South Dakota State University

Open PRAIRIE: Open Public Research Access Institutional
Repository and Information Exchange

Electronic Theses and Dissertations

1982

Blood Ethoprop Retention and Interaction

Ebenezer Bonsu Adjaye-Adjaye

Follow this and additional works at: https://openprairie.sdstate.edu/etd

Recommended Citation

Adjaye-Adjaye, Ebenezer Bonsu, "Blood Ethoprop Retention and Interaction" (1982). *Electronic Theses and Dissertations*. 4125.
https://openprairie.sdstate.edu/etd/4125

BLOOD ETHOPROP RETENTION AND INTERACTION

BY

EBENEZER BONSU ASAFU-ADJAYE

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science
Major in Chemistry
South Dakota State University
1982

BLOOD ETHOPROP RETENTION AND INTERACTION

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

David C. Hilderbrand   Date
Thesis Advisor

David C. Hilderbrand   Date
Head, Dept. of Chemistry

ACKNOWLEDGEMENTS

Table of Contents

INTRODUCTION

O-ethyl-S,S-dipropyl phosphorodithioate, technically known as Ethoprop, is a newly introduced insecticide-nematicide manufactured by Mobil Corporation. It is applied directly to the soil where it is believed to exercise a broad spectrum, non-fumigant effect. It has good soil movement and residual properties, and has been used to control rootworms, cutworms, nematodes, wireworms, fleabeetles and symphylans in corn, tobacco, potatoes, peanuts, bananas, and other crops [1].

Ethoprop comes in various formulations commercially such as:

Mocap 10G - 10% granular

Mocap 15G - 15% granular

Mocap EC - 6 lbs. ethoprop per gallon (719g/1)

Mocap Plus 10-5 Granular - 10% ethoprop and 5% Di-Syston

Mocap Plus 4-2EC - 4 lbs. ethoprop and 2 lbs. Di-Syston per gallon

Mocap PCNB 3-10 Granular - 3% ethoprop and 10% PCNB

Some physical and chemical properties of ethoprop are listed below [1]:

Physical State: light yellow liquid

Vapor Pressure: 3.5×10^{-4} torr (26°C)

Boiling Point: 86-91°C (0.2 torr)

Specific Gravity: 1.094g/ml (26°C)

Odor: slight mercaptan

Flash Point: 140°C (sealed)

Partition Coefficient: 140 (octanol/distilled water)

Solubility: water - 750ppm at 25°C

organic solvents - very soluble

Stability: thermally stable at 50°C for at least 12 weeks;
hydrolytically stable in acid medium; hydrolyzes
in basic medium;   commercial formulations are
stable under normal use conditions.

The uses of ethoprop are primarily agricultural, thus most of the work done on it to elucidate its properties has been in that area. The little research that has been performed on the biological properties was mainly to determine the probable products of metabolism in plants [2] and animals [3].

Though ethoprop, like other soil insecticides, is applied to the soil in which it exercises its effect, it is not uncommon for animals and other living organisms for whom the insecticide is not intended, to come in contact with it accidentally. The consequences of such contact may be harmful or even fatal to the victims themselves, or indirectly, to other living organisms. It is therefore very necessary to study the biological properties of the insecticide and its unintended effects on zoological species. Menzer et al[2], and Iqbal et al [3] identified some of the products of ethoprop metabolism in plants and animals respectively, and suggested pathways leading to such products. Subsequently it was thought to be of interest to study the retention and interaction of ethoprop in blood with cholinesterases

since most organophosphorus insecticides are known to be choline-
esterase inhibitors. The study was also considered important since
ethoprop is different from most insecticides in its class which
generally must be oxidized to phosphates to gain significant biologi-
cal activity. Ethoprop is expected to behave differently [3].

LITERATURE REVIEW

Ethoprop, also known commercially as Mocap[®] in the United States and ethoprophos in Europe, is an organophosphorus insecticide with the chemical name O-ethyl-S,S-dipropyl phosphorodithioate. It's structure is shown below:

$$H_7C_3 - S - \underset{\underset{C_2H_5}{\overset{|}{O}}}{\overset{\overset{C_3H_7}{\overset{|}{S}}}{\underset{|}{P}}} = 0$$
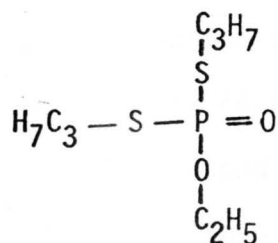
Unlike other organophosphorus insecticides, ethoprop is structurally different in that neither of the two alkyl-sulfur groups involved in the ester linkage with phosphorus is doubly bonded.

Since its introduction, most of the work done on ethoprop has been limited to evaluating its usefulness as an insecticide in relation to crop cultivation. It has been shown to be mainly effective on contact, with a relatively short residual life; but some root systemic activity has been detected [4]. It has also been shown to give excellent control of nematodes attacking nursery and ornamental plants [5,6,7,8], cotton [9], tobacco and potatoes [10], sweet potatoes [11], and onions [12]. As a soil insecticide-nematicide, it has been registered with the E.P.A. for use on field and sweet corn, white (Irish) potatoes, tobacco, peanuts, sweet potatoes, bananas and plantain, sugar-cane, snap and lima beans, cabbage, cucumbers, pineapple and commercial tuft [1].

