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THE TOXICITY OF PARATHION TO <u>ORCONECTES</u>

<u>RUSTICUS</u> AND <u>VIVIPARUS</u> <u>MALLEATUS</u>

BY

LESLIE ALAN GOLDSMITH

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1978

PARATHION TOXICITY TO

ORCONECTES & VIVIPARUS

Abstract

Parathion is an organophosphate pesticide used in great quantities in the United States and around the world. The mechanism of toxicity for parathion in mammals has been attributed to its enzymatic desulfuration to its oxygen analog paraoxon which subsequently forms a covalent bond with acetylcholinesterase (AChE), inhibiting the activity of that enzyme and precipitating cholinergic toxicity. The mechanism by which parathion produces its toxic effects in insects has not been completely determined, but it is believed to be due to the same mechanism. The effect parathion exposure has on fresh water invertebrates has not been investigated to any great extent, and a goal of this project was to determine the effects of parathion exposure and the relationship of this toxicity to the metabolism of parathion by the fresh water snail Viviparus malleatus and the crayfish Orconectes rusticus.

The determination of the toxicity of parathion in Orconectes and Viviparus was made by exposing the organisms to different concentrations of parathion or by the direct injection of parathion. The possibility that any toxicity exhibited by parathion was produced by paraoxon was determined by observing the effect the oxygen analog of parathion would have when exposed to the species, and determining the metabolism of parathion by the two species.

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The metabolism of parathion was determined in vitro and in vivo. The efficient and specific separation and identification of parathion and its metabolites were accomplished through the use of thin layer chromatography.

Parathion has been shown to be metabolized by different species by a multitude of pathways. Three of the important pathways produce p-nitrophenol as an end product. A spectrophotometric assay was used to measure the in vitro production of p-nitrophenol from homogenates of crayfish and snail tissues incubated with parathion.

The excretion of parathion metabolites by Orconectes and Viviparus was determined by identifying the compounds extracted from water samples that had contained crayfish or snails exposed to parathion. The accumulation of parathion or metabolites in the species was determined by extracting the parathion exposed tissues of crayfish or snails and identifying and quantifying the parathion and metabolites present. The excretation and accumulation experiments were accomplished with the use of ^{14}C labeled parathion which was labeled either in the ring or ethyl position.

The possible metabolism of parathion was also investigated by the direct determination of the formation of paraoxon, p-nitrophenol, diethyl phosphate and diethyl phosphorothionate by the homogenates of crayfish or snail tissues incubated with parathion and appropriate cofactors. This determination was also aided by the use of labeled parathion.

The snail Viviparus malleatus did not metabolize parathion or

paraoxon and did not accumulate parathion in its tissues. The snail did not exhibit any toxic reaction to parathion or paraoxon by exposure or direct injection. Concentrations of parathion that equaled the compound's solubility in water produced no effect in exposure experiments while injections of 250 mg/kg parathion had no untoward effect.

Acknowledgment

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I. Introduction

The organophosphates are a very large class of compounds as there are over 50,000 compounds in that category today. Originally developed in Germany as nerve gases, the most important ones being tabun, sarin, and soman, they have since been found to be of great use to mankind as pesticides.

Parathion (O,O-diethyl O-p-nitrophenyl phosphorothionate) was introduced in 1944 by Gerhard Schrader (Neal 1971), it is an ester of the acid $(HO)_3$ P=S with two molecules of ethanol and one molecule of the weakly acidic alcohol p-nitrophenol.
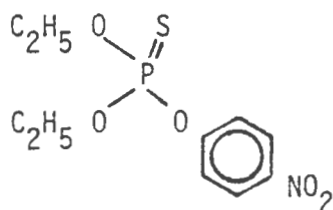
Unquestionably the organophosphates elicit their pharmaco-logical activity in vertebrates predominatly via an inhibition of the activity of esterase enzymes, especially acetyl cholinesterase (AChE) (EC 3.3.3.7) (Neal 1971). The extent to which enzyme inhibition is the mechanism of toxicity to other susceptible species is still being investigated, and it is not established that AChE inhibition is the

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only factor of its toxicity, O'Brian (1967) and Matsumura (1975).

Parathion requires enzymatic activiation to its oxygen analog to exhibit significant anti-esterase activity. This is supported by the work of Alary and Brodeur (1970), Bartels and Nachmansohn (1965), Neal (1971), Ptashne and Neal (1972), Roth and Neal (1972). The anti-cholinesterase effect of parathion is, therefore, dependent on the amount of free paraoxon generated. Thus the conversion of parathion to its oxygen analog paraoxon is of great toxicological importance. The relative degree of formation of alternative products, p-nitrophenol, paraoxon, diethyl phosphate and diethyl phosphorothionate produced by reactions with parathion are interesting because of their divergent toxicities, environmental contamination potentials. and use as a marker for parathion contamination.

The possible accumulation of parathion and/or its metabolites in species has been investigated. Miller et al. (1966) showed that some fish and the freshwater mussel accumulate parathion. No accumulation of parathion in crayfish or lobster tissues has been reported, and Yu and Sanborn (1975) could not show any accumulation of parathion in the snail used in their investigations.

Parathion has been shown to be toxic to many different species of animals. However, those species differ in their degree of susceptibility. The specifics of parathion toxicity in each individual species is considered in the literature review section as parathion has been shown to be toxic in the hamster, guinea pig,

mouse, rat, rabbit, cow, dog, pig, cat (Whitehouse and Ecobichon 1975) sunfish (Benke and Murphy 1974) mosquito fish (Chambers and Yarbough 1973) fathead minnow (Faust 1964) pink shrimp (Coppage and Mathews 1974) and lobster (Carlson 1973).

Not only is there a difference in species susceptibility to parathion but the age and sex of the animal also plays a role in the susceptibility of the species (Neal and DuBois 1965). The factor relating the differential toxicities produced by parathion exposure is the differential rate of metabolism of parathion to toxic or nontoxic compounds and the detoxification of the toxic metabolite paraoxon (Benke and Murphy 1975).

The chemical reactions and metabolism of this dialkyl phosphoric acid triester, parathion, are thus the essence of its toxicological importance. The two most important of the chemical reactions of parathion are hydrolysis and isomeriztion.

Little research into the toxicity and metabolism of parathion in crayfish and snails has been done. However, some interesting data have been gathered. Albaugh (1972) and Muncy and Oliver (1963) both have shown that crayfish are very sensitive to parathion exposure, perishing at concentrations below 1 ppm. However, when Carlson (1973) and Elmamlouk and Gessner (1976) investigated the necessary toxic conversion of parathion to paraoxon in lobsters neither investigator could demonstrate any such metabolism.

Important goals of this research were to determine the accumulation of parathion and metabolites in Orconectes rusticus and

<u>Viviparus</u> <u>malleatus</u> and to determine the metabolism of parathion by these species with particular emphasis on investigating the conversion of parathion to paraoxon by the crayfish. These data along with the toxicity exhibited by parathion in these species would enlighten the toxicological and environmental significance of parathion exposure to <u>Orconectes</u> and <u>Viviparus</u>.

II. Literature Review

The mandatory conversion of parathion to paraoxon to produce AChE inhibition in mammals points out the importance of the chemical reactions possible with parathion. Hydrolysis of organophosphates is one of the important chemical reactions that has been investigated.

Hydrolysis of organophosphates occurs in alkaline environments via an attack on the phosphours by OH^-. This is a nucleophilc attack where the phosphorus is positively charged as the result of the electron withdrawing capacity of the R groups of the molecule, and the dipolar and double bond character of the molecule. This concept of the electrophilic character of the R group attached to the phosphorus affecting the ease of hydrolysis is important. The conversion of parathion to paraoxon creates a double bonded oxygen in paraoxon which is more electrophilic than the double bonded sulfur of parathion. Thus the phosphorus of paraoxon is more positively charged and hydrolyzable in alkali medium than that of parathion (O'Brian 1967). The hydrolysis of parathion or paraoxon in an alkaline environment results in cleavage of the aryl side group and its replacement by an H atom and the production of a free p-nitrophenol moiety (Faust et al. 1972). Acid environments would lead to a rupture of the OCH_2CH_3 moiety as the initial step (Faust et al.

1972). The hydrolysis of parathion or paraoxon is dependent on pH
and temperature, and the hydrolysis of both compounds is faster at
higher pH and temperature (Faust et al. 1972). The method by which
hydrolysis of parathion occurs chemically is important because in
the living system the biochemical hydrolysis of paraoxon by AChE
causes a covalent bonding of the enzyme and compound which leads to
enzyme inhibition. There are other materials that promote organo-
phosphorus hydrolysis, they include, many amino acids, hydroxamic
acid, cholrine, inorganic phosphate, copper and molybdate ions
(O'Brian 1967).

The different isomerization reactions of organophosphates
have been investigated and shown to be very important. The most im-
portant conversion reaction is the thione to OXO conversion. This
reaction is important to parathion as it is catalyzed in mammals by
enzymes which transfer the nontoxic parathion into the potent AChE
inhibitor paraoxon as shown below.
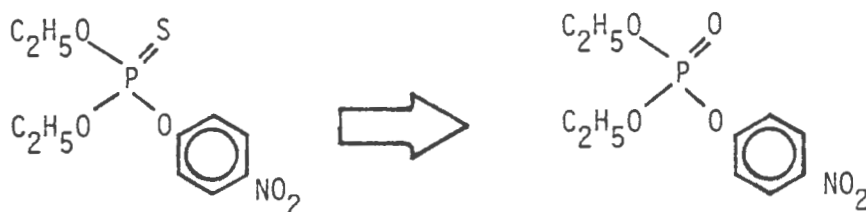
Controlled cholinergic nervous transmission depends on the
presence of the enzyme AChE which catalyzes the hydrolysis of
acetylcholine (ACh) to choline and acetic acid at all cholinergic
synapses, neuramuscular juctions and centrally. AChE catalyzes this
reaction by reacting with acetylcholine via two active sites on the

globular protein enzyme. The sites are designated anionic and esteratic. The anionic site is characterized by a negative charge attributed to a free carboxyl group of a dicarboxylic amino acid. This site attracts the positive charged quaternary nitrogen atom of the ACh molecule by electrostatic and other weak molecular forces. The esteratic site is composed of two components, 2.5 and 5.0 angstoms from the anionic site. The closer site is a basic nucleophilic group which is the imidazole group of the amino acid histidine and at the distant site is a hydroxyl group from the amino acid serine. Because of the proximity and charge of the imidazole group it exerts hydrogen bonding forces to draw the H atom of the serine molecule toward the histidine. This creates a situation where the O of serine is strongly nucleophilic and will readily react with the electrophilic carboxyl atom of ACh eventually creating a covalent bond. The nucleophilic character of the esteratic site is also responsible for the reaction between the O of serine of the AChE and the phosphorus of paraoxon which is electrophilic. The phosphorylated enzyme is very stable and hydrolytic cleavage of the organophosphate enzyme complex is very slow. Parathion will undergo limited hydrolysis of the complex while the two ethyl groups are still a component of the organophosphate. However, aging of the compound occurs within hours when one of the alkyl groups is cleaved off the complex. The resulting aged organo-phosphate enzyme complex is stable and the only source of viable enzyme after aging is new synthesis. When sufficient AChE is

inhibited by this mechanism the animal will exhibit signs of cholinergic toxicity as endogenously released ACh is not inactivated.

Metabolism

The metabolism of parathion is the essence of its toxicological importance, as it is the conversion of parathion to its oxygen analog paraoxon that produces the toxic AChE inhibitor. The details of the pathways by which parathion is metabolized have been researched extensively in the past decade. Figure 1 has been produced by combining all the routes of metabolism referred to in this section of the review. Neal and DuBois (1965) reported that parathion was more toxic to female rats than male rats but that the highly toxic desulfurated metabolite of parathion, paraoxon did not exhibit this sex difference. The conversion of parathion to paraoxon by desulfuration was then tested in vitro in livers of both male and female rats, and it was discovered that male rats converted parathion to paraoxon faster than females did. These data set the stage for the future work on parathion metabolism by establishing that not only must parathion be metabolized to paraoxon to exhibit toxic effects in mammals, but there must also be some kind of detoxification mechanism for both parathion and paraoxon.

Experiments by Hollingworth (1969), Kamataki et al. (1967 a, b), Lichtenstein et al. (1973), Neal (1964, 1967a, 1967b, 1971), Neskovic et al. (1973), Ptashne et al. (1971), Villeneuve et al. (1970), Whitehouse and Ecobochon (1975), and Wolcott et al. (1970)

established that liver microsomal enzymes were involved with the metabolism of parathion to paraoxon or diethyl phosphorothionate plus p-nitrophenol.

