eluate collected just before and just after the tubes found to contain radiation were combined. The pooled samples were concentrated by lyophilizing. After lyophilizing, each sample was dissolved in a small amount of distilled water and approximately 300 lambda were stripped on Whatman No. 1 chromatography paper.

Three chromatographs of each solution were done in order to obtain enough radiation to count. The chromatographs were developed with a formic acid solvent of the following composition: 46.6 ml. isopropanol, 2.6 ml. formic acid, 23.3 ml. ethanol, 27.2 ml. water, and 20.0 ml. pyridine for each 100.0 ml. of solvent. After the solvent front had moved within about one inch of the top of the paper, the chromatographs were removed from the solvent, allowed to dry, and then sprayed with a platinum iodide spray of the following composition: 0.4 ml. 20 per cent platinum chloride, 24.6 ml. water, and 25.0 ml. 6 per cent potassium iodide solution. The corresponding bands of each sample were combined and analyzed for ^{35}S activity.

II. METHODS

Preparation of Radioactive Compounds

Radioactive $H_2{}^{35}SO_4$[5] was used to prepare both the $Na_2{}^{35}SO_4$ injected subcutaneously in experiment 1 and the $Ca^{35}SO_4$ used in the diets. The carrier free $H_2{}^{35}SO_4$ was neutralized with NaOH and diluted to 15 cc. with isotonic saline solution when purchased. The $Na_2{}^{35}SO_4$ was prepared by dissolving Na_2SO_4 in distilled water, adding sufficient neutralized $H_2{}^{35}SO_4$ to obtain the desired specific activity, and drying. The $Ca^{35}SO_4$ (diets A and C, Table 2, page 30) was prepared by adding stoichiometric amounts of the $H_2{}^{35}SO_4$ to a saturated solution of calcium lactate. The resulting precipitate of $Ca^{35}SO_4$ was collected, washed,

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[5]Abbott Laboratory, Oak Ridge, Tennessee.

and dried by suction filtration. The ^{35}S-cysteine (diets B and D, Table 2, page 30) was prepared by dissolving high specific activity ^{35}S-cysteine[6] in a solution of non-radioactive cysteine and recovering the amino acid by evaporation of the water. The purity of the ^{35}S-cysteine was confirmed by paper chromatography.

Determination of Acetylcholine Esterase Inhibition

The Warburg manometric method (102) was used to measure the depression of acetylcholine esterase (AcE) activity by malathion in brain tissue. Each brain was homogenized in approximately 30.0 ml. of Ringer's buffer solution by using a motor driven Potter-Elvehjem homogenizer. A teflon pestle was used in a 15.0 ml. glass homogenizing tube. In order to prevent loss of enzyme activity, the homogenizing tube was immersed in an ice water bath during the homogenization. The brain homogenates were stored at approximately 4°C. until analysis was completed, usually within three days after the animals were sacrificed.

The bicarbonate Ringer's solution (102) is made by mixing together 100.0 ml. of 0.90 per cent NaCl, 30.0 ml. of 1.26 per cent $NaHCO_3$, and 2.0 ml. of 1.76 per cent $MgCl_2 \cdot 6H_2O$. The solution is adjusted to pH 7.4 by bubbling a 95 per cent N_2-5 per cent CO_2 gas mixture through it. The components of Ringer's buffer solution provide optimum conditions for acetylcholine esterase activity. The HCO_3^- acts as a buffer while the Na^+ and the Mg^{++} serve to activate the

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[6]Schwartz Bioresearch, Inc., Mount Vernon, New York.

enzyme (103). The stock solutions were refrigerated and a fresh buffer solution made up each second or third day.

Warburg flasks with one side arm were used for the analysis. They were placed in an ice bath while preparation was made for beginning the reaction. The reaction mixture in each flask is shown in Table 4. Ringer's buffer solution was added to all flasks so that the total volume in each flask was 3.0 ml. The brain homogenate and 10^{-5} M. eserine were pipetted into the main compartment of the flask avoiding the center well. Eserine was used in order to separate pseudocholine esterase activity from acetylcholine esterase activity. The substrate, acetylcholine iodide (7.52 per cent) or acetylcholine bromide (9.35 per cent), was pipetted into the side arm. The substrate was prepared 50.0 ml. at a time by dissolving the pure salt in diluted HCl of pH 4. Immediately before use, these stock solutions were diluted with four parts of the Ringer's buffer solution. The stable stock solutions were stored at approximately 4°C.

Since Baron (6) reported a depression in esterase activity when oil was given to animals, the AcE activity of the without ($\bar{s}$) oil rats was compared to the AcE activity of the with ($\bar{c}$) oil rats. The AcE activity of the $\bar{c}$ oil rats was also compared with the AcE activity of the rats receiving malathion. The AcE activities of brain homogenates of rats from a particular period were determined in the same run in order to have a valid comparison. With each run of two enzyme preparations to be compared, a thermobarometer was used for correction due to alterations in temperature and atmospheric pressure.

TABLE 4

REACTION MIXTURES FOR ACETYLCHOLINE ESTERASE DETERMINATION[1,2]

Flask No.	Homogenate	Substrate	Eserine	Buffer	H_2O	Total
	ml.	ml.	ml.	ml.	ml.	ml.
1	1.0	0.4	0.0	1.6	0.0	3.0
2	1.0	0.4	0.0	1.6	0.0	3.0
3	0.5	0.4	0.0	2.1	0.0	3.0
4	0.5	0.4	0.0	2.1	0.0	3.0
5	1.0	0.4	0.1	1.5	0.0	3.0
6	0.5	0.4	0.1	2.0	0.0	3.0
7	1.0	0.0	0.0	2.0	0.0	3.0
8	0.5	0.0	0.0	2.5	0.0	3.0
9	0.0	0.0	0.0	0.0	3.0	3.0

[1]Gas phase: 95 per cent N_2-5 per cent CO_2.