The fate of ethoprop upon soil application is variable,

depending on soil type, temperature and moisture content [13,14,15]. Smelt et al [14] measured the rates of degradation and downward movement of ethoprop in four soil types in 40cm long aluminum columns, under field conditions. A 10% formulation was incorporated in the top 10cm at a rate of 10.0-10.5kg active ingredient per hectare of soil. Under outdoor conditions during Spring and Summer, loss of ethoprop corresponded approximately to first order kinetics. The half-life was 87 days in humic sand and in peaty sand, with pH values of 4.5 and 4.6 respectively. In a sandy loam and loam soil with pH values of 7.2 and 7.3 respectively, the half-life ranged between 14 and 28 days. Under experimental conditions with fallow soils and 35.3cm rainfall, the downward movement of a substantial concentration of ethoprop by leaching and diffusion was restricted to a few centimeters. Studies performed by Leistra [16] on soil in which the field conditions were simulated in a computation model, showed that soil movement of ethoprop varied, with little movement in high organic soils; (adsorption coefficient 4.8×10^{-3} mmol kg^{-1} soil/mmol m^{-3} solution for humic soil of pH 4.5 and 7.4×10^{-3} mmol kg^{-1}/mmol m^{-3} for peaty soil of pH 4.6). In sandy loam and loamy soils with low organic matter (1.7% and 2.6% respectively) movement was considerable; (adsorption coefficient of 0.78×10^{-3} mmol kg^{-1} soil/mmol m^{-3} solution for sandy loam and 0.74×10^{-3} mmol kg^{-1} soil/mmol m^{-3} solution for loamy soil). The spreading of the insecticide was attributed mainly to convective dispersion since diffusion through the liquid and gas phases contributed little to the large scale spreading.

Volatilization of the insecticide from the soil was estimated to be limited to a small percentage (1%) of the dosage. Similar findings had been reported by Brodie [17] in 1971. Ethoprop has also been shown to move with irrigation water [19].

Uptake of ethoprop by some vegetables grown in soil treated for the control of nematodes was investigated by Argauer and Feldmaster [20]. In the process, they developed a simple and rapid method of determining ethoprop residues in vegetable crops. Their method involved blending 100g portions of the chopped or slurried vegetable, 10ml of 10% sulfuric acid and 250ml of methylene chloride in a Waring blender for 3 minutes. The blend was filtered by gravity into a flask containing anhydrous granular sodium sulfate. An 83ml portion of the filtrate was concentrated to near dryness on an evaporator at about 20°C under a water aspirator vacuum. The concentrate was then dissolved in 5ml ethylacetate and centrifuged to remove insoluble matter, and 5μl of the supernatant was injected into a gas chromatograph. The response was compared with that of an ethoprop standard. Recovery of ethoprop was 100% for samples fortified with as little as 0.01ppm. Ethoprop levels of 0.01ppm and above were detected in some of the vegetables. Beets, cabbage, cantaloupe, peas and tomatoes did not show any trace of ethoprop(0.01ppm). The crops were grown in soil treated one week before planting with 30,60, and 120 lbs of ethoprop (10% granules)per acre, (3.4, 6.7 and 13.4kg active ingredient/hectare).

The toxicolgy of ethoprop (95%) and of the various formulations

has been determined in rats and rabbits [1]. Specific values found are given in the table below. The effect of ethoprop on nematodes has been described as nematicidal at high to moderate concentrations, and nematistatic at low concentrations [4].

Formulation	Oral LD_{50} mg/kg-rat	Dermal LD_{50} mg/kg-rabbit	Inhalation LC_{50}^{*} µg/liter-rat
Ethoprop (95% Technical)	47-62	3.8-26	123
		330 (swine)	
Mocap 10G	355	510	>20,000
Mocap 15G	347	1000	2000
Mocap 6EC	85	25	120

* Duration of exposure not stated.

A hundred ppm of ethoprop(95%) in the diet of a 90 day rat and 122 ppm in the diet of a 180 day mouse caused no adverse effect other than the initial depression of cholinesterase levels [1].

Ethoprop, like other organophosphorus insecticides, is believed to interfere with the activity of cholinesterases and the control of insects and nematodes is believed to be accomplished through this mechanism [1]. Some cholinesterases, like acetylcholinesterase, are very important in nerve function. Nerve cells along which impulses are conducted, are connected with one another through gaps called the synapse. In the case of muscle fiber, the gap is referred to as the neuromuscular junction. The nerve impulses cannot jump from one cell to another across the gap. Continuity of transmission is achieved through the mediation of chemical substances called

transmitters or neurotransmitters [24]. The transmitter is released from the nerve-endings of the nerve cell (pre-synaptic cell) along which the impulses are travelling. The transmitter then migrates to receptors on the adjoining nerve cell (post-synaptic cell), and the interaction of the transmitter with the receptors triggers off nerve impulses in this cell. In this way, the nerve impulses are carried from one cell to another until the final destination in the central nervous system or in an organ. The released transmitter, once it has accomplished its task of triggering impulses, is rapidly destroyed by enzymes before the next impulses arrive [24].

Most organophosphorus insecticides interfere with this system of transmitter release, its impulse triggering action and its subsequent destruction [24]. The interference caused is usually harmful to the organism and often fatal. The insecticides mainly exhibit their action by either inactivating the enzyme, in which case it is believed to do so by the phosphorylation of the enzyme, or by blocking the receptor site on the post-synaptic cell. The usual cause of death resulting from the interference, is known to be due to respiratory paralysis which in turn may be due to the blockage of the neuromuscular transmission or malfunction of the respiratory center in the brain or by a combination of both factors [24].