The experiments of Neal (1967) showed that the metabolism of parathion by rat liver microsomes was typical of a mixed function oxidase (MFO) system as inhibitors of MFO microsomal systems reduced the metabolism of parathion. Neal (1967) also showed that p-chloromercuribenzoate, Cu^{2+} and 8-hydroxyquinoline inhibited the conversion of parathion to paraoxon more than the conversion of parathion to nontoxic diethyl phosphorothionate and p-nitrophenol. This type of metabolism was also stimulated by reduced sulfur, EDTA, and Ca^{2+}. These results led Neal to the conclusion that the metabolism of parathion to both paraoxon and diethyl phosphoro-thionate was being catalyzed by an enzyme or enzymes in the liver microsomes by a MFO system.

Experiments by Neal (1971) established that reduced nicotin-amide adenine dinucleotide phosphate (NADPH) and O_2 were needed for the metabolism of parathion to the oxygen analog paraoxon (pathway 1, figure 1) or to diethyl phosphorothionate and p-nitrophenol (pathway 3, figure 1), and that this metabolism was inhibited by carbon monoxide. These results further strengthened the contention that parathion was metabolized to these substances by a MFO system associated with cytochrome P-450. The enzymatic mechanism for the metabolism of parathion was also found in lung, kidney, and brain tissues (Neal 1971), but the greatest enzyme source was liver.
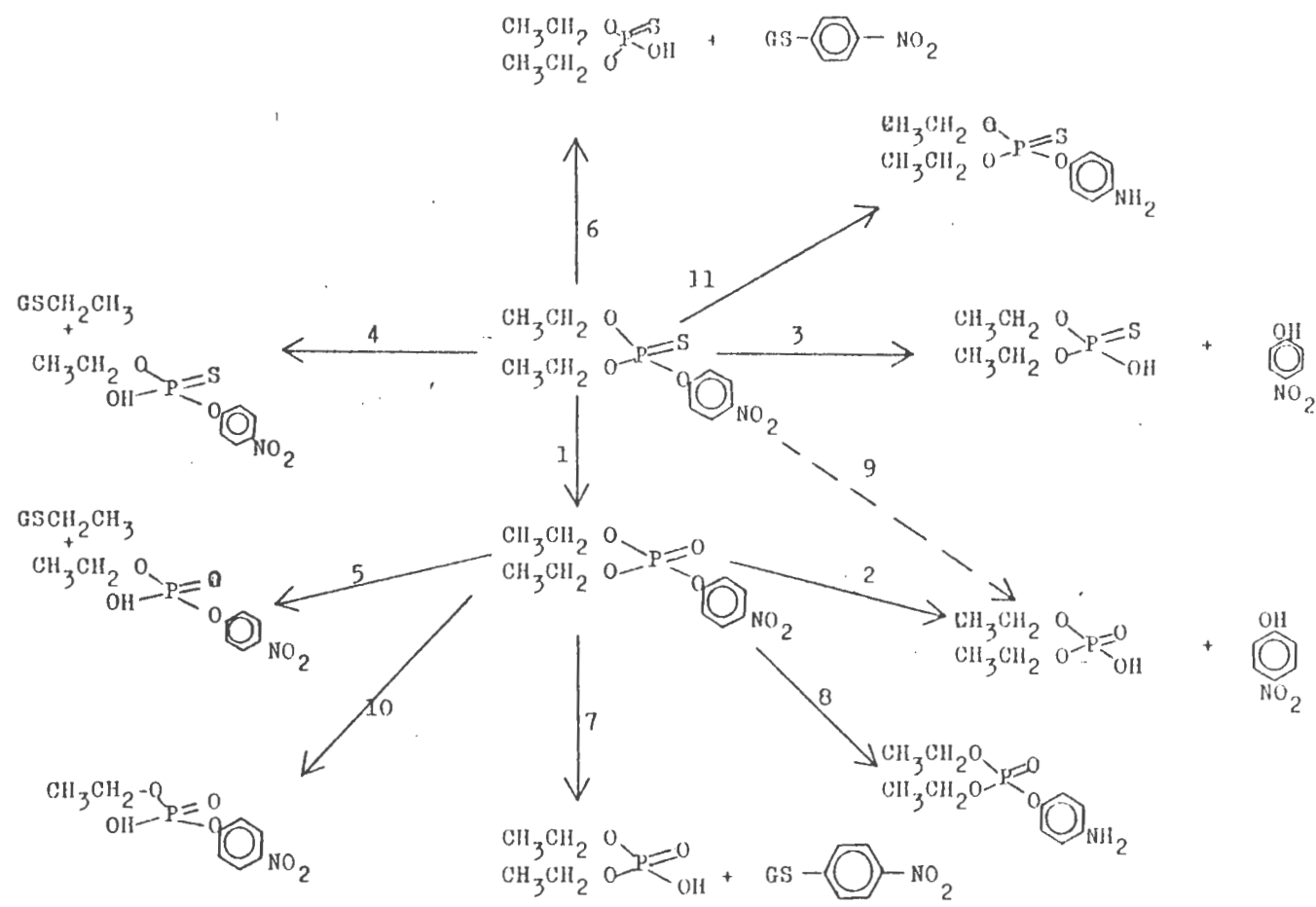
Figure 1.   Pathways of Parathion Metabolism.

Key to Figure 1 Reactions

1. Via MFO microsomal P-450 linked system in liver (Neal 1967; Ptashne et al. 1971) and lung, kidney and brain (Neal 1971).

2. Via paraoxonase in liver and blood and other tissue (Benke and Murphy 1974; Lichtenstein et al. 1973; Neal 1971). Via paraoxonase in sera (Lenz 1973).

3. Via microsomal enzyme MFO system in liver (Neal 1967; Ptashne et al. 1971).

4. Glutathione dependent S-alkyltransferase (methyl parathion) (Benke and Murphy 1975; Hollingworth 1973).

5. (Same as above)

6. Glutathione dependent aryl transferase (Dauterman 1971).

7. Glutathione dependent aryl transferase in rat liver soluble fraction (Benke and Murphy 1975; Hollingworth 1974).

8. Soluble liver fraction reduces paraoxon to amino paraoxon (Lichtenstein et al. 1973).

9. Direct reaction via intermediate (Kamataki et al. 1976b).

10. O-dealkylation via microsomal enzymes, NADPH and O_2 (Ku and Dahm 1973; Whitehouse and Ecobichon 1973).

11. Soluble liver fraction reduces parathion to aminoparathion (Lichtenstein et al. 1973).

The metabolism of parathion by soluble liver fractions (pathway 1, figure 1) was investigated by Dauterman (1971, Ku and Kahm 1973), Lichtenstein et al. (1973), and Neskovic et al. (1973).

The procedure used by Neal and DuBois (1964) to demonstrate the relationship between the MFO and parathion metabolism was to measure the production of p-nitrophenol by incubation mixtures of differentially contrifuged liver homogenates with parathion and appropriate cofactors. The production of colored p-nitrophenol was measured spectrophotometrically. Neal continued his investigation of parathion metabolism (Neal 1967) evaluating his contention, at that time, that the metabolism of parathion to either paraoxon or diethyl phosphorothionate and p-nitrophenol employed two different MFO enzymes. The p-nitrophenol determination, assay has been used by many investigators to follow the metabolism of parathion and to show that liver microsomal enzymes of the MFO system are involved with parathion metabolism via these two pathways in the vertebrate.

Benke and Murphy (1975) used the p-nitrophenol assay in their investigation on the influence of age and sex on the toxicity of parathion and methyl parathion in the rat. Their work also showed that the toxicity of parathion varied depending on the age and sex of the rat. Whitehouse and Ecobichon (1975) reported data analogous to that of many investigators, that there is a great disparity among species concerning the rate of metabolism of parathion.

They found that the desulfurating ability of the hamster, guinea pig, mouse, rat, rabbit, cow, pig, and cat declined in that order. Differential reaction rates shown by Benke and Murphy (1975) for the metabolism of parathion were shown to be the cause of the sex and age toxicity differences. The work also supported the previous finding that liver microsomal MFO was important in the metabolism of parathion. Similarly use of the p-nitrophenol method for parathion metabolism detection in vertebrate microsomal MFO P-450 systems was previously done by Ptashne et al. (1971) who showed the same association for MFO catalyzed desulfuration of parathion (pathway 1, figure 1) or oxidation to diethyl phosphorothionate plus p-nitrophenol (pathway 3, figure 1) and also a dearylation reaction.

Hollingworth (1969) demonstrated that the rate of dealkylation of organophosphates (pathway 4, figure 1) was dependent on the R groups of the molecule. He showed that the dealkylation by mouse hepatic enzymes of diethyl organophosphates preceded much faster than the dealkylation of diethyl compounds. He also showed that these O-dealkylation reactions in vivo proceeded to a large extent through alkylation of reduced glutathione.

Cramer and Hollingworth (1976) investigated the dearylation reaction of a series of paraoxon analogs by mouse liver homogenates (pathway 2, figure 1) and determined that the reaction was NADPH independent, and preceeded via an A-esterase. They showed that the

determining factor as to whether the reaction would proceed by a
MFO enzyme or by the A-esterase pathway was the specific length
of the alkyl side chain. The ethyl side chain of the paraoxon
molecule favored the A-esterase reaction.

Villeneuve et al. (1970) used the p-nitrophenol assay to
investigate the MFO role in parathion metabolism by liver microsomes.
They evaluated plasma esterase activity, and liver carboxylesterase
(A-esterase) activity to releate the effect of hepatic microsomal
enzyme induction and inhibition on altering the toxicity and meta-
bolism of parathion. They used SKF-525A, 3, 4-benzpyrene, DDT and
phenobarbital for the pretreatment of the rats. They determined that
DDT decreased the acute toxicity of parathion but increased carboxy-
lesterase activity and parathion metabolism while plasma esterase
levels were not affected. SKF-525A pretreatment caused an increase
in carboxylesterase activity but no change in the LD_{50} of parathion,
plasma esterase activity or parathion metabolism. Benze[a]pyrene
decreased the toxicity of parathion and increased plasma esterase
carboxylesterase and metabolism of parathion.

Alary and Broudeur (1970) also investigated the association
between microsomal metabolism of parathion and toxicity. They de-
termined that the ability of microsomal fractions to metabolize
parathion to diethyl phosphoric acid and diethyl phosphorothioinic
acid was a good index for the estimation of the in vivo toxicity of
parathion in adult rats. They concluded that phenobarbital stimulates

only the direct degradation of parathion to diethyl phosphoro-
thioic acid and p-nitrophenol.

Lichtenstein et al. (1973) showed that the liver microsomal
fraction had a high rate of activity for converting paraoxon to
p-nitrophenol and diethyl phosphate (pathway 2, figure 1).
Lichtenstein et al. (1973) also showed that the majority of the
paraoxon produced from parathion was from the soluble liver fraction,
a basic difference from the work of Neal's group. Lichtenstein,
et al. (1973) did not use the p-nitrophenol spectrophotometric
assay technique but employed gas liquid chromatography, thin layer
chromatography, colorimetric assays and radioactive parathion and
paraoxon to quantify their results. These methods will be discussed
in detail in the detection section of the literature review.

Whitehouse and Ecobichon (1975) investigated the metabolism of
parathion adopting techniques comparable to those of Lichtenstein
et al. (1973) analyzing extracted products from incubation mixtures
of liver and parathion. However, they also used the spectrophoto-
metric p-nitrophenol assay similar to the one previously discussed.

Kamataki et al. (1976b) investigated whether parathion
oxidation to paraoxon and diethyl phosphorothionate plus p-nitro-
phenol was catalyzed by two different MFO enzymes or utilized differ-
ent cytochrome P-450 systems or a combination of the both. Previous
work by Neal (1967) had contended that various inhibitors of hepatic
MFO enzymes had a differential effect on the products of parathion
metabolism Pretreatment of animals with inducers of hepatic MFO

indicated that the metabolism of parathion to paraoxon and diethyl phosphorothionate and p-nitrophenol were catalyzed by separate MFO enzymes (Neal 1967, Norman et al. 1974, Ptashne et al. 1971). Utilizing a homogenous preparation of rabbit liver cytochrome P-450 Kawataki et al. (1976b) presented evidence that parathion could be metabolized to not only both paraoxon (pathway 1, figure 1) and diethyl phosphorothionate plus p-nitrophenol (pathway 3, figure 1) but also diethyl phosphoric acid (pathway 9, figure 1) a product that had previously been reported to occur only following the hydrolysis of paraoxon. These data indicate that paraoxon, diethyl phosphorothionate and diethyl phosphoric acid are all capable of being formed utilizing the same species of cytochrome P-450. This would suggest that the three compounds are the result of a common intermediate for the single species P-450 metabolism of parathion is a compound analogous to a sulfine.