[2]Incubation temperature: 25°C.

The water bath of the Warburg apparatus was preheated to 25°C. The side arm and main opening of the flasks were made air tight by greasing the stem and manometer before inserting into the flasks. After the flasks were connected to the manometers, they were placed in the water bath and gassed for approximately 5 minutes with a 95 per cent N_2-5 per cent CO_2 gas mixture in order to drive out any oxygen present in the atmosphere of the flasks. The system was open during the gassing but was closed immediately after. Before the contents of the flasks were mixed, the Brodie's solution in the manometers was adjusted to read 150 mm. on both sides of the scale. This was the zero reading. The contents of the flasks were then mixed and readings were recorded at 5 minute intervals for 35 minutes by adjusting the right-hand scale to 150 mm. and reading the left-hand scale.

After the readings were completed, the system was opened and the flasks were removed from the manometers and degreased with acetone. The flasks were cleaned by the permanganate method of Ryan[7] who considered it superior to the usual dichromate method. This solution was prepared by dissolving 20.0 g. of potassium permanganate and 50.0 g. of sodium hydroxide in 1 liter of water. After degreasing, the flasks were rinsed in distilled water and soaked in the cleaning solution for at least 2 hours. The flasks were then rinsed in distilled water again and bleached by transferring to a saturated oxalic acid solution for a

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[7]Ryan, W. L. 1953 A metabolic study of cold shock in mammalian spermatozoa. Unpublished Ph. D. thesis. The University of Missouri.

few minutes. After bleaching, the flasks were rinsed and boiled by covering with distilled water in a pyrex dish or beaker and placing on a hot plate at about 400°F. After boiling, the flasks were allowed to cool, were rinsed in distilled and demineralized water, and were placed in an oven to dry.

The data from the AcE activity determinations were expressed as µl. CO_2 produced/mg. N_2. This was calculated as follows. The gas production at each reading minus the reading at zero time equals the mm. change produced as a result of the action of pseudocholine esterase and acetylcholine esterase. This figure minus the mm. change produced by the corresponding amount of enzyme preparation in the flask containing eserine is the mm. change produced as a result of acetylcholine esterase activity. The flask constant plus the manometer constant multiplied by the mm. change produced from the cleavage of acetylcholine equals the µl. CO_2 produced. The value obtained for the 30 minute reading was divided by the mg. N_2 in the corresponding quantity of brain homogenate to obtain µl. CO_2 produced in 30 minutes/mg. N_2.

Calibration of Flasks and Manometers

The flasks and manometers were calibrated by filling with mercury according to the method of Umbriet et al. (104). The flask and manometer constants were determined by the use of the following formula:

$$K_{CO_2} = \text{constant } CO_2 = \frac{Vg \cdot 273/T + Vf \cdot a}{P_o} ,$$

where Vg = total volume of the flask or manometer minus the fluid volume, T = 273 + 25, Vf·a = the fluid volume in the flask or manometer

times the solubility of CO_2, and P_o = 760 x 13.60/1.033 = 10,000 for Brodie's solution with a density of 1.033.

Nitrogen Determination

The nitrogen content of the liver and brain of each rat was determined by the micro-Kjeldahl method (105). One ml. of brain homogenate and 0.5 ml. of liver homogenate were analyzed in duplicate for each rat. In each case the homogenate was pipetted into a 30 ml. Kjeldahl digestion flask containing 1.25-1.35 g. of K_2SO_4 and 35-45 mg. of HgO. Two ml. of concentrated H_2SO_4 were added and the flask contents digested until the solution became clear. About 5 ml. of water were added to dissolve the material and, after the flask had cooled, the contents were quantitatively transferred to a steam distillation apparatus. Eight ml. of sodium hydroxide-sodium thiosulfate solution were then added and distillation was begun. Approximately 50 ml. were distilled into 5.0 ml. of saturated boric acid solution containing about 4 drops of either the methyl red-methylene blue or methyl red-bromcresol green indicator. The amount of nitrogen was determined by titration with 0.01 N. HCl prepared with constant boiling HCl. The mg. of N_2 were calculated by the following formula:

$$(\text{ml. acid}) \cdot (\underline{N}.\ \text{acid}) \cdot (\text{meq. wt. } N_2) = \text{mg. } N_2 .$$

The sodium hydroxide-sodium thiosulfate solution was prepared by dissolving 500 mg. of NaOH and 50 g. of $Na_2S_2O_3 \cdot 5H_2O$ in sufficient water to make one liter. The saturated boric acid solution was prepared by dissolving 40 g. of H_3BO_3 in about 900 ml. of distilled water

by heating, cooling the solution, and diluting to a final volume of one liter. The methyl red-methylene blue indicator was prepared by mixing two parts of 0.2 per cent methyl red with one part of 0.2 per cent methylene blue, both in 95 per cent ethanol. The methyl red-bromcresol green indicator was prepared by mixing five parts of 0.2 per cent bromcresol green with one part of 0.2 per cent methyl red, both in 95 per cent alcohol.