The best known transmitters are acetylcholine and noradrenaline (norepinephrine). In addition, other amines such as dopamine and 5-hydroxytryptamine (5-HT; serotonin), and amino acids such as

γ-aminobutyric acid and glycine have been recognized to act as possible transmitters in certain synapses. In insects, acetylcholine is accepted as the transmitter in the synapses of the central nervous system, [24] and the enzyme responsible for the hydrolysis (destruction) of the acetylcholine is acetylcholinesterase. Apart from acetylcholine, other cholinergic neurotransmitters are known in vertebrates, and are mostly located in the synapses of the central nervous system, the neuromuscular junctions of motor nerves, sensory nerve-endings, ganglionic synapses of both sympathetic and parasympathetic nerves, all post-ganglionic parasympathetic nerve terminals, and sympathetic nerve terminals on sweat glands, blood vessels and the adrenal medulla [24].

The physiological effects of ethoprop have been studied by workers such as Solly [21], who published in 1972 that no ill effects were found in sheep grazed on pasture top-dressed with 10% ethoprop at a rate of 2.24kg active ingredient per hectare, after withholding periods of 0, 3, 7 and 14 days. However, he reported that red blood cell cholinesterase activity was reduced in most animals. Samples of omental fat taken from sheep that had grazed on top-dressed pasture for six days contained no detectable residues (0.01ppm) of ethoprop.

In a recent study [22], performed at the Mobil Chemical Co., Mount Pleasant Plant in Tennessee, it was reported that laboratory technicians exposed to vapors of ethoprop from gas chromatograph exhaust ports showed no adverse health effects. This was after extensive interviews with the personnel, and evaluation of the

ventilation systems had been carried out.

In a study with plants, Epstein and Bravdo [23] reported that ethoprop reduced photosynthesis and transpiration in _Meliodogyna hapla_ infected rose leaves treated with the insecticide. Photosynthesis measured in terms of CO_2 uptake was reduced from 3.62mg CO_2 $dm^{-2}hr^{-1}$, (equivalent to 1.41mg CO_2mg^{-1} chlorophyll), to 2.32mg CO_2 $dm^{-2}hr^{-1}$, (equivalent to 1.14mg CO_2mg^{-1} of chlorophyll), a reduction of 36.3%. This was observed to be more than the calculated reduction due to stomatal closure. Transpiration measured by the rate of water loss, was reduced from 357.39mg H_2O $dm^{-2}hr^{-1}$, (equivalent to 4.08 sec. cm^{-1} resistance of leaf to water vapor loss) to 280.26mg H_2O $dm^{-2}hr^{-1}$, (equivalent to 10.70 sec. cm^{-1} leaf resistance to water vapor loss), a reduction of 21.0%. This reduction in net photosynthesis was reflected also by the reduction in flower yield.

The metabolic fate of ethoprop in some plants and animals has been investigated by Menzer _et al_ [2] and Iqbal _et al_[3]. Using labelled ethoprop, they were able to identify ethyl propyl sulfide, ethyl propyl sulfoxide, ethyl propyl sulfone and propyl disulfide in methylene chloride extracts of bean and corn plants grown in soil which has been treated with the insecticide. They also identified a water-soluble metabolite to be O-ethyl-S-propyl phosphorothioic acid. Only a small fraction of the ethoprop administered was found to have been taken up by the plants, the rest supposedly remaining in the soil. For the bean plant, recovery was 3.59% and in corn the percentage was 3.86. A significant portion of this activity was

found in the methanol-water extract in the form of hydrolytic products. For the portion taken up, only a small portion was extractable as ethoprop or its metabolies [2]. The proposed scheme of degradation in plants is shown in Figure I.

In the scheme, the role of the propyl thiolate ion ($C_3H_7S^-$) is thought to be very critical since it has been found to be a good leaving group in displacement with hydroxide ion. The propyl thiolate ion liberated in this type of reaction can attack a phosphorothioic ester since it is a strong nucleophile.

To account for the formation of ethyl propyl sulfide, which was found in significant quantities in the methylene chloride extracts, the mechanism proposed involved the formation of propyl thiolate ion which subsequently attacked intact ethoprop molecules. However, the possibility of an intramolecular reaction leading to the production of ethyl propyl sulfide was not entirely discounted. Greater emphasis on the former mechanism was considered because of the fact that the presence of other products of metabolism could be explained by reactions involving the propyl thiolate ion. Propyl disulfide was thought to be formed by the combination of two thiolate ions since thiolate ion was known to be strongly thiophilic. Further evidence pointing to the critical role of propyl thiolate ion was the detection of O-ethyl-S-propyl phosphorotioic acid and O-ethyl phosphoric acid as major components of the aqueous fraction of extracts from corn. The phosphoric acid was believed to be formed from the phosphorothioic acid by the release of propyl thiolate.