Glutathione dependent metabolism of parathion has been investigated by Hollingworth (1969), Benke and Murphy (1975), Lichtenstein et al. (1973), and Whitehouse and Ecobichon (1975). The method used by Hollingworth, and Benke and Murphy employed the soluble fraction of liver homogenate and radioactive (35_S) glutathione (GSH). Incubation of this mixture with parathion was followed by the identification of the metabolities by thin layer chromatography and quantification by scintillation counting. Benke and Murphy (1975) determined that parathion did not undergo glutathiond dependent dearylation (pathway 6, figure 1) in either male or female rats

as had been shown for mice (Hollingworth 1969). Glutathione
dependent dealkylation of paraoxon did not occur, but GSH depen-
dent dearylation of paraoxon (pathway 7, figure 1) did occur,
and that reaction varied with the age and sex of the rat.

Hollingworth (1973) also found that the GSH dependent deary-
lation of paraoxon in rat livers (pathway 7, figure 1) was more
rapid than deethylation. His previous work showed GSH dependent
O-dealkylation of methyl organophosphates but little or no
O-dealkylation of ethyl organophosphates, (pathways 4, 5, figure 1)
(Dauterman 1971, Hollingworth 1973). Ku and Dahm (1973) demon-
strated that O-dealkylation by liver microsomal enzymes was either
not effected or only slightly effected by reduced glutatione,
while Lichtenstein et al. (1973) reported that even though liver
microsomal MFO was the major route for arylphosphate cleavage of
parathion there was some glutatione dependent reaction. Whitehouse
and Ecobichon (1975) concluded that paraoxon detoxification by
liver homogenates involves hydrolytic dearylation by arylesterase
glutathione mediated dealkylation, oxodative dealkylation and
dearylation of nonspecific binding to tissue proteins. They believe
the role of GSH dependent detoxification is slight in organopho-
sphates that contain other then methyl side groups, but Benke and
Murphy (1975) concluded that GSH dependent detoxification of para-
thion in rats may be more important than previously thought.

Studies by Roth and Neal (1972) established that parathion bound to cytochrome P-450 creating both type I and II difference spectra. Their experiments also showed that there were at least three separate type I spectral binding sites for parathion and hexobarbital on cytochrome P-450 as evidenced by a hexobarbital and parathion. Stevens (1974) investigated the binding of parathion to cytochrome P-450 and found that both parathion and paraoxon would bind to reduced P-450 from rats and mice.

A question that had been unanswered for years was the fate of the sulfur released when parathion was converted to paraoxon. Norman et al. (1974) used radioactively labeled sulfur (35_S) and showed that the sulfur would covalently bind to the macromolecules of the microsomal membrane and would do so maximally in the presence of NADPH. This binding led to a decrease in cytochrome P-450 in the microsomes. Kamataki and Neal (1976) showed that the sulfur released from the metabolism of parathion to paraoxon became covalently bound predominately to cytochrome P-450. They showed that 50% of the bound sulfur attached to the side chain of the cysteine in the P-450 apoenzyme to form hydrodisulfide.

The detoxification of paraoxon to p-nitrophenol plus diethyl phosphate is a critical reaction in controlling the toxicity of parathion in the species discussed. Unlike the reaction of parathion to paraoxon or to p-nitrophenol and diethyl phosphorothionate which is catalyzed very rapidly by the MFO system, requires NADPH and O_2, and is inhibited by CO, this reaction proceeds rapidly in

particulate liver fractions or serum without NADPH and is carried
out via esteratic enzymes (pathway 2, figure 1) (Neal 1971). The
activity of the esterase enzyme that catalyzes paraoxon to p-
nitrophenol and diethyl phosphate was further investigated by
Neal (1967) showing that this esteratic enzyme required Ca^{++}
for optimal activity. Benke and Murphy (1975) showed the toxico-
logical importance of the esterase (paraoxonase) reaction con-
verting paraoxon to diethyl phosphate plus p-nitrophenol by demon-
strating that the LD_{50} of parathion in rats correlated with the
different rates of the reaction associated with age and sex in the
rat rather than to the activation reaction or detoxification of
parathion directly.

The association between compounds that effect liver micro-
somal enzyme induction such as phenobarbital, 3-methylcholanthrene,
DDT, dieldrin or endrin and organophosphate metabolism has been
investigated by Alary and Brodeur (1969), Harbison (1975), Ku and
Dahm (1973), and Villeneuve et al. (1970).

Alary and Brodeur (1969) showed that phenobarbital pre-
treatment increased liver paraoxonase activity 1.5 fold but had little
effect on serum paraoxonase activity, both of which convert paraoxon
to diethyl phosphate plus p-nitrophenol. They also showed that pheno-
barbital pretreatment led to a greater increase in the reaction rate
for parathion conversion to diethyl phosphorothionate plus p-nitro-
phenol (pathway 3, figure 1) than to paraoxon (pathway 1, figure 1),
although the reaction to paraoxon was increased. Villeneuve et al.

(1970) demonstrated that DDT, phenobarbital and benzo [a] pyrene
decreased the toxicity of parathion in rats three fold. Harbison
(1975) investigated the phenomena of phenobarbital induced pro-
tection against parathion toxicity in mice, and showed that
phenobarbital protected mice fetuses against parathion.

Ku and Dahm (1973) showed that phenobarbital, DDT, dieldrin
and endrin, all compounds that induce liver microsomal enzymes, led
to an increase in the NADPH-independent paraoxonase (pathway 2, figure
1). Ku and Dahm (1973) also noted that the O-dealkylation detoxifi-
cation of paraoxon (pathway 10, figure 1) is dependent on NADPH and
O_2 and inhibited by CO.

Work designed to specifically investigate liver microsomal
paraoxonase activity was done by Neal and DuBois (1965) and more
recently by Whitehouse and Ecobichon (1975) who showed that only rats
exhibited a sex difference in the hydrolysis of paraoxon, males pos-
sessing higher activity then females, and that various species did
exhibit different rates of paraoxon hydrolytic activity. The highest
activity was found in mice followed by cow, rat, guinea pig, rabbit,
hamster, cat, dog, and pig in decreasing order. They showed that
the hydrolysis of paraoxon could be by paraoxonases from various
body tissues including liver or by a MFO system requiring NADPH and
O_2, but the hepatic paraoxonase was the major route for paraoxon
detoxification.

The nature of the serum enzyme capable of catalyzing the
hydrolysis of paraoxon to diethyl phosphate and p-nitrophenol in

mammals was reviewed and investigated by Lenz (1973). Lenz found that the reaction was not susceptible to substrate or product inhibition and that the enzyme contained one active group which was hydrophobic and required an electron withdrawing group in the substracte for binding.

In summary the most important discovery brought about by this type of research was that the phosphorothionate organophosphates must be metabolized by desulfuration to an active toxic compound (paraxon in the instance of parathion) to exhibit anti-acetylcholinesterase activity. Mammals evaluated in the preceeding papers predominatly accomplish this metabolism by a liver microsomal MFO cytochrome P-450 dependent system but this desulfuration can also be produced by soluble fraction enzymes. The detoxification of paraoxon has been shown to be associated with liver microsomal paraoxonase, and the rates of production of the different metabolites of parathion are species, and for rats, sex dependent. There is also direct detoxification of parathion by a microsomal MFO system to diethyl phosphorothionate plus p-nitrophenol and the rate of this reaction does not respond to enzymatic inhibition or induction the same as the microsomal MFO reaction that converts parathion to paraoxon.

Toxicity

As stated above, the toxicity of parathion in mammals has been shown to be due to its metabolite paraoxon. Parathion is very toxic

to adult rats, Faust and Gomaa (1972) reported LD_{50} values in the rat of 3-30 mg/kg orally and 4-200 mg/kg dermally. The results for the oral LD_{50} agree with those of Villeneuve et al. (1970) who reported oral LD_{50} values for parathion of 6.3 to 12.0 mg/kg. Villeneuve et al. (1970) also showed that DDT, benzo[a]pyrene and phenobarbital pretreatment increased that LD_{50} value to greater than 20 mg/kg. DuBois et al. (1968) showed that male rats were more resistant to organophosphate toxicity through different rates of liver enzyme activity when compared to female rats. Benke and Murphy (1975) showed that male rats were more resistant to parathion than females and that an LD_{50} in adults (greater than 56 days old) was 2-6 mg/kg i.p. Benke and Murphy (1975) noted that the toxicity steadily declined as the rats aged, reporting LD_{50} values of 2-4 mg/kg i.p. for rats 23-24 days old and only 1 mg/kg for rats 12 days old. Single lethal dose ranges for parathion via i.p. administration in mice were reported by Benke and Murphy (1974) to be 13-15 mg/kg. The work of Alary and Brodeur (1970) agrees with the preceding data concerning acute LD_{50} values in rats and the effects of phenobarbital pretreatment. Parathion toxicity in dogs has been reported (Faust and Gamaa 1972) as 10 mg/kg i.v.

Research concerning the toxicity of parathion in nonmammals and invertebrate species in particular is scarce compared to the data available concerning mammals (Carlson 1973). Research has been done in the area and work by Benke and Murphy (1974, 1975) showed that sunfish were much more resistant to parathion on a mg/kg basis than the mammals studied. The sunfish LD_{50} values of 10-200 mg/kg was

determined, but they also concluded that the rate of onset of
AChE inhibition did not explain all of their toxicological findings.
Benke et al. in 1974 continued their investigation of parathion
toxicity in sunfish (Lepomis gibbosus) and calculated an LD_{50}
of 110 mg/kg. They showed that fish cholinesterase was inhibited
by paraoxon, but that the fish had a lower sensitivity to paraoxon
than mice did. Glutathione dependent metabolism of methyl para-
thion and methyl paraoxon could be detected in liver homogenates
but not for paraoxon or parathion. Other investigators that have
delved into the toxicity of parathion in fish include, Faust (1964),
Chambers and Yarbough (1973, 1974). Chambers and Yarbough
investigated the toxicity of parathion in the mosquito fish
(Gambusia affinis). They found that methyl parathion was more
toxic in the fish than parathion. They also found that resistant
fish had a higher level of microsomal MFO. Their data indicate that
the toxicity of parathion varied depending on whether the fish were
in the susceptable or resistant group and the time of year, but in
all cases the compound was toxic in water at concentrations of
1.0 ppm or less. The toxicity of parathion in fathead minnows was
investigated by Faust (1964) who recorded that parathion was acutely
toxic to the fatheads at 1.4 ppm in 96 hours. Miller et al. (1966)
showed that the estuarine fish (Fundulus heteroclitus) would accumu-
late parathion from a model ecosystem experimental apparatus. Ex-
periments have thus shown that parathion is toxic at low concentra-
tions in fish, and that the toxin can accumulate in fish. The

involvement of the cholinesterase inhibiting action of parathion in its toxic manifestations has been shown by Potter and O'Brian (1964), Benke and Murphy (1974), and Benke et al. (1974) who reported cholinesterase inhibition in exposed animals.

The toxicity of parathion in nonvertebrates other than insects has received the least attention. Research in this area has been done by Miller et al. (1966) who showed that using a model ecosystem developed to simulate a cranberry bog they could demonstrate the accumulation of parathion in the freshwater mussel (Elliptio complanatus). They concluded that since it has been shown that oysters and mussels accumulate pesticides, there may be an environmental hazard. They also concluded that the mussel had a much slower rate of parathion metabolism as measured by the TLC identification of radioactive metabolites of parathion than fish or mammals.

Yu and Sanborn (1975) also used a model ecosystem to study parathion toxicity and metabolism. Employing radioactive parathion and utilizing extraction techniques, they concluded that parathion did not accumulate in the snail used in their study.

The toxicity of parathion to crustaceans has been investigated to a limited extent. The anti-AChE mechanism of toxicity being credited for the toxic effects of parathion has been supported by Coppage and Matthews (1974) who showed a reduction of AChE activity following organophosphate exposure to pink shrimp (Penaeus

duorarum). Carlson (1973) investigated the toxicity and metabolism
of parathion in lobsters and obtained very interesting data. Point-
ing out that phosphorothionates must be metabolized to their oxygen
analog to be toxic, and with the knowledge that Brodie and Maickel
had shown that the lobster hepatopancreas metabolized many drugs,
he attempted to demonstrate the correlation between the lobster
hepatopancreas' ability to metabolize or detoxify parathion and
the susceptability of the lobster to parathion. Elmamlouk et al.
(1974) have since shown that the lobster hepatopancreas does
contain cytochrome P-450. Carlson determined that after inject-
ing parathion into the cheliped sinus of the lobsters an acute
dose LD_{50} of 0.3 mg/kg could be calculated, a value considerably
lower than that for any of the mammals as yet studied. Carlson
investigated the ability of the lobster hepatopancreas to de-
sulfurate the parathion to paraoxon. Measuring the ability of
incubated samples of hepatopancreas homogenate or microsomes
with parathion to inhibit AChE activity he was not able to de-
monstrate that the lobster hepatopancreas formed paraoxon.
Carlson also used the spectrophotometric p-nitrophenol assay to
investigate the effect lobster heptopancreas had on parathion
metabolism to paraoxon and subsequently to diethylphosphate plus
p-nitrophenol or directly to p-nitrophenol plus diethyl phosphoro-
thionate. The lobster did demonstrate a temperature dependent pro-
duction of p-nitrophenol from parathion. Carlson concluded that
since the lobsters were susceptible to parathion but no paraoxon

could be detected in the in vitro experiments then, the in vitro experiments did not reflect the in situ reaction rates or that other organs in the lobster were responsible for parathion conversion to paraoxon.