Determination of the Specific Activity of Costal Cartilage Sulfomucopolysaccharides

A sulfomucopolysaccharide fraction was prepared from the costal cartilage according to the modified method of Bostrom (97). The excised ribs were boiled in distilled water for approximately 5 minutes. The cartilage was completely freed of muscle and weighed. The cartilage was then sonified with approximately 5 ml. acetone by using a Bronwell, Biosonik probe. The acetone was discarded, and the cartilage sonified with 4.0 ml. of 0.5 N. NaOH for 4 minutes. The liquid portion was decanted into a 15 ml. centrifuge tube. The cartilage was then sonified a third time using 2.0 ml. of 0.5 N. NaOH for 2 minutes. The liquid was decanted, combining it with the product of the first extraction. The pH of the decanted liquid was adjusted to 6 with 10 per cent acetic acid, and the solution was centrifuged for 10 minutes at 2000 r.p.m. in an International Model SBV centrifuge. The supernatant fluid was poured into a 50 ml. centrifuge tube, 3 or 4 drops of 20 per cent sodium acetate were added, and the solution was precipitated

overnight at -20°C. with three volumes of 95 per cent ethanol. The next day, the mixture was centrifuged as above for 10 minutes, and the supernatant fluid was discarded. The residue was dissolved with stirring in 3.0 ml. of 0.5 N. NaOH and again centrifuged. The supernatant fluid was poured into a 15.0 ml. centrifuge tube, adjusted to pH 6, 20 per cent sodium acetate added, and precipitated overnight as described above. The following day, the solution was centrifuged, the supernatant fluid discarded, and the precipitate hydrolyzed with 6.0 N. HCl for 3 hours at 100°C. according to the method of Dodgson and Rice (106) for hydrolyzing the sulfate ester linkage. If a black precipitate formed during this procedure, the sample was filtered through Whatman No. 2 filter paper followed by washing with about 5 ml. of distilled water.

The sample was precipitated, collected, and counted by the method of Katz and Golden (107). One ml. of carrier sulfate solution was added followed by 1 ml. of 10 per cent barium chloride. The carrier sulfate solution, calculated to yield 12 mg. of barium sulfate, was prepared by diluting 2.85 ml. of concentrated sulfuric acid to 1 liter with distilled water. The resulting precipitate was collected as $BaSO_4$ on a weighed glass-fiber filter paper disc. During the collection process the 24 mm. filter paper disc was supported by a small piece of Whatman No. 1 filter paper and secured between a perforated rubber disc and a glass funnel cut from glass tubing. The side arms of the glass funnel were attached to a vacuum flask with springs. The precipitate was washed onto the paper with 5 to 10 ml. portions of distilled

water, 0.5 N. HCl, and 95 per cent ethanol followed by acetone. A dry cake of barium sulfate of constant area was formed. The filter paper containing the precipitate was weighed, attached to the center of an aluminum planchet with a small quantity of rubber cement, put in a drying oven for about 3 minutes, allowed to cool, and counted for 4000 counts with a Nuclear-Chicago, windowless, automatic, gas-flow chamber. The counts per minute (c.p.m.) per mM. of sulfate were calculated and recorded.

^{35}S-Sulfate Incorporation by Liver

The effect of malathion on ^{35}S-sulfate incorporation by the liver was measured by homogenizing the livers with a motor-driven Potter-Elvehjem homogenizer using a teflon pestle and a glass tube. The livers were homogenized individually in 3.0 ml. of medium A per gram of tissue. Medium A was prepared by dissolving 231.40 g. sucrose (0.350 M.), 7.00 g. $KHCO_3$ (0.035 M.), 1.63 g. $MgCl_2 \cdot 6H_2O$ (0.004 M.), and 3.72 g. KCl (0.025 M.) in 2 liters of distilled water. This solution caused difficulty in combusting and digesting because of the sugar content. Later in the study, Ringer's buffer solution was used with more success.

Duplicate 0.5 ml. samples of each liver were combusted in 5.0 ml. 50 per cent HNO_3 and then in 5.0 ml. combustion mixture composed of 600 ml. concentrated HNO_3, 300 ml. 70 per cent perchloric acid, and 9.0 g. cupric nitrate. The samples were dissolved in 10.0 ml. distilled water and precipitated, collected, and counted as previously described.

The counts per minute were calculated and the data expressed as counts per minute per mg. N_2.

Determination of Specific Activity of Diets and of Total Activity of Radioactive Bands from Chromatography Experiments

The specific activity of each radioactive diet was determined by combusting duplicate 0.5 g. samples in a Parr, series 1900, oxygen bomb sulfur apparatus containing 10.0 ml. distilled water. The bomb was charged with oxygen to a pressure of 35 atmospheres. The bomb was then ignited and allowed to cool in a water bath for 15 minutes. The sample was transferred from the bomb to a 25 ml. erlenmeyer flask, precipitated, collected, and counted as previously described. The counts per minute per gram of diet were calculated. The corresponding bands of each sample chromatographed (experiment 7) were combined and combusted 1.0 g. at a time as described above. The total c.p.m./band were calculated.