Figure I. Proposed Scheme of Metabolic Degradation of Ethoprop
in Bean and Corn Plants [2].
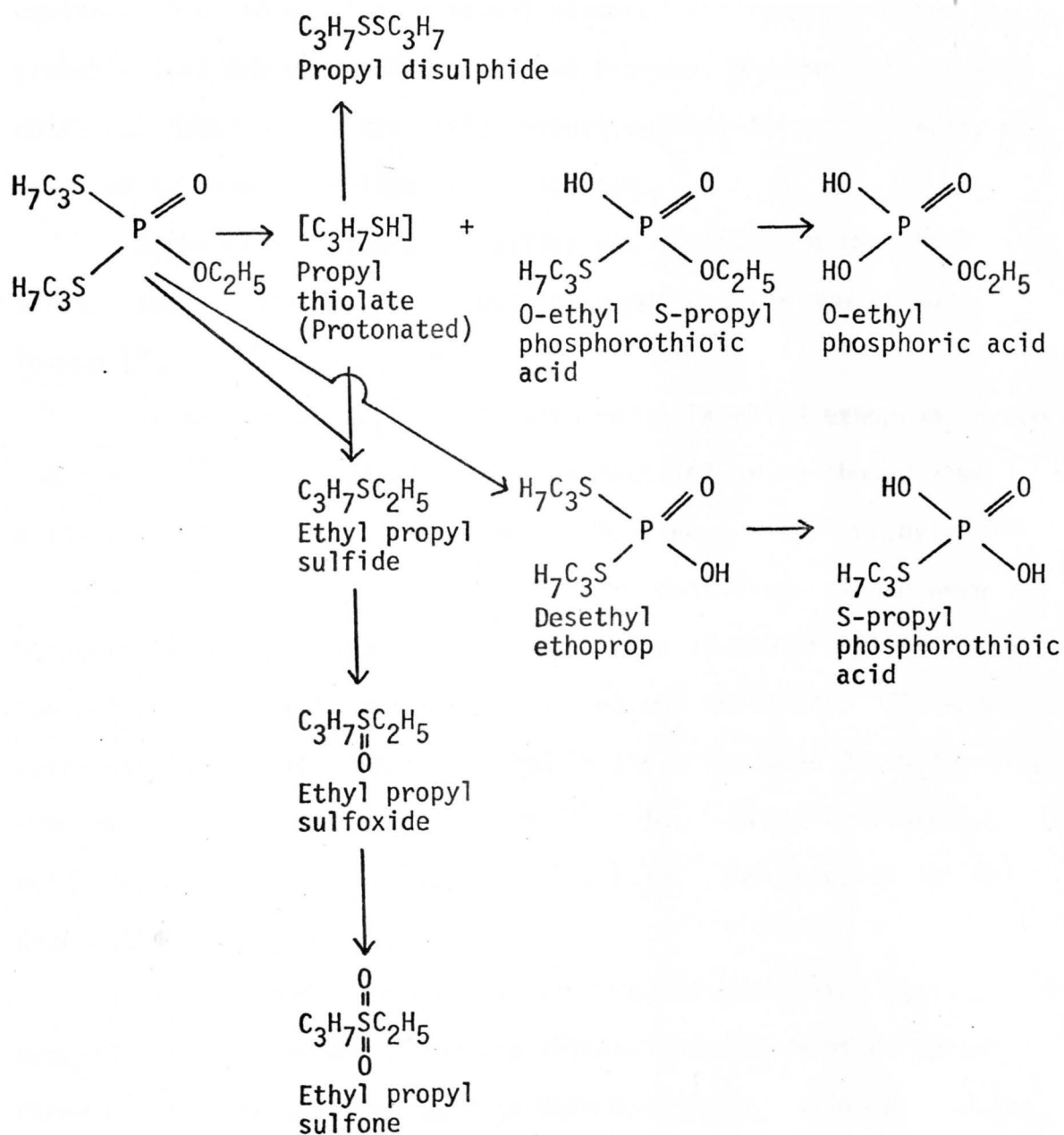
Since ethyl propyl sulfide was considered to be formed from the deethylation of ethoprop by propyl thiolate the presence of desethyl ethoprop was expected. However, this was not found. The explanation given was that desethyl ethoprop was very unstable; it probably lost propyl mercaptan to give S-propyl phosphorothioic acid which was found in plants. Ethyl propyl sulfide formed in plants was oxidized to form its sulfoxide and sulfone.

Although methyl propyl sulfide was detected in the plant extracts, investigations carried out could not resolve how it was formed [2].

In the animal experiments with radio labelled ethoprop, Menzer and Iqbal [3] found that chloroform extracts of urine showed some activity, with traces of methyl propyl sulfide. Methyl propyl sulfoxide and methyl propyl sulfone were also identified. These were believed to be the products of S-methylation of the propyl thiolate ion released from ethoprop and its subsequent oxidation. The major water-soluble metabolites identified in the urine were O-ethyl S-propyl phosphorothioic acid, O-ethyl phosphoric acid, S-propyl phosphorothioic acid and S,S-dipropyl phosphorodithioic acid. The proposed scheme of degradation is given in Figure II.

In the scheme, propyl thiolate ion was postulated again to be essential to the mechanism for the initial metabolism of ethoprop. Other metabolites were proposed to form after the initial production of the thiolate ion. The initial formation of propyl thiolate, as in plants, was supported by the detection of methyl propyl sulfide which