The toxicity of organophosphates to crayfish has been investigated by Muncy and Oliver (1963) and Albaugh (1972). Albaugh (1972) showed that there was a difference in the susceptibility of two different crayfish populations to methyl parathion. The crayfish from an enviromental area that was exposed to insecticides capable of causing microsomal enzyme induction produced a toxicity value of 3.4 parts per billion while those from an area not exposed to microsomal inducing pesticides produced a value of 2.4 parts per billion. Muncy and Oliver (1963) used a median tolerance limit technique (TL_m) to establish the toxicity in red crawfish (Procambarus clarki) by ten insecticides. They determined a 24 hour TL_m value of 0.05 ppm and 48 and 72 hour TL_m value, of 0.04 ppm for methyl parathion.

The perplexing problem of potent parathion toxicity to crustaceans but their apparent lack of ability to produce paraoxon was further investigated by Elmamlouk and Gessner (1976). They tested the ability of lobster hepatopancreas derived microsomes to convert parathion to paraoxon and compared that with the ability of mouse liver microsomes to carry out the same reaction. They concluded that no metabolism of parathion to paraoxon or to p-nitrophenol occured in hepatopancreas preparation. They do mention that

it is possible for the conversion to have taken place below the
level of their detection capabilities.

Detection

The determination of metabolites of parathion in snails and
crayfish would be impossible without using effective extraction
techniques for separating parathion and its metabolites from both
tissue and water samples. The extraction, concentration, and
clean up techniques that can be used for organophosphates in water
environments have been throughly investigated by Appleton and
Nakatsugawa (1972), Burchfield and Storrs (1975), Burkhard and Voss
(1972), Coburn and Chau (1974), Faust and Gomaa (1972) Faust and
Suffer (1969, 1972), Gomaa and Faust (1972), Faust (1964),
Nakatsugawa (1972), Ripley et al. (1974), Suffet and Faust (1972a)
The direct measurment of parathion in water samples is impossible
due to sensitivity and specificity limitations (Faust and Suffet
1969, 1972 and Suffet and Faust 1972a).

Extraction Techniques

Carbon/Absorption

The carbon absorption method is applicable to large
samples of water from natural sources in the field or for the preli-
minary cleanup of water samples in the labroatory. This method of
organophosphate contaminated water sample through activated charcoal
and then the extraction of the organophosphate that has bound to the
activated charcoal with solvents (Faust and Suffet 1969, Nicholson et al.

1962). Nicholson et al. (1962) reported that this technique was inadequate for the extraction of parathion from a farm pond.

Liquid-Liquid Extraction

Liquid-liquid extraction has replaced carbon absorbtion (Faust and Suffet 1969) for most organophosphate extraction requirements. The choice of solvent to be used in liquid-liquid extraction is critical, and there are a large number to choose from.

There are many important general criteria when choosing the proper liquid-liquid extraction solvents. They include, the pH of the water to be extracted and whether that will effect the organophosphate and/or the extraction procedure; if the compound being extracted is volatile, if it undergoes spontaneous reactions under the conditions which it is being extracted; and the ultimate goal of the extraction procedure. There are also important specific criteria that must be evaluated when determining liquid-liquid extraction solvents and procedures, such as: the solubility of the solvent in water; whether the solvent is polar or nonpolar, aromatic or aliphatic; its ability to be used with the eventual detection system; the availability of pure solvents that are pesticide quality and the ease of solvent handling, toxicity and flamability (Faust and Suffet 1972).

The pH of the water in which the organophosphate is dissolved is important. Parathion will hydrolyze in alkali environments to p-nitrophenol and diethyl phosphorothionate (Faust and Gomaa 1972).

Therefore, it is important that the pH of the water be known or adjusted, and any extraction procedure that would greatly increase

the pH of the sample be avoided. Faust and Suffet (1969) determined that neutral pH values are the best for maintaining the integrity of parathion samples as the sample will also hydrolyze under extremely acid conditions.

Volatilization of parathion samples may occur under some condition (Faust and Suffet 1969). It has been suggested (E.P.A. Training Manual 1974) that when drying parathion samples on thin layer chromatography (tlc) plates, the use of hot air be avoided.

One of the problems of extracting parathion from water samples is that parathion itself is not indefinately stable in the water environment and undergoes hydrolysis and desulfuration (Faust and Suffet 1969, 1972a). To determine the extent to which parathion is being metabolized to paraoxon or other compounds, it is mandatory to recover all of the compounds. This necessitates that the pH and solvent be evaluated critically.

Experiments by Faust and Gomaa (1972) show that both parathion and paraoxon are more resistant to hydrolysis under acid condition than alkali conditions. They reported rate constants for hydrolysis and half lives for the two compounds under different pH conditions (table 1).

TABLE 1

RATE CONSTANTS FOR HYDROLYSIS AND HALF LIVES FOR
PARATHION AND PARAOXON AT 20° C[a]

pH	K_{ob} (hr^{-1})	T 1/2 (Hours)
Parathion		
3.1	1.65 x 10^{-4}	4182
5.0	1.88 x 10^{-4}	3670
7.4	2.66 x 10^{-4}	2594
9.0	1.32 x 10^{-3}	523
10.4	2.08 x 10^{-2}	33.2
Paraoxon		
3.1	1.46 x 10^{-4}	4726
5.0	1.66 x 10^{-4}	4156
7.4	2.00 x 10^{-4}	3450
9.0	9.87 x 10^{-3}	69.9
10.4	1.15 x 10^{-1}	6.0

[a]Faust and Gomaa (1972)

Different methods for the determination of the extent to which the organophosphates will partition between the water and solvent phase of the extraction fluid have been tried. A thermodynamic partition coefficient determination system that measures the fraction of solute that partitions into the nonpolar solvent is the best system. That system is called the p value determination approach.

Coburn and Chau (1974) showed that benzene could be used as a solvent for extracting parathion and metabolites. Benzene extraction was also done by Ripley et al. (1974) who reported 95 to 100% recovery of pesticide from water samples using that method. Kliger and Yaron (1975) showed that by extracting water samples with a mixture of water and hexane 5:2 the parathion could be completely partitioned into the solvent phase while p-nitrophenol and diethylphosphoric acid would remain in the aqueous phase. White et al. (1973) showed that in extracting parathion from bean plants 5% ethyl ether in benzene was an acceptable solvent, but to determine the solvent that would yield the best return the investigator should use the p value approach.

The p value determination approach demonstrated that parathion and paraoxon in an acid (pH 3.1) environment are effectively extracted by hexane, benzene, ethyl acetate, and ether, and that ether is the superior solvent due to it's having the highest p value (Faust and Suffet 1972a). When p-nitrophenol was to be determined, ether with a p value of 0.98 was again the best solvent, especially

when compared to hexane which only had a p value of 0.20 or
benzene with a value of 0.60 (Suffet and Faust 1972b).

Extraction from Tissue

The extraction of parathion and metabolites from samples
containing animal cells or cell fragments may require different
solvents or procedures than those used in the extraction of these
compounds from water samples.

Lichtenstein et al. (1973) found it necessary to extract
parathion and metabolites from homogenate or microsomal samples.
They transferred the 2 ml contents of their incubation flasks to
a separatory funnel with two 5 ml portions of water and 10 ml
of acetone without acidification. They then extracted the sample
with two 10 ml portions of hexane and two ml portions of diethyl
ether added to the hexane. They determined that 98% of the p-
nitrophenol was in the solvent phase with amino parathion. P-
aminophenol went totally into the water phase with a large portion
of the amino paraoxon. The extracted components were analyzed by
liquid scintillation counting, TLC,   and autoradiography.

Burkhard and Voss (1972) extracted the organophosphate iodo-
fenphos and iodofenoxon from milk samples. They used methylene chlo-
ride to extract the samples followed by concentration on a water bath
using a Kuderna-Danish evaporator. They also extracted muscle and
liver samples by first chopping and then grinding the tissue and
using 25 g aliquots for extraction. The sample was ground with

anhydrous sodium sulfate (70-100 grams). That mixture was then

mixed at high speed for three minutes with benzene (200 ml)

followed by filtering under vacuum and concentration by rotary

evaporation. Fat samples were cut and macerated as were the muscle

and liver samples and then ground with anhydrous sodium sulfate

and Celite. That mixture was then extracted for three hours with

300 ml hexane. The solution that was produced was partitioned

with acetonitrile in a separatory funnel, and the acetonitrile

phases were re-extracted with hexane. The product was evaporated

to dryness and then dissolved with benzene.

Black et al. (1973) extracted parathion from rat hepatic

tissue samples. The parathion was extracted from homogenates

equivalent to 2g of liver with 4 ml of hexane for 5 minutes in a

Thomas tissue grinder. The mixture was centrifuged at 6000 xg

at $0^{o}C$ for 10 minutes. The hexane supernatant, which was subse-

quently analyzed by gas chromatography, exhibited an extraction

efficiency of 90.0 ± 0.9%.

Hasselberg and Johnson (1972) extracted pesticides from

fish tissue samples using a technique similar to the one used by

Burkhard and Voss (1977) for extracting organophosphates from milk.

Their procedure included the grinding of their samples with anhydrous

sodium sulfate.

Burchfield and Storrs (1975) described methods for the ex-

traction of organophosphates and metabolites from nonfatty foods

using acetonitrile. The chopped samples were mixed with acetonitrile

and Celite and blended at high speed. Eventually the acetonitrile solution containing the pesticide was extracted in a separatory funnel with petroleum ether. Burchfield and Storrs (1975) also described a method for the cleanup and isolation of organophosphates from animal tissues. A few grams of tissue were chopped and blended with 15 ml of cold acetone. Benzene (30 ml) was added with 25 grams deactivated siloxid. This slury was put in a chromatography column and phosphates eluted with a 2:1 mixture of benzene and acetone followed by drying with sodium sulfate and rotary evaporation.

Detection

The qualitative and quantitative detection of parathion and its metabolites required detection techniques that were sensitive to very low concentrations of the desired compounds. The techniques reviewed here include thin layer chromatography and monitoring of radioactive compounds.

Thin Layer Chromatography

Thin layer chromatography (TLC) is a sensitive and convenient method for the detection and separation of organophosphates and their metabolites (Joiner and Baetcke 1973, Stahl 1965).

Several stationary phases have been evaluated in TLC systems. Silica gels H, G, and G-HR are used to a great extent as are neutral aluminum oxide G and adsorbosil g-1 (Burchfield and Storrs 1975), Silca gel is the most popular stationary phase and it was used by

Gunther et al. (1970), Norman et al. (1974), Joiner and Baetcke (1973), Lichtenstein et al. (1973). However, there are some unusual phases such as cellulose MN300 that have been used (Hollingworth 1969).

The possible choices for developing solvent system, absorbent, eluent, and method of visualization of the migration spot are numerous. The Environmental Protection Agency (EPA) Training Manual (1974) recommends the use of 250 micron thick Silica Gel plates and visualization aided by Rhodamine B (0.1 mg/ml ethanol) spray, and elution recovery with a mixture of ethyl and petroleum ethers. The work of many investigators agrees with these recommendations. Rhodamine B spray was the visualizing spray that was used by Joiner and Baetcke (1973) to identify organophosphates. However, other investigators use different techniques, e.g. Neal (1967) eluted parathion off silica TLC plates with 2 ml volumes of methanol followed by one 10 ml portion of chloroform methanol 1:1.

The choice of the developing solvent system to use is complicated by the large number of systems that have been employed. Joiner and Baetcke (1973) evaluated 12 different systems containing differing amount of three of the following solvents: ether, petroleum ether, ethyl ether, galcial acetic acid, absolute methanol, chloroform, ammonium hydroxide, and heptane. They concluded that the best separation of photoalteration products (including parathion, paraoxon, p-nitrophenol and diethyl phosphate, was by the following four systems: petroleum ether, ethyl ether, glacial acetic acid (80:15:5) and

and (50:45:5) or methanol, chloroform, ammonium hydroxide (24:75:3.5) and methanol, chloroform, petroleum ether (10:20:70).