Determination of the Specific Activity of the Brains Obtained from Rats Fed Malathion-^{35}S

Many of the pesticides now used are reported to be stored in lipid; therefore, the specific activity of the lipid and protein portion of brains of rats fed malathion-^{35}S was determined separately. Each brain was homogenized in 10 ml. of 2:1 chloroform-methanol. The homogenate was then transferred to a 15 ml. centrifuge tube, covered, and heated in a 60°C. water bath for about 10 minutes. The mixture was centrifuged at 2000 r.p.m. in an International Centrifuge, Model

SBV, and the liquid containing lipid was decanted into a 25 ml. volumetric flask. The protein portion was homogenized, heated, and centrifuged a second time and the liquid decanted into the 25 ml. volumetric flask. Sufficient 2:1 chloroform-methanol was added to the flask to bring the liquid to volume. The protein portion was homogenized in medium A, and the lipid and protein fractions of each sample were combusted, precipitated, collected, and counted as described previously. Five ml. duplicate samples of each fraction were combusted. Most of the chloroform-methanol in the lipid samples was allowed to evaporate on low heat before the combustion mixture was added.

Determination of the Specific Activity of Urine

The urine samples collected were diluted to a known volume and duplicate 1.0 ml. samples were combusted in 25 ml. flasks with 3.0 ml. combustion fluid. After combusting, the samples were precipitated, collected, and counted as previously described. The c.p.m. in the total urine sample collected were calculated.

Determination of the Specific Activity of Feces

The fecal samples were dried in a 60°C. blowing oven for about 4 hours and were weighed. Each sample was ground to a fine powder with a mortar and pestle. Duplicate 0.3 g. samples were combusted, precipitated, collected, and counted. The c.p.m. total fecal sample were calculated.

Statistics

The methods of paired or unpaired comparisons and Student's *t* test as described by Steel and Torrie (108) were used to determine the statistical significance of results. The method of unpaired comparisons was used when data were missing due to death of experimental animals.

CHAPTER IV

RESULTS AND DISCUSSION

As stated previously, this investigation was begun in order to compare the effects of malathion administration on acetylcholine esterase and the fixation of sulfate by cartilage mucopolysaccharides. The original hypothesis was that interference with sulfur metabolism would be shown by an inhibition of sulfate fixation which would be more sensitive to malathion administration than acetylcholine esterase inhibition. In the process of investigating this hypothesis, other effects of malathion on sulfur metabolism were found and will be discussed. In addition, an investigation of the relationship of these effects to the level of inorganic sulfur in the diet also evolved from investigating the original hypothesis. The fixation of sulfate by cartilage mucopolysaccharides has been shown to be sufficiently sensitive for the assay of growth hormone (8); therefore, it seemed plausible to assume that if sulfate fixation were affected by malathion administration, an inhibition of sulfate fixation might be used to detect malathion toxicity and to study any dietary related changes in malathion intoxication.

Since the optimum level of malathion for investigation of these effects was not known, a survey was conducted by giving rats levels of malathion ranging from 100 to 1000 mg./kg. of rat (experiment 1). The levels shown in Table 5 were the ones used most successfully in the

TABLE 5

EFFECT OF MALATHION ADMINISTRATION ON ACETYLCHOLINE ESTERASE ACTIVITY AND ^{35}S-SULFATE FIXATION

No. of Animals	Additions mg. Malathion/ Kg. of Rat	Acetylcholine Esterase[1]		Sulfate Fixation[2]	
		μl. CO_2/mg. N_2	Per Cent Inhibition	c.p.m./mM. x 10^{-4}	Per Cent Inhibition
		Male			
20	None	192 ± 10	--	7.7 ± 2.3	--
5	250	189 ± 37	1.5	17.9 ± 9.3	--
6	300	197 ± 9	--	3.0 ± 0.8	61.0
8	500	169 ± 8	11.9	6.4 ± 1.7	16.8
		Female			
21	None	173 ± 9	--	9.9 ± 0.9	--
8	250	135 ± 17	21.9	3.7 ± 1.1	62.6
6	300	162 ± 11	6.3	1.8 ± 0.0	81.8
11	500	144 ± 10	16.7	9.4 ± 4.0	5.0

[1]Data are averages ± the standard error of the mean of values obtained from assay of rat brain homogenates.

[2]Data are averages ± the standard error of the mean of values obtained from assay of rib cartilage mucopolysaccharides.

survey. Levels of malathion below 250 mg./kg. were found to be of little value in this type of study, and illness or death occurred when levels higher than 500 mg./kg. were attempted. Therefore, levels of malathion ranging from 250 to 500 mg./kg. were used throughout the study as a result of this initial survey.

A comparison of the data for depression of acetylcholine esterase activity and sulfate fixation presented in Table 5 shows that sulfate fixation is depressed more than acetylcholine esterase activity. These data are particularly exciting since assay of acetylcholine esterase is the classic method of detecting organophosphorus poisoning and since Murphy (6) has shown that inhibition of esterases other than acetylcholine esterase are more sensitive to poisoning by these compounds. When comparing the control with the values for rats receiving malathion, there are much greater depressions in sulfate fixation than in acetylcholine esterase inhibition. The greatest inhibition of acetylcholine esterase activity was 22 per cent, the value obtained by comparing the control value with the value for 250 mg. malathion/kg. for the females. A 63 per cent depression in sulfate fixation by females was obtained by comparing these same values. The greatest inhibition of sulfate fixation (82 per cent) was obtained by comparing the control value with the value for 300 mg./kg., again in female rats.