Figure II.   Proposed Scheme of Metabolic Degradation of Ethoprop

in Rats and in Rat and Rabbit Liver Microsomes and

Supernatant Preparations [3].
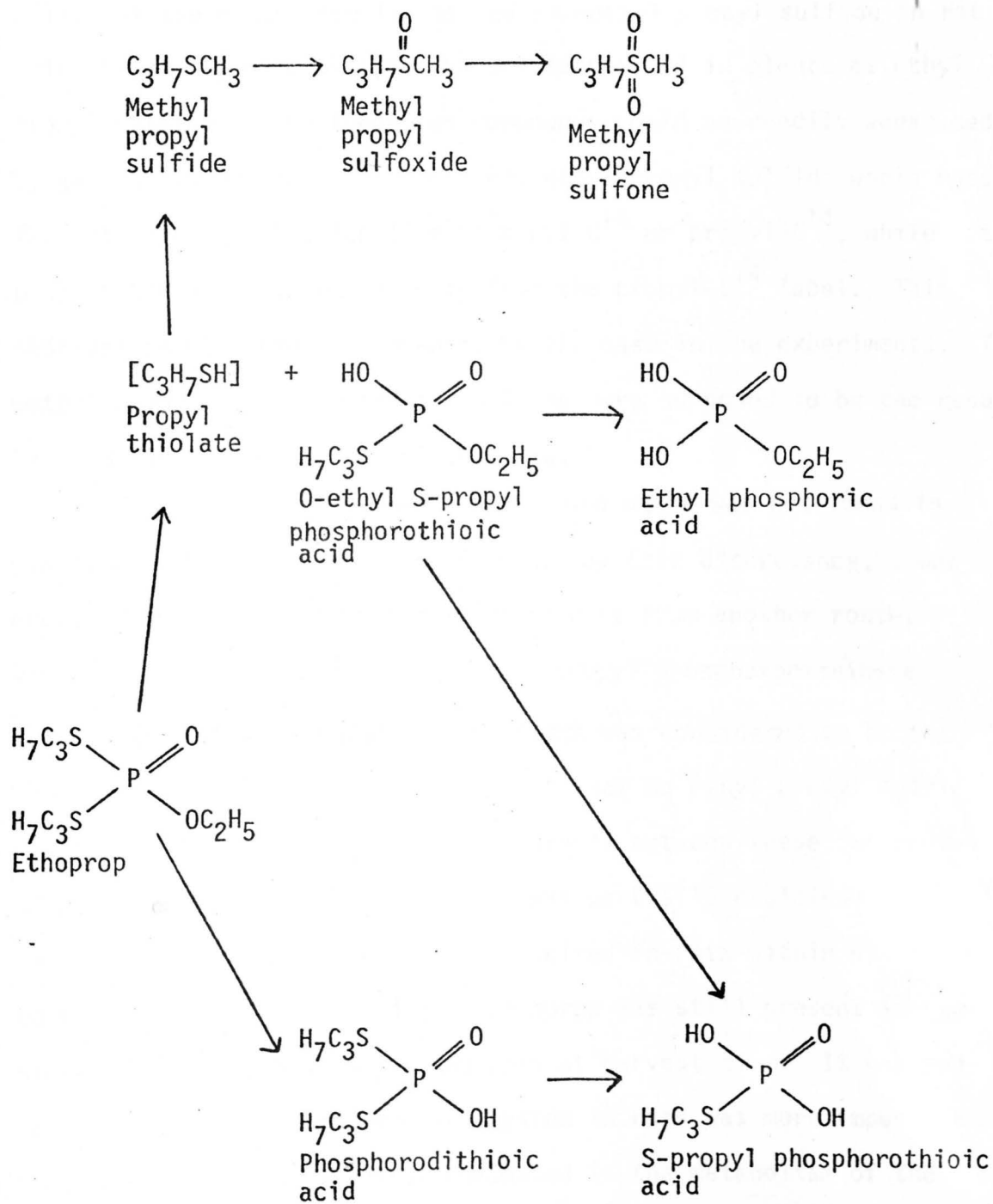
was believed to be formed by methylation of propyl thiolate by S-methyl transferase systems.

Iqbal et al [3] also pionted out the remoteness of the possibility of the metabolite identified as methyl propyl sulfide in rat urine being the same as the compound identified in plants as ethyl propyl sulfide, since these two compounds could be readily separated by gas chromatography. In addition, ethyl propyl sulfide would result from ethoprop whether labelled as ethyl-C^{14} or propyl-C^{14}, while methyl propyl sulfide would result only from the propyl-C^{14} label. This observation was actually found to be the case in the experiments. The methyl propyl sulfoxide and the sulfone were supposed to be the result of oxidation of methyl propyl thiolate.

S,S-dipropyl phosphorodithioic acid which was not found in plants was present in rats. To account for this discrepancy, it was proposed that this metabolite arose in rats from another route. Direct de-ethylation of O-ethyl-S,S-dipropyl phosphorodithioate not involving propyl thiolate ion-attack was considered to be the route. This was supported by the fact that no ethyl propyl sulfide was isolated in rat urine. The difference between these two routes of metabolism in plants and animals was partially explained by the fact that ethoprop was completely oxidized in rats within six hours. On the other hand, unmetabolized ethoprop was still present in signicant amounts in both beans and corn at harvest time. It was suggested that S-methyl transferase system in rats was more important than that in plants, and thus dominated in the metabolism of the

insecticide in rats. The system responsible for the formation of ethyl propyl sulfide in plants was considered to be relatively more important there. Further, it was thought that the propyl thiolate ion, if formed, was probably present for a very short time in rats before being methylated while in plants there was time enough for it to react in a number of other ways.

Finally, in in vitro experiments with rat liver microsomal enzymes, the latter were found to O-deethylate ethoprop in the presence of reduced glutathione. The glutathione acted as an ethyl group acceptor. This further lends credence to the above supposition that ethoprop metabolism in plants and animals may proceed by slightly different routes.

On the whole, the metabolism of ethoprop was considered to be quite rapid and resulted in only metabolites which were not expected to present any toxic hazard to man.

In this work the effect of ethoprop, for various lengths of time, on cholinesterase activity in rat, cow and human blood,under different temperature conditions is studied in detail. In the case of rats, in vivo time-inhibition relationship is also studied. A simple procedure for the extraction of ethoprop from blood and analysis of the extract is carried out. Attempts are made to analyze blood extracts of rats fed with ethoprop to determine levels of the insecticide at various times after administration of the insecticide. Analysis is by gas chromatography.

EXPERIMENTAL

A. Materials

1. Spraque Dawly white male rats weighing between 160g and 180g were kept in seperate cages and fed a regular diet of Lab Blox, manufactured by Allied Mils Inc., and water. The food and the water were replenished every two days.

2. Fresh heparinized blood of rats, cows and humans. The rat blood was taken by heart-puncture with a needle and syringe, the human blood from the arm. The cow blood was supplied by the Veterinary Department, South Dakota State University.