There have been many other mobile phases used for organo-phosphate analysis. Burchfield and Storrs (1975) used the following mobile phases: benzene, benzene, cyclohoexane (3:1), hexane, acetone (20:80), acetone, hexane (15:85). Hollingworth (1969) used a solution of 2-propanol; water; concentrated ammonium hydroxide (75:24:1).

The most popular mobile phase developing mixture is hexane, chloroform, methanol, (7:2:1). This solution has been used by Gunther et al. (1970) who reported Rf values for paraoxon of 0.55 and for parathion of 0.75. Lichtenstein et al. (1973) used this solvent system and reported aminoparaoxon at an Rf value of 0.07 and p-aminophenol at 0.00. This system was also used by Elmamlouk and Gessner (1976).

Autoradiography was one technique used by Hollingworth (1969), White et al. (1973) and Lichtenstein et al. (1973) to quantify the amounts of compound that were present on the plates. Gas liquid chromatography was also a very popular technique used to quantify the amounts of compounds present, and Gunther (1970) and Lichtenstein et al. (1973) used that procedure. Gunther used the thermionic detector while Lichtenstein et al. used a flame photometric +5250 or phosphorus filter, or an electron affinity detector.

The procedure of scraping the coating from the TLC plate that contained the compound in question or cutting out that spot from paper

strips used in TLC was a technique used with great success by Benke and Murphy (1975), Elmamlouk and Gessner (1976) Lichtenstein et al. (1973), and MacNeil and Frei (1975). A technique for recovering the coating by using an eye dropper plugged with glass wool connected to a vacuum was presented in the EPA manual (1974) and was reported to be extremely effective. The possible interference that could be caused by the coating in the counting process was evaluated by MacNeil and Frei (1975) who showed that the quenching effect of silica gel in liquid scintillation counting was shown to be an excellent technique to quantify compounds that were radioactive.

III. Experimental

A. Materials and Methods

Chemicals: Parathion (O,O-diethyl-p-nitrophenyl phosphoro-
thionate) and paraoxon (O,O-diethyl-p-nitrophenyl phosphate) were
obtained from New England Nuclear, 575 Albany Street, Boston,
Massachusetts 02118. New England Nuclear was also the source of the
radioactive parathion samples used. Two differently labeled radio-
active parathion samples were used, parathion (ethyl-1-^{14}C) and
parathion (2,6 ^{14}C).

The radioactive parathion samples were diluted with nonradio-
active parathion to obtain concentrations of parathion in the ppm or
ppb range that were desired for that experiment and to produce a
specific activity for the diluted radioactive sample that would produce
cpm in a range that would be quantitative without using samples of
unnecessarily high specific activity.

Diethyl phosphorothionate and diethyl phosphate were synthe-
sized and generously provided by Dr. R. M. Hollingworth, Purdue
University.

All other chemicals and solvents used were either analytical or
pesticide grade.

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Radioactive Parathion Samples[a]

	Parathion 2,6^{14}C Ring Label	Parathion [ethyl-1-^{14}C]
Batch	C-37	C-34
MW	291.27	291.27
Sp. Activity mCi/mM	2.2	19
ml/sample	0.5	0.51
total activity	50 uCi	50 µCi
DPM/µ Mole	4.88×10^6	4.21×10^7
DPM/mg	1.67×10^7	144×10^8
DPM/µl	2.22×10^5	2.17×10^5
µg/µl	13.212	1.50

[a]Supplied by New England Nuclear.

B. Animals

Crayfish used in this project were the species *Orconectes rusticus* and were obtained from Connecticut Valley Biological Supply Co. Inc. (Southampton, Massachusetts). The crayfish were stored in fresh water aquariums containing glass distilled aerated water.

Adult male rats were obtained from Charles River Breeding Laboratories (Wilmington, Massachusetts). They were housed in an air conditioned room and supplied food and water *ad libitum*.

The snails used in the project were *Viviparus malleatus* which were collected from Silver Lake in North Kingston, Rhode Island. Snails were housed in fresh water aquariums in the animal quarters at the University of Rhode Island.

C. Analytical Procedures

1. Toxicity Due to Parathion and Paraoxon Exposure in Water in *Viviparus* and *Orconectes*

The effect exposure to parathion or paraoxon in the water environment had on *Orconectes* or *Viviparus* was determined by placing the crayfish individually or snails in groups of three in 1 liter beakers containing 500 ml of glass distilled water. Tygon tubing connected to a "Silent Giant" air pump produced a constant gentle air stream to the beakers. Parathion or paraoxon dissolved in ethanol was then added to the beakers to produce different nominal concentration levels, where some of the beaker concentratons were theoretically greater than the solubility of parathion in the water. Control

experiments were done to determine the effects of ethanol, air being pumped, maximum possible number of animals per beaker and the possibility of parathion leaching into and then out of the glassware. The animals were observed for periods of 24, 48 and 96 hours and recordings made of toxic signs and/or death if they occurred.

2. Snail Toxicity to Parathion Due to Direct Injection

The possibility that the route of administration may alter toxicity was investigated by the direct injection or parathion into the snail Viviparus malleatus rather than through exposure in the water environment. A small hole was made in the shell of the snail by filing down the shell and then boring a hole in the shell with a dissecting needle. Samples of parathion dissolved in ethanol were injected into the snail, and observations made after 24, 48 and 96 hours.

3. p-Nitrophenol Spectrophotometric Assay

Livers from rats, hepatopancreas and gills from crayfish (Orconectes rusticus) and whole intestinal tissues of snails (Viviparus malleatus) were removed, blotted and weighed. Three grams of tissue were then homogenized in 9 ml of a cold solution of 0.15M NaCl, 0.015M $MgSO_4$, and 0.008M nicotinamide. The homogenate was centrifuged at 6,000 × g for 15 minutes in a Sorvall refrigerated centrifuge.

The 6,000 × g supernatant fraction was used in the following assay procedure to determine the production of p-nitrophenol. The incubation mixture consisted of 1.5 ml supernatant fraction, 0.18 ml of

phosphate buffer pH 8.0 (buffer made from 5.3 ml of 0.05M monobasic
soldium phosphate plus 94.7 ml of 0.05M dibasic sodium phosphate
diluted to 200 ml containing 0.1% Triton X-100), 0.12 ml H_2O, 0.12 ml
NADP (100 mg/10 ml), 0.17 ml glucose 6 phosphate (85 mg/10 ml), and
0.10 ml parathion (12.0 mg/ml in ethanol) or, when the metabolism of
paraoxon to diethyl phosphate and p-nitrophenol was being tested 100 µg
of paraoxon in 10 µl of ethanol. Thus the 2.19 ml incubation flask
contained 9.8 mM monobasic sodium phosphate 0.21 M dibasic sodium
phosphate, 2.5 mM glucose-6-phosphate 0.188 mM parathion and 0.737 mM
NADP or 0.735 mM NADPH. The reaction was started by addition of the
substrate. This mixture was incubated in 20 ml glass beakers aero-
bically at 37° C for either 30 or 60 minutes in a Dubnoff precision
scientific metabolic shaker.

In some experiments the incubation mixture contained
dithiothreitol (10^{-4}M) to protect sulfhydryl groups. On other occasions
NADP was replaced by NADPH in the incubation mixture for comparison
purposes. Appropriate tissue blanks were also included. Inactivation
of supernatant fractions for control value determinations (spontaneous
hydrolysis) was accomplished by boiling the sample for 15 minutes.

The reaction was terminated by addition of 5.0 ml of cold
acetone. The samples were then alkalinized by the addition of 0.5 ml
of 0.5 M glycine-NaCH buffer (pH 9.5). The mixture was left to stand
in test tubes at -10° C for 30 minutes. The samples were centrifuged
to remove the acetone precipitated protein. The optical density of the
supernatant was determined at 410 nm using a Beckman model DBG

spectrophotometer. The amount of p-nitrophenol produced was determined by comparing absorbance values with those obtained from standards. Corrections were made for nonenzymatic hydrolysis by incorporating appropriate control (heat deactivated homogenate) and blank samples. The results were expressed as µg of p-nitrophenol formed/hour/gram of tissue.

4. Procedure for Spotting,
   Scraping, and Eluting
   Samples off Thin Layer
   Chromatography Plates

Thin layer chromatography was used to separate, identify and quantify parathion and its metabolic products.

Silica Gel-G coated TLC plates 250 microns in thickness were used. The plates were spotted with microliter quantities of parathion, paraoxon, p-nitrophenol, diethyl phosphate, diethyl phosphorothionate or unknown above the level of the solvent bath present in the chroma-tography tank. The solvent system employed consisted of hexane, chloroform, and methanol 7:2:1, all pesticide grade. One hundred ml of solvent mixture was used with this amount forming a 1 cm deep pool of solvent in the tank. A large square of filter paper was placed in the tank to assure saturation.

The solvent front was allowed to develop to 10 cm before the plate was removed from the tank to dry. Identification of the separated spots was aided by the use of Rhodamine B 0.1 mg/ml in ethanol which was sprayed on the plate until a light pink color covered the plate. The plate was then viewed under ultraviolent light where the parathion

and metabolite spots were plainly visible against a light background.

When radioactive compounds were to be recovered, the sprayed plates were marked to isolate the desired area of the plate to be scraped by tracing around the area with a dissecting needle. That area of the coating was scraped from the plate with a small spatula. The silia gell coating was then retrieved with the use of a glass wool plugged eye dropped attached to a vacuum hose. Care was taken to be sure no significant amount of coating was lost in this process and controls were run to determine the effectiveness and reliability of this process. The glass wool containing the Silica Gel G and compounds was then deposited into a scintillation vial containing Hydromix Packard Liquid Scintillation Counter. Blanks and controls were included to determine the effect of the glass wool, Silica Gel G and Rhodamine B spray. Raw counts were used for all subsequent data calculations.

5. Elution Procedure

Parathion and metabolites were recovered from the TLC plates in liquid form for subsequent gas liquid chromatograph characterization or as a comparison to the above procedure by scraping the area of the plate and then using the eye dropper technique to transfer the coating along with glass wool to a vial containing 5 ml of anhydrous ethyl ether. This mixture was shaken and poured into a funnel with filter paper. The filtering funnel was rinsed with 5 ml more of ether, and the liquid containing the parathion or metabolites was evaporated with

suction down to less than 4 ml. The ether was then placed into a scintillation counting vial containing 10 ml of Hydromix and counted or analyzed with the gas chromatograph.

6. Extraction of Parathion and
   Metabolites from Water
   Samples

The extraction of parathion, paraoxon, p-nitrophenol, diethyl phosphate and diethyl phosphorothionate from 500 ml samples of water was done using anhydrous ethyl ether as the sole extracting solvent.

The water sample was placed in a large (1000 ml) separatory funnel and extracted with 40 ml of ether. The sample was shaken for 2 minutes. The ether layers from three consecutive extractions were pooled and passed through a glass column containing 3 inches of anhydrous sodium sulfate to remove any water. Ether layers that were in a semigel state had additional 20 ml ether portions added to re-establish the more liquid state before passage through the column. The dried sample was then placed in rotary evaporator flasks and the volume reduced under suction but without heat. The sample was then either spotted on a thin layer chromatography plate or injected into the gas chromatograph.

7. Extraction of Parathion and
   Metabolites Excreted into
   Water Samples by Crayfish

Four crayfish were individually placed into 1 liter beakers containing 500 ml of glass distilled water. The water in two of the beakers then had ^{14}C parathion labeled in the ethyl group added while

the other two beakers had ^{14}C parathion ring labeled added. The
amounts of parathion added to all of the beakers were such that a
concentration of 100 ppb was attained. One and one-half hours after
exposure, the crayfish were removed from the water and frozen for
subsequent analysis of their tissues for parathion and metabolites.
The water in which they were exposed was immediately extracted employ-
ing the water extraction procedure to determine if any metabolites
could be detected.

8. Extraction of Parathion and
   Metabolites from Crayfish
   and Snail Tissue

The recovery of parathion and metabolites from the tissues
of crayfish following exposure to parathion in water was accomplished
using the crayfish that had been exposed to 100 ppb parathion and then
frozen. The same procedure for extraction from tissue was performed on
the whole snail tissue from the snails that were exposed to radio-
activity labeled parathion at a concentration of 320 ppm for 48 hours.

The day following the exposure, the hepatopancreas and tail
muscles of the ^{14}C-ethyl parathion exposed crayfish tissue were pooled
as were the tissues from the crayfish exposed to the ring labeled
compound. The tissues were homogenized with a blade homogenizer in
9 ml of ether for 30 seconds. The homogenate was then scraped into
centrifuge tubes containing 9 ml of ether. The tissue attached to the
homogenizer blades was rinsed into the tube with additional ether. The
centrifuge tube containing the tissue and ether was shaken for 2 minutes

and then centrifuged at low speed to separate the tissues from ether layer.