In agreement with data collected by other investigators (27, 32, 33) using other strains of rats, the data in Table 5 show that the female rats used were more sensitive than were the males. There is a significant inhibition ($.05 > P > .02$) of acetylcholine esterase

activity by malathion when comparing the values obtained from female rats receiving no malathion with those given 250 mg./kg. while there is no significant decrease in the corresponding values for male rats. The same is true for the inhibition of sulfate fixation when the values for the control and 250 mg./kg. are compared in both males and females. There is a highly significant decrease ($.01 > P > .001$) in the inhibition of sulfate fixation in females when comparing the control value with the value for 250 mg./kg. The higher degree of significance obtained for sulfate fixation inhibition in females is additional evidence that inhibition of sulfate fixation is more sensitive to malathion administration than acetylcholine esterase inhibition. Although no statistical significance was found when comparing the control values with the values for malathion for either type of inhibition in males, there is a significant decrease ($.05 > p > .02$) when the values for 300 and 500 mg./kg. for acetylcholine esterase inhibition are compared.

Since Baron (6) has shown a significant depression of esterase activity when 5 to 10 ml. of corn oil were given to rats, a comparison of the data obtained for acetylcholine esterase activity and ^{35}S-fixation by rib cartilage from rats receiving no corn oil and rats receiving oil was made (Table 6). Statistical analysis showed that the levels of oil used in this study cause no significant decrease in either acetylcholine esterase activity or ^{35}S-fixation by rib cartilage mucopolysaccharides.

In order to observe the effects of feeding malathion as a constituent of the diet on acetylcholine esterase activity and ^{35}S-fixation, malathion was fed at the levels of 5 and 10 mg. of malathion/g. of diet

TABLE 6

EFFECT OF CORN OIL ON ACETYLCHOLINE ESTERASE ACTIVITY AND ^{35}S-FIXATION

Treatment[1]	Acetylcholine Esterase[2]	^{35}S-Fixation[3]
	μl. CO_2/mg. N_2	c.p.m./mM. x 10^{-4}
$\bar{s}$ oil (9)	157 ± 22	14.0 ± 15.0
$\bar{c}$ oil (9)	138 ± 12	14.8 ± 4.3

[1]Numbers in parentheses indicate number of animals per treatment.

[2]Averages ± the standard error of the mean of data obtained from brain homogenates.

[3]Averages ± the standard error of the mean of data obtained from rib cartilage mucopolysaccharides.

(experiment 2). The data obtained from this study are shown in Table 7. There were no significant decreases in acetylcholine esterase activity or ^{35}S-fixation as a result of feeding diets containing malathion, although the malathion was given for 8 to 10 days as compared with 3 days in the previous experiment. When the diet containing 5 mg. of malathion/g. of diet was fed, the rats received the same or a little more malathion (about 250 mg./kg.) than in the first experiment since they ate about 10 g. of diet/day. At the level of 10 mg. of malathion/g. of diet, the rats received about twice the amount of malathion given in the previous experiment or 500 mg. of malathion/kg. of rat. Metcalf (31) and Negherbon (14) have reported that the tolerance of rats for malathion in the diet is relatively great. Symptoms are few or none as was observed with these rats. Five thousand parts per million (p.p.m.) has a slight effect on survival, feed intake, and growth. Some males have survived 10,000 p.p.m. for a year and some females have survived 20,000 p.p.m. for 2 years, but with serious effects on growth, feed intake, and general health.

The rats in the above experiment showed no rejection of the diet and did not lose weight. The difference in the toxicity of malathion given in the diet as compared with that given by stomach tube is probably due to a difference in absorption of the malathion. As previously discussed, corn oil facilitates the absorption of malathion (23). Poor absorption of malathion when given as a constituent of the diet could have been due to the concentration of oil in the diet, since there was much less oil associated with the malathion in the diet than when given by stomach tube.

TABLE 7

EFFECT OF FEEDING MALATHION AS A DIETARY CONSTITUENT ON ACETYLCHOLINE ESTERASE ACTIVITY AND ^{35}S-FIXATION

Mg. Malathion/ g. of Diet[1]	Acetylcholine Esterase[2]	^{35}S-Fixation[3]
	μl. CO_2/mg. N_2	c.p.m./mM. x 10^{-4}
None (11)	165.0 ± 6.5	4.0 ± 0.7
5 (6)	121.3 ± 28.1	5.7 ± 0.6
10 (5)	188.2 ± 10.6	4.1 ± 0.2

[1]Numbers in parentheses indicate number of animals per treatment.

[2]Averages ± the standard error of the mean of data obtained from brain homogenates.

[3]Averages ± the standard error of the mean of data obtained from rib cartilage mucopolysaccharides.

Randomly labeled malathion-^{35}S was given to four rats in order to investigate the metabolism of the sulfur in malathion (experiment 3). Several organs and the excreta were analyzed for ^{35}S-activity. The rats were killed 24 hours after the last malathion administration. The data in Table 8 show that at this time very little ^{35}S-activity is found in the tissues such as liver, brain, and cartilage and that most of a malathion dose is excreted in the urine with an appreciable amount being excreted in the feces. From these data, the location of malathion in brain tissue is difficult to determine. The data indicate that the malathion may be in the lipid portion, although the literature says it is not stored in lipid as are DDT and some other pesticides. Of the three tissues assayed, the liver contained the greatest amount of malathion. This could be expected since malathion is detoxified as well as made more toxic in the liver of mammals.