3. Set of syringes and needles.

4. Gas Chromatograph. Varian Aerograph Series 2100. Conditions: Column - length, 6ft.; inside diameter, 1/8 in.; packing, 1:1 15% QF-1 and 10% DC-200 on Chromosorb W; temperature, 200°C.
Carrier gas - Nitrogen; flow rate, 40-50ml/min.
Detector - Ni^{63} electron-capture; temperature, 300°C.
Injector - temperature, 240°C.

5. Recorder. Sargent Welch Model XKR; chart speed 1cm/min. span, 1mV.

6. pH meter. Corning Model 12 Research Meter.

7. Thermostatic Water Bath.

8. Vortex Mixer.

9. Centrifuge.

B. Reagents

All reagents were pesticide grade and obtained from Fischer

Scientific Company unless otherwise indicated. Reagents that had to be of a specific concentration were prepared and kept in a refrigerator.

1.   Ethoprop - 95% technical, was obtained from Mobil Chemicals, Insecticide Division. A stock solution containing 0.5g/100ml of ethoprop was prepared by suspending the insecticide in water with the aid of Tween-80.

2.   Ethoprop standard solution - A stock solution containing 0.1004g/100ml in hexane, approximately equal to 1000ppm was prepared and kept refrigerated. Working standards of 1ppm and 0.1ppm were also prepared. For the 1ppm solution 0.1ml of the stock solution was diluted with hexane to 100ml. One milliliter of this solution was diluted to 10ml with hexane to obtain the 0.1ppm standard solution.

3.   n-Hexane.

4.   Acetone.

5.   Acetone-Hexane mixture, 1:9 by volume.

6.   Heparin solution - 10mg/ml in distilled water.

7.   Sulfuric acid - 60% by volume.

8.   Saponin - 0.01g/ml solution in water.

9.   Acetylcholine - 0.2g/10ml in water, prepared at the time of use.

10.   Buffer solution - 0.02M sodium barbital (4.123g).

$$0.004M \ KH_2PO_4 \ (0.5446g).$$

$$0.60M \ KCl \ (44.730g).$$

For a liter of solution, reagents were dissolved in 900ml distilled water; 28.0ml of 0.01N HCl was added while shaking, and volume was

finally made up to the mark with distilled water.

C. Procedures

All the experiments were performed in the pesticide laboratory and the small animals lab of the Station Biochemistry Section of the Chemistry Department at South Dakota State University. The pesticide laboratory besides carrying out research work also performs routine analyses for pesticide residues in a variety of materials including plant and animal material, as a service to farmers and various agencies in and around Brookings, South Dakota. This service is being done under certification from the U.S. Environmental Protection Agency.

1. Extraction of ethoprop from rat blood incubated at 0°C, 37°C and room temperature for various lengths of time.

One milliliter samples of heparinized whole rat blood were fortified with 0.1ml of 0.05mg/ml aqueous ethoprop suspension. The treated smaples were incubated for 1, 6, 18, 24 or 168 hours respectively. All treatments were prepared and analyzed in triplicate. In addition, a control sample was prepared for each treatment condition. The control was identical to the treated samples except that no ethoprop was added.

Prior to extraction, the samples were treated with sulfuric acid to precipitate protein. Two successive 0.75ml portions of 60% H_2SO_4 were added to each sample. The sample was allowed to cool between additions, and prior to addition of a final 1ml aliquot of 60% H_2SO_4. The cooled samples were extracted with three 5ml aliquots of a 1:9 acetone-hexane mixture. The samples were shaken for 2-3

minutes for each extraction and the upper organic phase was removed after each extraction using a medicine dropper.

The lower aqueous phase was then centrifuged at 2000rpm for 5 minutes and the upper organic layer obtained was removed and added to the previous extracts. The combined extract for each sample was concentrated under a stream of nitrogen gas to 10ml. Five microliters of each sample was chromatographed on a gas chromatograph. Peaks obtained were compared to peaks from 5µl standard solution of 0.0005mg/ml ethoprop. The percentage of ethoprop extracted was calculated as follows:

$$\% \text{ Extracted} = \frac{\text{Sample peak height}}{\text{Standard peak height}} \times 100 \qquad (a)$$

2. Extraction of ethoprop from blood of rats treated with ethoprop.

Five rats with average weight of about 170g were fed with the ethoprop at a dose of 25mg/kg of body weight by means of a stomach tube. A sixth rat, not treated with ethoprop, was kept as a control. At time intervals of 1, 2, 4, 6, 8, 10, 12 and 24 hours, 1 ml blood samples were taken by heart puncture. The samples were extracted using the method described in 1. above. Five microliters of each 1ml concentrate of the final extract was chromatographed and the peaks obtained were compared to that of a standard.

In a second similar experiment, a second dose of ethoprop was given 24 hours after the first and blood samples were analyzed as above. Chromatographic peaks obtained were compared to that of a

standard ethoprop solution.

3. Extraction of ethoprop from urine of rats treated with ethoprop.

In addition to the analysis of rat blood for ethoprop after treatment, urine samples of three rats fed with ethoprop at a dose of 25mg/kg of body weight in an earlier experiment were collected at 0-12hr, 12-24hr and 24-48hr and analyzed. An extraction method described by Iqbal and Menzer [3] was used. Each urine sample was extracted three times with equal volumes of chloroform. The combined extracts from each sample was concentrated under vacuum in a rotary evaporator to 1ml. The samples were then subjected to a clean up procedure [25] on a 6% deactivated florisil column topped with a 1in. layer of anhydrous Na_2SO_4. The column was washed with 200ml of hexane prior to the introduction of the sample concentrates. Elution of the samples was performed with 250ml of 15% ethyl ether in petroleum ether, followed by 100ml of 50% ethyl ether in petroleum ether. The eluate collected for each sample was concentrated in the evaporator under vacuum to 3-5ml and then picked up in hexane to a volume of 10ml. Five microliters of this final extract was chromatographed.