Following the centrifugation the ether layer was removed with a Pasteur pipet and transferred to a rotary evaporator flask. The sample was then evaporated without heat to a volume small enough to be spotted onto TLC plates. The plates were developed and sprayed as described along with standards for identification of the products. The spots were removed and counted in the liquid scintillation counter to quantify the amount of product recovered.

9. Production of p-Nitrophenol,
    Paraoxon, Diethyl Phosphate
    or Diethyl Phosphorothionate
    from Parathion via in vitro
    Metabolism by Orconectes or
    Viviparus Tissues

The procedure for this assay was almost identical to that for the p-nitrophenol spectrophotometric assay.

Tissues from snail or crayfish were removed and homogenized in NaCl, $MgSO_4$, nicotinamide in the same way. The tissue was incubated in the same way with the parathion added to initiated the reaction containing a known amount of radioactive parathion. Ring labeled ^{14}C parathion was used to follow the fate of parathion, paraoxon and p-nitrophenol while ^{14}C ethyl label was employed to investigate the possible production of diethyl phosphate and diethyl phosphorothionate.

The reaction was stopped by the addition of 5 ml of cold ether and the mixture was stored in glass stoppered test tubes. The mixture

was extracted with two 5 ml portions of ether by shaking the sample with ether for 2 minutes and removing the ether layer and depositing it into flash evaporator flask. The extraction solvent was then evaporated under suction without heat to a small volume (300 μl) which was subsequently spotted on thin layer chromatography plates. The plates were developed and sprayed as previously described and the spots corresponding to metabolites were scraped and placed in scintillation vials. The amount of parathion that was metabolized to the other products was determined by the relative amounts of radioactivity found in parathion spot versus the p-nitrophenol, paraoxon, diethyl phosphate and diethyl phosphorothionate spots.

IV. Results

A. Parathion Toxicity

The fresh water snail Viviparus malleatus was not sensitive to parathion in the dose range to which the animal was exposed in these experiments. A level of 1000 ppm failed to exert any observable toxic effects on the snail (table 3). The solubility of parathion in water is only 20 ppm so the attempt to create a 1000 ppm concentration was to be certain that saturation was reached.

The crayfish Orconectes rusticus demonstrated extreme sensitivity to parathion exposure in its water environment. Concentrations as low as 1.0 parts per billion (ppb) produced death in 100% of the crayfish exposed in less than 24 hours (table 2). The toxicity was evidenced by twitching movements and exaggerated muscle contractions in response to provocation immediately before death.

The concentration of 0.1 ppb was the only concentration that did not produce 100% lethality in 24 hours. Three of the four crayfish tested at that concentration did die in 24 hours. The remaining animal dies in less than 48 hours.

It should be noted that it was necessary to employ new tygon tubing, new air stones and new beakers for this study as it was shown that beakers that had contained parathion and were subsequently cleaned could still leach enough parathion to cause death to crayfish.

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TABLE 2

SUSCEPTIBILITY OF ORCONECTES RUSTICUS TO
PARATHION EXPOSURE IN WATER

Exposure Concentration (ppb)	N^a	Number dying in 24 hours	Number dying in 48 hours
0	4	0	0
0.1	4	3	1
1.0	4	4	-
10.0	4	4	-
100.0	4	4	-

[a]Number of animals individually exposed.

TABLE 3

SUSCEPTIBILITY OF VIVIPARUS MALLEATUS TO
PARATHION EXPOSURE IN WATER

Exposure Concentration (ppm)	N^a	Number dying in 24 hours	Number dying in 48 hours
0	6	0	0
32[b]	6	0	0
100	6	0	0
320	6	0	0
500	6	0	0
1000	6	0	0

[a]Number of animals exposed.

[b]Parathion solubility in water is 20 ppm.

The calculation of TL_m50 values for these data was not possible because of the lack of snail susceptibility and extreme crayfish sensitivity.

The resistance of the snails to parathion was further studied by injecting the snail directly with parathion. The two snails that were each injected with 5.0 mg (approximately 250 mg/kg) of parathion showed no toxic sign in 96 hours.

B. Paraoxon Toxicity

The susceptibility of Viviparus malleatus or Orconectes rusticus was determined for the oxygen analog of parathion, paraoxon. These data show no snail mortability due to paraoxon exposure at 10 ppm exposure in 96 hours (table 5).

The crayfish was extremely senstitive to paraoxon (table 4). All of the animals exposed to concentrations ranging from 0.1 to 20 ppb died in less than 24 hours and exhibited the same toxic signs (e.g., twitching movements and exaggerated muscle contractions in response to provocation) as those that dies in the parathion exposure experiment.

The resistance of the snails to paraoxon was studied by injecting paraoxon directly into two snails. The snails that were each injected with 1.0 mg (approximately 50 mg/kg) of paraoxon showed no toxic signs in 96 hours.

C. p-Nitrophenol Spectrophotometric Assay

The spectrophotometric p-nitrophenol assay measured the production of p-nitrophenol from parathion by crayfish hepatopancreas,

TABLE 4

SUSCEPTIBILITY OF ORCONECTES RUSTICUS TO
PARAOXON EXPOSURE IN WATER

Exposure Concentration (ppb)	N[a]	Number dying in 24 hours
0.1	2	2
0.33	4	4
0.5	10	10
1.0	10	10
3.3	10	10
10	10	10
20	2	2

[a]Number of animals individually exposed.

TABLE 5

SUSCEPTIBILITY OF VIVIPARUS MALLEATUS TO
PARAOXON EXPOSURE IN WATER

Exposure Concentration (ppb)	N[a]	Number dying in		
		24 hours	48 hours	96 hours
1.0	3	0	0	0
1.8	3	0	0	0
3.2	3	0	0	0
5.6	3	0	0	0
10	3	0	0	0

[a]Number of animals exposed.

crayfish gill, snail whole intestine and rat liver homogenates.

The results of these experiments are presented in table 6. These data show that rat liver homogenates produce p-nitrophenol from parathion at a rate of 32.40 µg/hour/gram tissue when determined over a 1 hour incubation period. The male rat is a species known to be susceptible to parathion toxicity at a dose of 7.0 mg/kg ip (Benke and Murphy, 1975).

The homogenate of whole snail intestine did not produce p-nitrophenol at a level that could be measured by this technique. The possibility that the reaction that could produce p-nitrophenol by these tissues was NADPH dependent and that the NADPH generating system employed in the assay procedure was ineffective in the incubation mixture was tested by the addition of NADPH in place of NADP. No p-nitrophenol was detected. Dithiothreitol at 10^{-4} M was also tested for its effect on p-nitrophenol production. However, no p-nitrophenol production was discernible.

The hepatopancreas of crayfish Orconectes rusticus was evaluated using the p-nitrophenol assay for its ability to metabolize parathion. The data of table 6 show that no conversion of parathion to p-nitrophenol by Orconectes hepatopancreas homogenates could be detected.

The effect the addition of NADPH and/or DTT would have on the production of p-nitrophenol was also tested. No conversion of parathion to p-nitrophenol could be detected using the spectrophotometric assay.

TABLE 6

IN VITRO METABOLISM OF PARATHION WITH THE PRODUCTION OF P-NITROPHENOL
MEASURED SPECTROPHOTOMETRICALLY

Tissue[a]	N[b]	Incubation Time (hour)	NADP	NADPH	DTT[c]	μg p-Nitrophenol g Tissue/Hour
Rat liver	6	1.0	+[d]	−[e]	−	32.4 ± 1.9
Crayfish gill	7	1.0	+	−	−	none
Crayfish gill	2	0.5	+	−	−	none
Crayfish hepatopancreas	21	1.0	+	−	−	none
Crayfish hepatopancreas	7	0.5	+	−	−	none
Crayfish hepatopancreas	2	1.0	−	+	−	none
Crayfish hepatopancreas	2	1.0	−	+	+	none
Crayfish hepatopancreas	2	1.0	+	−	+	none
Snail intestine	33	1.0	+	−	−	none
Snail intestine	2	1.0	−	+	−	none
Snail intestine	2	1.0	−	−	+	none
Snail intestine	2	1.0	+	−	+	none

[a]Three grams of tissue used.

[b]Number of Animals.

[c]Dithiothreitol 10^{-4} M.

[d]Present in incubation mixture.

[e]Absent from incubation mixture.

The lungs of mammals have the ability to metabolize parathion, are in ready contact with the material and are capable of lipid absorption, so the gills of Orconectes were tested for parathion metabolizing ability as a comparison. The data in table 6 show that no p-nitrophenol production could be detected using that tissue and the spectrophotometric assay.

D. Thin Layer Chromatography

The use of Rhodamine B spray (0.1 mg/ml ethanol) made it possible to visually identify parathion, p-nitrophenol, paraoxon, and diethyl phosphorothionate spots under ultraviolet light. Initial experiments determined that 1.0 μg of parathion spotted on a TLC plate and subsequently developed with the hexane; chloroform, methanol, solvent mixture (7:2:1) could be visualized. Amounts of parathion below 1.0 μg were occasionally difficult to visualize so that identification was inconsistent.

The efficiency and reliability of the techniques used in this study that required TLC were evaluated. The Rf values for p-nitrophenol, paraoxon, parathion and diethyl phosphorothionate were 0.17, 0.42, 0.69 and 0.75 respectively, using the hexane, chloroform, methanol (7:2:1) system with the 250 μ Silica Gel-G plate, but because the Rf value of a compound may change with slight alterations in the solvent mixture, standards were run concurrently with the unknowns to facilitate accurate identification.

Radioactive parathion and its metabolites were separated,

identified and quantitatively recovered by TLC using techniques that required knowledge of the effect glass wool, Silica Gel-G and Rhodamine B spray would have on recovery and counting efficiency. Preliminary tests determined that glass wool, scraped Silica Gel-G coating and Rhodamine B spray in the amounts used in these experiments did not interfere with the liquid scintillation counting procedure.

The efficiency of the procedure used to recover parathion and its metabolites from TLC plates is presented in table 7. These data show that TLC followed by liquid scintillation counting could be effectively used as a procedure for the separation, identification, recovery and quantification of parathion and its metabolites.

E. Extraction of Parathion and Metabolites From Water Samples

The extraction of parathion and metabolites from water samples was necessary to evaluate the excretion of parathion metabolites from crayfish and snails exposed to the pesticide. Prior to experiments to determine if parathion and/or metabolites could be recovered, the efficiency and reproducibility of the extraction process was determined (table 8). The ether extraction procedure of water samples extracted 77% of the parathion in the sample with less than 10% standard error.

The ability of crayfish to metabolize parathion and then to excrete those metabolites into their water environment was tested by exposing the crayfish for 1-1/2 hours to water samples containing 100 ppb parathion (labeled in the ring or ethyl position) and then determining the metabolites present in the water samples. The ether

TABLE 7

EFFICIENCY OF THIN LAYER CHROMATOGRAPHY PROCEDURES

Procedure	N[a]	CPM	Percent[b] Recovery
Scrape sprayed plate (background)	4	23 ± 0.4	---
Spot radioactive parathion on plate, no development, spray and scrape	4	224 ± 5.7	100
Spot radioactive parathion on plate, develop plate[c] spray and scrape	4	196 ± 3.7	81
Spot radioactive parathion on plate, no development, spray, scrape elute sample off coating[d] count	4	200 ± 5.8	92

[a]Number of samples run.

[b]Equal amount of stock radioactive parathion was placed directly into scintillation vials to compute percent recovery.

[c]Hexane, chloroform, methanol 7:2:1, was developing solvent, Silica Gel-G 250 μ thick plate.

[d]Ether used to elute sample (10 ml added and then evaporated to less than 4 ml for counting.

TABLE 8

EFFICIENCY OF THE ETHER EXTRACTION OF
PARATHION FROM WATER SAMPLES

Standard[a] CPM	Extracted Sample[b] CPM
346	
388	343
375	292
414	+ 260
+ 412	
$\bar{x}$ 387 ± 12.0 = 2.5 µg parathion	$\bar{x}$ 298 ± 24.0 = 1.9 µg parathion
	= 3.85 ppb

[a]Standards were the radioactive compounds placed directly into scintillation vials. The amount of radioactive parathion was equivalent to 2.5 µg or the amount of parathion in 500 ml of water to equal 5 ppb.

[b]Samples were 2.5 µg of radioactive parathion dissolved in 500 ml of water and then extracted using the water extraction procedure.

extraction, thin layer chromatographic separation and scintillation counter quantification technique was used for that determination. The data in table 9 show that unquestionably the vast majority of the total amount of compound recovered from the exposure was in the form of parathion.

The snails were exposed to 320 ppm of ring labeled parathion for 48 hours with subsequent extraction of the aquarium water as with the crayfish (table 10). The results presented in tables 9 and 10 show that for both the crayfish and the snail there was no excretion of parathion metabolite into the water environment. There was no production of p-nitrophenol with either species. However high values for p-nitrophenol were obtained which was shown to be due to spontaneous hydrolysis.