Experiment 4 was planned in order to further study ^{35}S excretion patterns and to investigate the effects of $Ca^{35}SO_4$ and ^{35}S-cysteine as radioactive compounds on ^{35}S excretion when malathion is given. Although the standard errors presented in Table 9 are quite large, they reflect the inability to prepare diets of the same specific activity from replication to replication rather than a biological variation. Therefore, although statistical verification is difficult, these and other data to be presented indicate that there is a mobilization of tissue sulfur following malathion administration. Based on the averages obtained, these data show that feeding 400 mg. of malathion/kg. of rat results in an approximate 2-fold increase in the total ^{35}S-excretion

TABLE 8

DISTRIBUTION OF ^{35}S-ACTIVITY IN URINE, FECES AND SELECTED TISSUES OF THE RAT FOLLOWING ADMINISTRATION OF RANDOMLY LABELED ^{35}S-MALATHION[1]

Mg. Malathion/ Kg. of Rat	Liver	Brain Fractions		Cartilage Mucopoly-saccharides	Urine	Feces
		Lipid	Protein			
	c.p.m./mg. N_2	total c.p.m.		c.p.m./mM. SO_4	Total c.p.m. x 10^{-5}	Total c.p.m. x 10^{-5}
250	50	51	100	864	14.8	0.8
250	42	225	152	576	16.6	1.1
500	38	413	207	1536	20.6[2]	0.6[2]
500	113	115	214	2112	--	--

[1]Numbers represent only one animal since there were four animals on the study.

[2]Data are missing because of death of one rat.

TABLE 9

EFFECT OF MALATHION ON ^{35}S EXCRETION IN FECES AND URINE OF FEMALE RATS[1]

Mg. Malathion/ Kg. of Rat[2]	Total ^{35}S Excretion in c.p.m. x 10^{-3}			
	Urine		Feces	
	$Ca^{35}SO_4$[3]	^{35}S-cysteine[3]	$Ca^{35}SO_4$[3]	^{35}S-cysteine[3]
None (7)	33.8 ± 14.4	26.7 ± 2.1	18.9 ± 4.2	18.9 ± 8.5
250 (5)	16.4 ± 7.6	34.0 ± 6.4	26.8 ± 16.8	21.3 ± 6.1
400 (2)	55.1 ± 48.6	71.5 ± 37.1	41.7 ± 38.2	69.1 ± 59.4
500 (5)	20.7 ± 4.2	26.4 ± 7.9	15.7 ± 2.6	27.6 ± 21.9

[1]Data are averages ± the standard error of the mean.

[2]Numbers in parentheses are number of animals per treatment.

[3]Source of ^{35}S-activity in the diet.

in both the feces and urine which is somewhat greater if the sulfur pools are labeled by feeding ^{35}S-cysteine. In spite of the large standard errors, there is a significant increase ($.05 > P > .02$) when the ^{35}S excretion in urine of the control rats is compared with the value obtained for rats receiving 400 mg./kg. and the ^{35}S-cysteine diet, and there is almost a significant increase ($.1 > P > .05$) in the corresponding values obtained from feces of rats fed this diet.

The data in Table 9 for rats given 500 mg. of malathion/kg. of rat did not show an increase in mobilization when compared with the data for 400 mg./kg. as might be expected. The data obtained from analysis of excreta of these rats may not be reliable since at 500 mg./kg. many of the rats became sick.

The cartilage mucopolysaccharides and livers of these rats were analyzed for ^{35}S in an attempt to determine the source of the mobilized sulfur found in urine and feces in the above experiment. Since feeding ^{35}S-cysteine would label tissue proteins as well as ester sulfates, the more than 20-fold increase in the specific activity of liver (Table 10) obtained when ^{35}S-cysteine was fed as compared with values obtained when rats were fed $Ca^{35}SO_4$ was not surprising. The higher specific activity may mask any mobilization of sulfur by malathion; however, these data indicate that tissue amino acids are not the source of the sulfur mobilized by malathion administration. In contrast, the reductions in specific activity of liver ester sulfate obtained when rats were fed 400 and 500 mg. of malathion/kg. and $Ca^{35}SO_4$ seem to indicate that ester sulfate is the source of the sulfur mobilized when malathion

TABLE 10

EFFECT OF MALATHION ON ^{35}S MOBILIZATION FROM LIVER OF FEMALE RATS

Mg. Malathion/ Kg. of Rat[2]	Counts/Min./Mg. N_2[1]	
	$Ca^{35}SO_4$[3]	^{35}S-Cysteine[3]
None (7)	0.73 ± 0.32	20.87 ± 1.20
250 (5)	0.88 ± 0.45	20.36 ± 2.03
400 (2)	0.52 ± 0.02	22.05 ± 1.05
500 (5)	0.60 ± 0.10	21.20 ± 2.20

[1]Data are averages ± the standard error of the mean.

[2]Numbers in parentheses are number of animals per treatment.

[3]Sources of ^{35}S in the diets.

is administered. There is a significant decrease ($.05 > P > .02$) in ^{35}S incorporation into liver ester sulfates when the values obtained from rats fed the $Ca^{35}SO_4$ diet and 250 and 400 mg./kg. are compared.

Although the reduction in specific activity is not drastic and the standard error is large, the data which are shown in Table 11 indicate a trend toward sulfate mobilization from the cartilage mucopolysaccharides. These data support the indication obtained from analysis of liver tissue that ester sulfate is the source of the sulfur mobilized following malathion administration. The failure to obtain a clear-cut indication of the source of the mobilized sulfur by analysis of these two tissues was disappointing; however, these data suggest that ester sulfate is probably the source of the mobilized sulfur and that sulfolipid is a possibility. This possibility is especially attractive in view of the high concentration of sulfolipid in the brain and the neurological symptoms of malathion toxicity. Although difficult to examine, the possibility of sulfolipids as a source of the mobilized sulfur seems worthy of investigation.