The concentration of ethoprop in each sample from 2. and 3. was calculated using the formula below.

$$\text{Ng. ethoprop per 1ml of extract} = \frac{\text{Sample peak height} \times \mu\ell \text{ of std. injected} \times \mu\ell \text{ of extract}}{\text{Std. peak height} \times \mu\ell \text{ sample injected} \times \text{Ng of standard}}$$

(b)

4. Determination of cholinesterase activity in rat blood fortified with ethoprop.

One milliliter samples of rat blood were drawn by heart puncture and placed in tubes containing a drop of heparin solution. These samples were fortified with 0.1ml of 0.05mg/ml (equivalent to 0.005mg/ml) ethoprop. The samples run in duplicates for each time duration, were incubated at 37°C for 1, 6, 24 and 168 hours. Samples, in duplicate for each time duration, containing 1ml blood but no ethoprop were set up as controls. At the end of each time period, the blood samples were removed from the bath and cholinesterase activity determined using the method described below. Two other similar experiments as above were set up, only this time, one incubation was carried out in a refrigerator with a temperature of about 0°C and the other at room temperature. Again cholinesterase activity was determined for each blood sample at the end of the selected time periods.

A modification of Michel's [26] method was used in the determination of cholinesterase activity. The method is based on the change in pH due to the production of acetic acid from the hydrolysis of acetylcholine by the enzyme in the blood. The change was measured by using a pH meter capable of reading to the nearest 0.001 units.

The 1ml blood sample was mixed with 9ml of 0.01% saponin solution. Two milliliters of the resulting solution were transferred to a test tube containing 2ml of the barbital buffer and mixed on a Vortex mixer. Each mixture thus obtained was incubated for 10 minutes at 25°C. The pH of each solution was determined after careful calibration of

the pH meter, and the pH (designated as pH_1) was recorded. Then 0.4ml of acetylcholine solution was added to each tube and mixed. A time interval of one minute between additions to each successive tube was allowed to compensate for the time to be used to read the pH. The tubes were incubated for 1hr. at 25°C. At the end of one hour, the pH for each sample was determined (designated as (pH_2).

The activity of acetylcholinesterase, measured as ΔpH/hr. was calculated as follows:

$$\Delta pH/hr. = (pH_1 - pH_2) - b \qquad\qquad (c)$$

where pH_1 and pH_2 are the initial and final pH's respectively, and b is a correction factor corresponding to non-enzymatic hydrolysis of substrate. b was determined by carrying out an identical choline-esterase determination as above but substituting 1ml of buffer for the blood, $b = (pH_1 - pH_2)$ for this blank sample.

The percentage inhibition of cholesterase by ethoprop was calculated using the equation below:

$$\% \text{ Inhibition} = \frac{\Delta pH/hr. \text{ control} - \Delta pH/hr. \text{ test}}{\Delta pH/hr. \text{ control}} \times 100 \qquad (d)$$

where ΔpH/hr. control is the activity of the enzyme in the absence of ethoprop and ΔpH/hr. test is the activity in the presence of ethoprop. The ΔpH control was determined in the same manner using blood known to be free from cholinesterase inhibition.

5. Determination of blood cholinesterase activity in rats treated with ethoprop.

Blood samples of rats that had been fed with ethoprop as in 2. at a dose of 25mg/kg body weight, were withdrawn by heart pucture at various time intervals over a two week period after the treatment. Cholinesterase activity was determined using the procedure described in 4. above. The percent inhibition of cholinesterase by ethoprop was calculated for each sample using equations (c) and (d).

6. Determination of blood cholinesterase acticity of rats fed with various concentrations of ethoprop.

The procedure used was as described in 4. or 5. above, except that 4 rats with weights ranging from 250g to 255g were fed with ethoprop by means of a stomach tube, at doses ranging from 20mg/kg to 35mg/kg body weight. Cholinesterase activity was determined in blood samples from each rat 2hr. and 6hr. after administration of the insecticide. The percent inhibition was calculated using equations (c) and (d).

7. Determination of cholinesterase activity in human and bovine blood fortified with ethoprop.

After the determination of cholinesterase activity in rat blood, the effect of ethoprop on human and bovine blood cholinesterase was studied. Conditions similar to the _in vitro_ experiments with rat blood were used. The procedure used was exactly as in 4. above, except that samples of human and bovine blood were used.

RESULTS AND DISCUSSION

A. Extraction and Analysis of Blood Containing Ethoprop

All analyses were performed on a Varian Aerograph Series 2100 gas chromatograph with an attached Sargent-Welch Model XKR recorder. Chromatograph and recorder conditions were as given under the "Materials" section of "Experimental". A typical chromatogram of an ethoprop standard and a sample incubated at room temperature for 1hr is shown in Figure III.

Retention time of the sample peak is 2.4min (chart speed, 1cm/min) which is identical with that of the standard The background noise was quite low and thus distinct peaks could be obtained for ethoprop. The peak height was measured from the base of the peak.

The result of ethoprop extraction from rat blood fortified with the insecticide is shown in Table I.