F. Evaluation of Parathion and Metabolites Accumulation in
     Crayfish and Snail Tissues Following Parathion
     Exposure in a Water Environment

To study the accumulation of parathion by tissues of the species animals were exposed to 100 ppb ^{14}C labeled parathion for one-half hour and were then sacrificed for subsequent analysis. The extraction of labeled ^{14}C parathion and metabolites from crayfish tissues showed that there was no indication of any accumulation of these compounds in hepatopancreas or muscle tissues. The data (table 11) show that when hepatopancreas or muscle samples from animals exposed to ring or ethyl labeled ^{14}C parathion were pooled, no accumulation could be detected.

TABLE 9

PARATHION METABOLISM AND EXCRETION INTO THE WATER ENVIRONMENT BY ORCONECTES RUSTICUS

Label[a]	Exposure Concentration ppb	CPM for Area of TLC Plate Corresponding to:[a]			
		p-Nitrophenol	Paraoxon	Parathion	diethyl-phosphorothionate
Ethyl	100	23[b]	23[b]	556	22[b]
Ethyl	100	24[b]	22[b]	396	23[b]
Ring	100	45[c]	34[b]	211	22[b]
Ring	100	54[c]	29[b]	283	26[b]

[a]Raw counts (not corrected for background).

[b]Value not above background (does not indicate compound production-test sensitivity 4.7 μg ethyl and 9.3 μg ring).

[c]High value due to spontaneous hydrolysis.

TABLE 10

PARATHION METABOLISM AND EXCRETION INTO THE WATER ENVIRONMENT BY <u>VIVIPARUS</u> <u>MALLEATUS</u>

Label[a]	Exposure Concentration ppm	CPM for Area of TLC Plate Corresponding to:			
		p-Nitrophenol	Paraoxon	Parathion	Diethyl-Phosphorothionate
Ring	320	121[c]	42[b]	393	21[b]
Ring	320	128[c]	38[b]	400	25[b]

[a] ^{14}C labeled parathion sample.

[b] Values do not indicate compound production (test sensitivity 16 mg).

[c] High value due to spontaneous hydrolysis.

TABLE 11

ACCULUATION OF PARATHION AND METABOLITES IN <u>ORCONECTES</u> <u>RUSTICUS</u> TISSUES FOLLOWING EXPOSURE[a]

Tissue	N[b]	CPM for Area of TLC Plate Corresponding to:			
		p-Nitrophenol	Paraoxon	Parathion	Diethyl-Phosphorothionate
Hepatopancreas[c]	2	25[e]	24[e]	25[e]	26[e]
Hepatopancreas[d]	2	24[e]	22[e]	25[e]	25[e]
Muscle[c]	2	25[e]	26[e]	26[e]	26[e]
Muscle[d]	2	24[e]	29[e]	30[e]	30[e]

[a]One and one-half hour exposure to 100 ppb.

[b]Number of animals pooled sample was taken from.

[c]^{14}C label used for exposure (Ring) (Test sensitivity 9.3 µg).

[d]^{14}C label used for exposure (Ethyl) (Test sensitivity 4.7 µg).

[e]Values do not indicate any production or accumulation of parathion or metabolite.

The accumulation of parathion or metabolites in snails was tested by exposing two snails to ^{14}C ring labeled parathion at a concentration of 320 ppm for 48 hours. The animals were then sacrificed and their tissues analyzed for parathion and metabolites. The data in table 12 show that there was no accumulation of parathion or metabolites in Viviparus malleatus.

G. Production of Parathion Metabolites by Crayfish
Hepatopancreas and Whole Snail
Intestinal Tissue

An assay was developed to utilize the procedures employed to determine if crayfish hepatopancreas or snail intestinal homogenates could metabolize parathion to p-nitrophenol, paraoxon or diethyl phosphorothionate in vitro. The results obtained by the incorporation of radioactive parathion into the p-nitrophenol spectrophotometric assay procedure followed by the extraction of the incubation mixture to recover parathion and any radioactive metabolites are presented in tables 13 and 14.

The results (table 13) show that there was no production of p-nitrophenol, paraoxon or diethyl phosphorothionate from snail tissues that were active or deactivated by boiling. That there was 120 μg of radioactive parathion present in the incubation mixture (4.0 cpm/μg for ring label and 3.0 cpm/μg for ethyl label) and a large percentage of that radioactivity was effectively extracted, separated and recovered is illustrated by the CPM values for parathion itself.

The results of the experiments with crayfish hepatopancreas

TABLE 12

ACCUMULATION OF PARATHION AND METABOLITES IN <u>VIVIPARUS</u> <u>MALLEATUS</u> TISSUES FOLLOWING EXPOSURE[a]

Tissue	N[b]	CPM for Area of TLC Plate Corresponding to:			
		p-Nitrophenol	Paraoxon	Parathion	Diethyl-Phosphorothionate
Whole Snail[c]	2	30[d]	26[d]	27[d]	27[d]

[a]Forty-eight hours exposure to 320 ppm parathion.

[b]Number of animals pooled sample was taken from.

[c]Ring ^{14}C label used for exposure (test sensitivity 16.0 µg).

[d]Values do not indicate any production or accumulation of parathion or metabolite.

TABLE 13

METABOLISM OF ^{14}C LABELED PARATHION[a] INTO P-NITROPHENOL, PARAOXON, AND
DIETHYL PHOSPHOROTHIONATE BY <u>VIVIPARUS</u> <u>MALLEATUS</u>

Tissue[b] Present	N[c]	Position of Label	Counts Per Minute			
			p-Nitrophenol	Paraoxon	Parathion	Diethyl Phosphorothionate
Deactivated[d]	4	Ethyl	19	19	105	20
Active	4	Ethyl	20	21	149	20
Deactivated	4	Ring	24	22	126	21
Active	4	Ring	23	20	170	20

[a]One hundred twenty µg (4.0 cpm/µg ring label, 3.0 cpm/µg ethyl label) (test sensitivity 11.5 µg
ring and 15.3 µg ethyl).

[b]Whole tissue homogenate equivalent to 0.5 g of tissue (1 hour incubation).

[c]Number of animals tested.

[d]Boiling 15 minutes.

TABLE 14

METABOLISM OF ^{14}C LABELED PARATHION[a] TO P-NITROPHENOL, PARAOXON, AND DIETHYL PHOSPHOROTHIONATE BY <u>ORCONECTES</u> <u>RUSTICUS</u>

Tissue Present[b]	N[c]	Position of Label	Counts Per Minute			
			p-Nitrophenol	Paraoxon	Parathion	Diethyl Phosphorothionate
None	4	Ethyl	X[d]	X	720.2±22.7[f]	27.0±2.6
Active	4	Ethyl	X	23.0 0.57	534.3±50.7[f]	22.6±1.2
Deactivated[e]	4	Ethyl	X	25.0 1.35	511.7±65.6[f]	22.7±1.2
Active	4	Ring	X	X	228.0±10.2[f]	X
Deactivated	4	Ring	X	X	189.5±09.3[f]	X

[a]Fifty µg (13 cpm/µg ring label, 30 cpm/µg ethyl label) (test sensitivity 3.5 µg Ring and 1.5 µg Ethyl).

[b]Hepatopancreas homogenate (1.5 ml) used in incubation (1 hour) mixture equivalent to 0.1 g of tissue.

[c]Number of animals tested.

[d]X = no spot on TLC plate or CPM was below blank.

[e]Boiling for 15 minutes.

[f]Significantly different from blank, using student's <u>t</u>-test ($p<0.05$).

incubation with labeled parathion are presented in table 14. These data show that there was no metabolism of parathion to diethyl phosphorothionate, paraoxon or p-nitrophenol in the active tissues or deactivated tissues. The fact that sufficient labeled parathion (50 μg, 13 cpm/μg ring label, 50 μg, 30 cpm/μg ethyl label) was added to the incubation mixture to determine metabolism of the organophosphate, if it were present, is shown by the high radioactive recovery of parathion from all the samples tested.

H.   In Vitro Metabolism of Paraoxon by Orconectes and Viviparus

The possibility that both crayfish and snails might be able to metabolize the oxygen analog of parathion, paraoxon, to diethyl phosphate and p-nitrophenol was evaluated by using the p-nitrophenol spectrophotometric assay with paraoxon added to initiate the reaction in place of parathion. The results from this experiment (table 15) show that in no instance did crayfish or snail tissue demonstrate any significant production of p-nitrophenol that could be measured by this method.

TABLE 15

IN VITRO METABOLISM OF PARAOXON TO P-NITROPHENOL BY ORCONECTES
AND VIVIPARUS DETERMINED SPECTROPHOTOMETRICALLY

Species	Tissue[a]	N[b]	Paraoxon[c]	Abs.±S.E.[d]
Viviparus	O	2	no	0.135±0.015
Viviparus	+	2	no	0.144±0.000
Viviparus	O	2	yes	0.208±0.002
Viviparus	+	2	yes	0.204±0.000
Orconectes	O	2	no	0.084±0.001
Orconectes	+	2	no	0.087±0.001
Orconectes	O	2	yes	0.151±0.000
Orconectes	+	2	yes	0.149±0.003
No Tissue		2	yes	0.074±0.004

[a]O = tissue (Viviparus intestine equivalent to 0.5 g, Orconectes
hepatopancreas equivalent to 0.1 g) deactivated by boiling 15 minutes.

+ = tissue active.

[b]Number of samples tested.

[c]yes = 10 μl of 10 μg/μl paraoxon in ethanol added to incubation
mixture.

no = paraoxon not added to incubation mixture.

[d]Absorbance measured at 410 nm ± standard error.

V. Discussion

The investigation of the metabolism of parathion by fresh water invertebrates is more than an academic exercise in pesticide metabolism and detection. The problems that followed the use of the chlorinated hydrocarbon pesticides were in part due to the lack in understanding of the environmental consequences of their use and ignorance as to the effects the compounds would have on organisms other than the target species and man.

The increase in use of the organophosphates along with a limited understanding of their toxicity and metabolism in nonmammals is a situation analogous to the one that led to problems with chlorinated hydrocarbon pesticides. Nicholson et al. (1962) did investigate the environmental exposure consequences of parathion and showed that parathion would accumulate in a farm pond, and that it would persist in the environment for at least nine months. These times are similar to those that were reported by Faust (1964).

Nicholson et al. (1962) showed that following the normal agricultural spraying of an orchard, concentrations of 1.22 ppb of parathion could be detected in a farm pond that was subject to rain runoff from that orchard. The concentration of parathion recovered from the bottom mud samples from that pond were even greater than the concentrations in the water. Nicholson's evaluation of the fauna

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present in the farm pond showed no crayfish present.

The environmental implications of parathion use were considered an integral part of the stimulus for this project, as the possible toxocity and/or metabolism of parathion by Orconectes rusticus and Viviparus malleatus would be a result of environmental exposure. The determination of parathion-induced toxicity to Orconectes or Viviparus was investigated with an awareness of the environmental exposures possible and an interest as to the possible accumulation of the toxin and/or metabolites in the species.

The results obtained by the exposure and injection toxicity experiments have shown that the snail Viviparus malleatus is not susceptible to acute parathion induced toxicity, and no accumulation of parathion or metabolites were detected in the snail. This correlates with the work of Yu and Sanborn (1975) who could not detect any accumulation of parathion in snail tissues following exposure to the pesticide. The toxicity data derived concerning the crayfish Orconectes rusticus demonstrates the opposite, that Orconectes is susceptible to acute parathion induced toxicity at the extremely low concentration of 1.0 ppb. Realizing that parathion concentrations of greater than 1.0 ppb can be attained in natural waters as a result of the normal agricultural use of parathion and that no crayfish were reported in the pond investigated by Nicholson et al.(1962), the possibility that the use of parathion may have deleterious effects on the crayfish populations gains credence.

The reasons why parathion exposure was toxic to Orconectes

and not to _Viviparus_ were evaluated by this project. The fact that
acute toxicity due to parathion exposure is due to its inhibition of
AChE had been shown by many investigators and that evidence has been
presented in the literature review section. It is important to
remember that parathion itself is not the compound that exerts the
inhibition of the AChE enzyme but rather the desulfurated metabolite of
parathion, paraoxon. Previous research has shown that not only must
parathion be metabolized to paraoxon to produce toxic effects but
parathion can be metabolized directly to relatively nontoxic meta-
bolites diethyl phosphorothionate plus _p_-nitrophenol or diethyl phos-
phate plus _p_-nitrophenol, through the same intermediate which gives
paraoxon. The toxic compound paraoxon can be detoxified prior to its
aging to diethyl phosphate and _p_-nitrophenol. Many routes of metabolism
of parathion and paraoxon other than these two important pathways have
also been elucidated, such as the glutathion dependent alkyl and aryl
transformations. These pathways are all presented in Figure 1. The
important point is that these reactions occur and that the rates at
which they compete for parathion and paraoxon influences the toxicity
of parathion exhibited in that particular species (Benke and Murphy,
1975).