Experiments 5 and 6 were conducted in order to investigate the effects of malathion administration on sulfur metabolism in rats fed diets containing varying levels of inorganic sulfur. The two experiments were identical except that 250 mg. of malathion/kg. was given in experiment 5 while 400 mg. of malathion/kg. was given in experiment 6. The diets were radioactively labeled as shown in Table 3, page 33. Previous

TABLE 11

EFFECT OF MALATHION ON ^{35}S MOBILIZATION FROM CARTILAGE OF FEMALE RATS[1]

Mg. Malathion/ Kg. of Rat[2]	Counts/Min./mM. x 10^{-3}	
	$Ca^{35}SO_4$[3]	^{35}S-Cysteine[3]
None (7)	7.52 ± 1.34	5.01 ± 1.64
250 (5)	7.18 ± 1.59	5.93 ± 1.44
400 (2)	7.10 ± 0.29	4.37 ± 0.91
500 (5)	8.77 ± 1.17	6.55 ± 1.87

[1]Data are averages ± the standard error of the mean.

[2]Numbers in parentheses are the number of animals per treatment.

[3]Source of ^{35}S in the diet.

work from this laboratory[1] has shown that rats fed a high level of inorganic ^{35}S-sulfur will incorporate a greater percentage of the sulfur into their cartilage mucopolysaccharides (Table 12). However, when the rats were given 250 mg. of malathion/kg. of rat, the largest per cent of the radioactivity appeared to be incorporated into the cartilage mucopolysaccharides of rats fed the lowest level of inorganic sulfur. The values obtained for the normal rat fed the diets containing 0.10 per cent inorganic sulfur as sulfate are not significantly different, while there are highly significant increases in the retention of labeling when rats were fed these diets and given 250 ($.02 > P > .01$) and 400 mg. of malathion/kg. of rat ($.001 > P$). There is a significant difference ($.02 > P > .01$) in the values obtained with normal rats fed the diets containing 0.42 per cent and 0.00 per cent inorganic sulfur as sulfate while there is no significant difference in the values obtained from rats fed the same diets but also receiving malathion.

These data show an interrelationship between dietary sulfur and the effects of malathion on sulfur metabolism and seem to fit the other data obtained. For example, why should malathion administration cause an increase in the specific activity of cartilage mucopolysaccharide sulfur? These rats received the radioactive diet before malathion administration was begun; therefore, if one assumes mobilization of sulfur from all mucopolysaccharides and resynthesis, the data obtained in Table

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[1]Gilmore, M. F. 1963 A comparison of the utilization of organic and inorganic sulfur by the rat. Unpublished M. S. thesis. The University of Tennessee.

TABLE 12

RADIOACTIVITY OF CARTILAGE MUCOPOLYSACCHARIDES[1]

Diet	Per Cent Dietary Sulfur as Sulfate		Level of Malathion[2]		
	Inorganic	Organic	None[3]	250	400
A	0.42 ($Ca^{35}SO_4$)	0.25	.78 ± .04	1.99 ± .26	2.17 ± .20[4]
B	0.10	0.57 (^{35}S-amino acid)	.64 ± .10	1.10 ± .20	1.28 ± .26
C	0.10 ($Ca^{35}SO_4$)	0.57	.56 ± .04	1.84 ± .29	4.48 ± .54
D	0.00	0.67 (^{35}S-amino acid)	.39 ± .04	2.37 ± .71	1.83 ± .63[4]

[1]All data are averages of 5 trials expressed as counts/min./mM. SO_4 as a percentage of the total ingested radioactivity ± the standard error of the mean, unless indicated otherwise.

[2]Mg. of malathion given/kg. of rat.

[3]Gilmore, M. F. 1963 A comparison of the utilization of organic and inorganic sulfur by the rat. Unpublished M. S. thesis. The University of Tennessee.

[4]Four animals per group.

11, page 62, and Table 12, page 64, could be obtained. Since rats fed a low sulfur diet have been shown to synthesize mucopolysaccharides slower than those with sufficient sulfate, the specific activity would not be diluted as fast by new synthesis; therefore, in order for this difference to show up there must be resynthesis of mucopolysaccharides. Malathion administration above 250 mg./kg. drastically inhibits new synthesis of mucopolysaccharides (Table 5, page 50), so new synthesis would not occur at a rate sufficiently rapid to overcome the difference in the original labeling of the mucopolysaccharides and the pattern of 250 mg./kg. of malathion would not be obtained with 400 mg./kg. as shown.

The next table (Table 13) shows that the highest level of ^{35}S-sulfur was found in the feces of the normal rats fed a 0.10 per cent of sulfate diet. In contrast, when the rats were given malathion the highest level of ^{35}S-sulfur was found in the feces of rats fed the diet low in sulfate (0.00 per cent). The data show a greater mobilization of ^{35}S-sulfur from the tissues of rats fed the low sulfate diet. Statistical analysis showed significant increases in ^{35}S excretion in feces of rats fed the 0.00 per cent of sulfate diet and 400 mg. of malathion/kg. as compared with the two 0.10 per cent of sulfate diets (^{35}S-cysteine, $.05 > P > .02$; $Ca^{35}SO_4$, $.01 > P > .001$). When rats were fed the 0.00 per cent of sulfate diet and 250 mg. of malathion/kg., there were significant increases in ^{35}S excretion in feces as compared with the values obtained when rats were fed the 0.10 per cent and the 0.42 per cent of sulfate diets ($Ca^{35}SO_4$, 0.10 per cent, $.05 > P > .02$;

TABLE 13

RADIOACTIVITY OF FECES[1]

Diet	Per Cent Dietary Sulfur as Sulfate		Level of Malathion[2]		
	Inorganic	Organic	None[3]	250	400
A	0.42 ($Ca^{35}SO_4$)	0.25	1.47 ± .23	0.46 ± .08	1.33 ± .27[4]
B	0.10	0.57 (^{35}S-amino acid)	1.52 ± .44	1.11 ± .19	1.14 ± .11
C	0.10 ($Ca^{35}SO_4$)	0.57	1.97 ± .40	0.73 ± .08	0.91 ± .10
D	0.00	0.67 (^{35}S-amino acid)	1.21 ± .23	1.24 ± .16	1.60 ± .18[4]

[1]All data are averages of 5 trials expressed as a percentage of the total ingested radioactivity ± the standard error of the mean, unless indicated otherwise.

[2]Mg. malathion/kg. of rat..

[3]Gilmore, M. F. 1963 A comparison of the utilization of organic and inorganic sulfur by the rat. Unpublished M. S. thesis. The University of Tennessee.

[4]Four animals per group.

0.42 per cent, $.02 > P > .01$). There was also a significant increase in ^{35}S excretion in the feces of rats fed the ^{35}S-cysteine 0.10 per cent of sulfate diet and 250 mg. of malathion/kg. as compared with the value for the 0.42 per cent of sulfate diet ($.01 > P > .001$).

The source of the organic sulfur in the work of Gilmore shown in Tables 12 and 13, pages 64 and 66, respectively, was methionine while cysteine was substituted for the amino acid in this study. Previous work[2] in this laboratory has shown that these sulfur amino acids are interchangeable as endogenous sources of inorganic sulfate.

An increased mobilization of ^{35}S as a result of malathion administration when a low sulfur diet is fed is also shown in Table 14. The value for the rats fed the low inorganic sulfur diet and given 250 mg. of malathion/kg. is over twice as large as the value for rats fed the diet high in inorganic sulfur. When rats were fed the diet containing a low level of inorganic sulfur (0.00 per cent) and given 250 mg./kg., there were significant increases in the ^{35}S excretion in the urine as compared with the values obtained when rats were fed diets containing high ($.01 > P > .001$) and normal ($Ca^{35}SO_4$, $.05 > P > .02$) levels of inorganic sulfur.

An attempt was made to identify the ^{35}S-excretory products in the urine and feces in order to determine the sources of the ^{35}S being mobilized. The ^{35}S compound isolated from both urine and feces was

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[2]Fulton, S. F. Unpublished observation.

TABLE 14

RADIOACTIVITY OF URINE[1]

Diet	Per Cent Dietary Sulfur as Sulfate		Level of Malathion[2]	
	Inorganic	Organic	250	400
A	0.42 ($Ca^{35}SO_4$)	0.25	2.28 ± .56	4.17 ± .71[3]
B	0.10	0.57 (^{35}S-amino acid)	3.05 ± 1.51	3.68 ± .34
C	0.10 ($Ca^{35}SO_4$)	0.57	2.51 ± .98	3.00 ± .55
D	0.00	0.67 (^{35}S-amino acid)	5.09 ± .56	3.82 ± .36[3]

[1]All data are averages of 5 trials expressed as a percentage of the total ingested radioactivity ± the standard error of the mean, unless indicated otherwise.

[2]Mg. malathion given/kg. of rat..

[3]Four animals per group.

found to be water soluble. The appearance of several radioactive bands on paper chromatographs of urine and fecal extracts indicates that more than one compound is present. Further research will have to be done before identification of these compounds is possible.

CHAPTER V

SUMMARY

The effect of malathion administration on the acetylcholine esterase activity and the fixation of a test dose of $^{35}SO_4^=$ was compared. The decrease in fixation of $^{35}SO_4^=$ was shown to be a more sensitive method of detecting toxicity than depression of acetylcholine esterase since the depression of the enzyme was never more than 22 per cent with the highest levels of malathion while an 82 per cent depression in sulfate fixation was obtained with female rats given 300 mg. of malathion/kg. of rat. By feeding malathion in the diet, the conclusion was made that rats have a high tolerance for malathion given in this manner since there was no significant depression in acetylcholine esterase activity or ^{35}S-fixation as obtained when malathion was given by stomach tube. As a result of administering malathion-^{35}S, malathion was found to be excreted mainly in the urine with some fecal excretion. A very small amount of malathion was found in the liver, brain, and rib cartilage with the largest concentration in these tissues being found in the liver.

If the sulfur pools of the rats were labeled by feeding diets containing either $Ca^{35}SO_4$ or ^{35}S-cysteine, feeding 400 mg. of malathion/kg. resulted in a two-fold increase in ^{35}S excretion in both the urine and feces. Data obtained from ^{35}S analysis of cartilage mucopolysaccharides and livers of these rats indicated that ester sulfate is

the source of the mobilized sulfur.

Malathion was shown to change the normal labeling pattern of cartilage mucopolysaccharides when diets varying in the level of inorganic sulfate were fed. A greater mobilization of ^{35}S resulted in an increase in ^{35}S excretory products in both urine and feces of rats given malathion and fed diets low in inorganic sulfur than when fed diets containing normal or high levels of inorganic sulfur.

Attempts were made to identify the compounds mobilized and excreted in urine and feces. Identification was not made but the ^{35}S compounds are believed to be water soluble and possibly to originate from more than one source.

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LITERATURE CITED

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