The parathion exposure experiments showed that _Viviparus_ was
not sensitive to parathion in its water environment even when the
solubility of parathion in the water was exceeded while _Orconectes_ was
sensitive to parathion in its water at 1 ppb. The question that pre-
sented itself was why there was such a species difference in

susceptibility. Refering to Figure 1 and with an understanding of the data from other investigators the possible mechanisms for these results were envisioned. The snail could be resistant to parathion exposure due to the following reasons:

1. Parathion was not entering the shell of the snail.

2. Parathion was entering the shell but not being metabolized to paraoxon, which if produced would cause toxicity.

3. The parathion was being metabolized extremely rapidly and efficiently via pathways 3, 4, 6, 9 or 11 (Fig. 1) to the nontoxic metabolites or being bound to tissues where no metabolism could occur.

4. The parathion was being metabolized to paraoxon (pathway 1, Fig. 1) very slowly while pathways 2, 5, 7, 8 or 10 were operating very quickly.

5. The snail may be converting parathion to paraoxon but be insensitive to paraoxon.

The goal of the investigation of the resistance of Viviparus malleatus to parathion was then to design experiments to determine which of these mechanisms was responsible for the lack of parathion toxicity in the snail.

The possibility that the parathion was not entering the shell of the snail was evaluated by injecting snails with 5.0 mg/kg. The results show that there were no toxic signs demonstrated by the snails. Paraoxon was also injected directly into snails. Two snails were exposed to 50 mg/kg of paraoxon by direct injection with no toxic

signs demonstrated in 96 hours. The results of these two experiments establish that the ability of the compound to enter the shell of the snail was not a limiting factor in its lack of toxicity. The results also show that the snail is resistant to paraoxon as well as parathion so that the absence of pathway 1 of Figure 1 would not be the mechanism responsible for the animals' resistance to the organophosphate.

The p-nitrophenol spectrophotometric assay was used with whole snail homogenates to determine if the snail was capable of metabolizing parathion via pathways 2, 3 or 9 of Figure 1. Since these pathways are the most important detoxification pathways for parathion resistance, the demonstration of measurable p-nitrophenol would show that the parathion or paraoxon was being detoxified and could possibly be the cause for the animals' resistance to parathion and paraoxon exposure. The p-nitrophenol spectrophotometric assay with whole snail intestine did not produce p-nitrophenol at a level that could be measured by this technique. The reason for the lack of production was not due to deficiency of NADPH for dependent enzyme reactions or the destruction of essential sulfhydryl groups of the membranes or enzymes, as NADPH and dithiothreitol were added. The same spectrophotometric assay incorporating paraoxon in the place of parathion was also done to determine if pathway 2 of Figure 1 was possible but not detected with parathion in the incubation due to the absence of the conversion of parathion to paraoxon (pathway 1, Fig. 1). Again, no p-nitrophenol was produced indicating the lack of pathway 2 route of metabolism.

The p-nitrophenol assay is designed to demonstrate the presence

of metabolism occurring but because the measured compound can be produced by numerous pathways if the compound is detected the exact pathway followed cannot be determined. A method to determine if specific pathways were used in the metabolism of parathion would be to determine the presence of the specific compounds paraoxon and diethyl phosphorthionate, along with p-nitrophenol and parathion.

The extraction of parathion and metabolites from water samples measured the excretion of metabolites of parathion into the water environments of snails. The results of that experiment (table 10) show again that there was no metabolism followed by excretion of parathion by the snail. To be certain that there was no metabolism of parathion occurring by Viviparus malleatus an experiment was designed where homogenates of snail tissue were incubated with radioactive parathion, that mixture subsequently being extracted and the metabolites separated by thin layer chromatography and quantified by liquid scintillation counting. The results (table 13) further substantiate the conclusion that parathion is not metabolized by Viviparus malleatus. The snail is demonstrating a biologic resistance to parathion, as the inhibition of AChE if present has no effect on the organism.

The crayfish demonstrated susceptibility to acute parathion toxicity, and the muscle twitching signs were consistent with AChE inhibition. The mechanism by which this toxicity was produced was assumed to follow the desulfuration of parathion to paraoxon via pathway 1 of Figure 1. This conversion would have to be at a rate high enough to account for a paraoxon concentration that would cause

toxicity. The reaction rates for the toxic conversion reaction and detoxification reactions has been shown to be an important factor in the toxicity exhibited by species to parathion exposure does not establish the existence or absence of detoxifying reactions by Orconectes, only the existence of a toxic conversion reaction

The goal, then, for the investigation of the Orconectes susceptibility to parathion was to demonstrate the presence of the reaction coverting parathion to paraoxon and any other metabolic pathways present as depicted in Figure 1. This is closely related to the question of the accumulation of parathion and/or its metabolites in crayfish tissues. The results presented in table 11 show that there was no accumulation of these compounds in the gill, muscle or hepatopancreas of crayfish following exposure to parathion.

The possibility that the crayfish toxicity was due to concentrations of paraoxon too low to detect could not be overlooked in this investigation considering the research of Carlson (1973) and Elmanlouk and Gessner (1976). They reported that the hepatopancreas of the lobster is the organ responsible for drug metabolism, but the hepatopancreas of the lobster had very little, if any, observable ability to convert parathion to paraoxon.

Establishing if paraoxon was capable of producing the toxicity in crayfish was done by exposing the crayfish to paraoxon in the aquarium water as had been done with parathion. The experimental results showed that the crayfish was sensitive to paraoxon and that the toxic signs exhibited by the crayfish following paraoxon exposure were

the same as those following parathion exposure. The concentration range that produced the toxicity in the crayfish due to paraoxon (table 4) was consistent with the theory that the parathion was exhibiting its toxicity through conversion to paraoxon and subsequent AChE inhibition.

The toxicity determination experiments did little to increase the understanding of the metabolism of parathion by Orconectes. Experimental procedures similar to the ones used to determine the metabolism of parathion in snails needed to be done to determine the pathways of metabolism of parathion and paraoxon in crayfish.

The p-nitrophenol spectrophotometric assay was used to ascertain if any metabolism of parathion or paraoxon could be determined with that technique. Rats are susceptible to parathion toxicity at 7.0 mg/kg i.p. (Benke and Murphy, 1975) so they were used as controls in these experiments to compare their metabolism of parathion to that in the crayfish. The p-nitrophenol assay was performed on rat liver homogenates as well as homogenates of crayfish hepatopancreas and gill (table 6). The results show that the rat liver homogenate caused the production of 32.4 µg/hour/gram of tissue when determined over a 1 hour incubation period, but the crayfish hepatopancreas and gill homogenates which would be expected to be the organs capable of metabolizing parathion produced no p-nitrophenol that could be measured spectrophotometrically. These results were not due to lack of NADPH for dependent enzyme processes or the destruction of vital enzymes since the addition both NADPH and dithiothreitol (10^{-4} M) added to

some incubation samples as had been done in the snail tissue experiments, were not effective.

Since the p-nitrophenol assay was done using both parathion and paraoxon in the incubation flasks, the assay had the capability of monitoring pathways 2, 3 and 9 of Figure 1 all of which cause the production of p-nitrophenol. No p-nitrophenol could be detected by this technique. However, because the crayfish were sensitive to the parathion and paraoxon exposures other experimental designs were used in an attempt to monitor some metabolism of parathion or paraoxon by the crayfish or its tissues.

The possibility that the crayfish was able to absorb parathion from its water environment and then to excrete metabolites of parathion back into the water was evaluated. ^{14}C Parathion labeled either in the ethyl or ring positions was used for these exposure-excretion experiments. Following the exposure of crayfish to labeled parathion at a concentration of 100 ppb for 1-1/2 hours, the water in which the crayfish were kept was extracted with ether, the samples separated by thin layer chromatography and the appropriate spots scraped and counted by liquid scintillation counting. The results showed that after the spontaneous hydrolysis of stock parathion is considered there was no excretion of parathion metabolites into the water that would be indicative of parathion metabolism.

The ability to produce even minute amounts of parathion metabolites by crayfish tissues was tested by the use of an incubation technique similar to the p-nitrophenol assay. Homogenates of crayfish

hepatopancreas were incubated with ^{14}C parathion in either the ring or ethyl positions. When the incubation was completed, the mixture was extracted, separated and quantified by thin layer chromatography and scintillation counting. The results (table 14) show that there was no production of p-nitrophenol or paraoxon both of which would be detected through the use of the ring label and no production of diethyl phosphate or diethyl phosphorothionate detectable through the use of the ethyl label.

Retrospectively, it is easy to propose a number of mechanisms by which the metabolism of parathion and paraoxon could be responsible for the crayfish exhibited toxicity without the metabolism being detected by sensitive mechanisms. The crayfish could be so exquisitely sensitive to paraoxon that a very small amount of metabolite below detectability could be responsible. The paraoxon that was produced could bind with the enzyme to cause AChE inhibition and remain attached to the enzyme. The in vivo experiments demonstrated that the crayfish are sensitive to very small quantities of paraoxon. Unfortunately the in vitro techniques for the detection of p-nitrophenol are not qualitative and quantitative enough to separate the nonspontaneous hydrolytic production of p-nitrophenol from the small amounts of p-nitrophenol that would accompany any paraoxon inhibition of AChE.

The question that rises from these results is how is the parathion producing its toxicity if no metabolism can be detected.

VI. Conclusions

The goal of determining the toxicity and metabolism of para-
thion in the fresh water invertebrates Orconectes rusticus and
Viviparus malleatus made the extraction, detection and quantification
of minute quantities of organophosphate and its metabolites necessary.

Groups of the crayfish Orconectes rusticus when exposed to
1.0 ppb or greater of parathion all died. The snail Viviparus
malleatus was resistant to parathion exposure at the level of parathion
solubility and to direct injection of parathion with a dose of
approximately 50 mg/kg. The crayfish demonstrated extreme sensitivity
to paraoxon exposure and showed the same signs of cholinergic stimu-
lation that were exhibited with parathion exposure, while the snail was
not effected.

The ether extraction of water samples for parathion and
metabolites was both efficient and reproducible. That technique
demonstrated that neither parathion or metabolites accumulated in the
tissues of Orconectes or Viviparus following exposure.

The thin layer chromatography technique used in the investi-
gation was simple, reliable, specific, and sensitive. The combination
of Silica Gel G 250 μ plates developed in hexane, chloroform, and
methanol (7:2:1) and sprayed with Rhodamine B (0.1 mg/ml in ethanol)
allowed for the ultraviolet visualization of as little as 1.0 μg of

organophosphate. Excellent separation of p-nitrophenol, paraoxon, parathion, and diethyl phosphorothionate was obtained.

The ability of the crayfish and the snail to metabolize parathion was determined through the use of the p-nitrophenol spectrophotometric assay, the investigation of excreted metabolites that were extractable from water samples, the determination of the presence of metabolites accumulating in tissue, detection of radioactive metabolites from tissue incubation experiments and the toxicity experiments. All of the experimental data support the conclusion that there was no metabolism of parathion by the snail and that this lack of metabolism and the insensitivity of Viviparus to paraoxon form the basis for the lack of toxicity exhibited by those compounds to the snail.

The data indicate no metabolism or accumulation of parathion or its metabolites by the crayfish, but the conclusion that no metabolism was taking place cannot be made as the toxicity experiments do not support that contention.

The data from the p-nitrophenol spectrophotometric assays with parathion and paraoxon using either crayfish or snail tissue revealed no production of p-nitrophenol by either the snail or the crayfish indicating that pathways 1, 2, 3 and 9 of Figure 1 could not be demonstrated by that technique.

The extraction experiments show that there was no detectable accumulation of parathion or metabolites in Orconectes or Viviparus and no detectable excretion of parathion metabolites into the water environment.

The experiments performed with radioactive parathion incubated with either crayfish or snail tissues were unable to demonstrate any metabolism of parathion, paraoxon, p-nitrophenol, diethyl phosphorothionate and diethyl phosphate by crayfish or snails.

The resistance to parathion and paraoxon demonstrated by the snail does not stem from enzyme systems capable of detoxifying the compounds. The snail is biologically resistant to the inhibition of AChE.

The mechanism by which parathion exhibits toxicity in Orconectes cannot be determined from the results obtained by these experiments. The extreme sensitivity to parathion and the clinical signs demonstrated by the crayfish following parathion exposure are consistent with the production of effective concentrations of paraoxon that can inhibit the AChE at synapse and neuromuscular junctions. However, until the production of some metabolism of parathion by some crayfish tissue can be demonstrated more definitive conclusion cannot be made.

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