The overall extraction procedure, as evidenced by the results in Table I, was very efficient. As would be expected, for longer time periods and the higher the temperature, the lower the percentage extraction. This may be due to the degradation of the insecticide by enzyme systems in the blood as well as by thermal decomposition. These two modes of loss of ethoprop were obviously not very significant considering the high yields obtained. Even over a period of 7 days, recovery from the 37°C and room temperature samples were considerable. This latter observation was not surprising since ethoprop is thermally stable even at 50°C for at least 12 weeks [1]. Thus, not much decomposition of the insecticide occurred over the time period of the study that can be attributed to the effect of temperature

Figure III. Chromatogram of Ethoprop Standard and Extract of Rat
Blood Fortified with 0.1ml of 0.05mg/ml of Ethoprop
and Incubated at Room Temperature for 1 Hour.

5µℓ blood extract - 0.1ml of 0.05mg/ml spike - 1hr. after incubation at room temperature. Total extract volume = 1ml

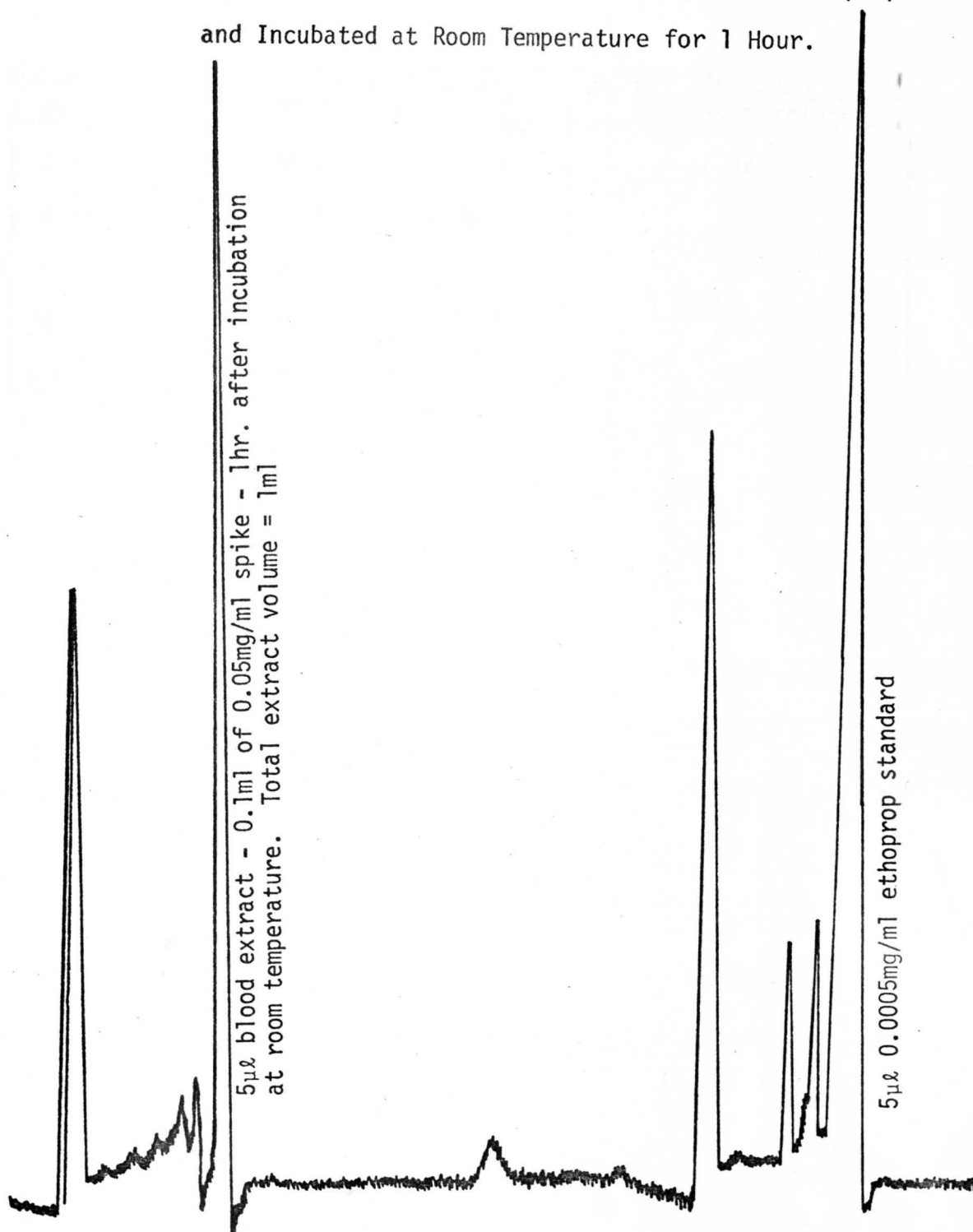5µℓ 0.0005mg/ml ethoprop standard

Table I. Average Percentage of Ethoprop Extracted from Rat Blood
Fortified with the Insecticide.

Incubation Period	Percent Ethoprop Extracted (Average)		
	0°C	37°C	Room Temperature
1 hr.	95.8	93.3	82.2
6 hr.	87.5	86.5	82.9
18 hr.	87.5	77.4	79.8
24 hr.	86.2	73.3	77.6
7 days	85.0	60.8	73.8

under the conditions of the experiment. It may therefore be suggested that the differences in the amount of insecticide extracted among the three temperatures were mainly due to differences in enzyme action at varying temperatures.

Within each temperature group, except for the 0°C ones, the decrease in the percent extracted could be attributed mainly to enzymatic action, with some minor contribution from thermal breakdown as time went on. Here too the overall reduction in the amount of insecticide extracted was not very large. In the case of the 0°C samples, apart from the initial fall within the first six hours, the percentage of insecticide extracted was constant with time. This observation results from the decreased enzymatic activity and rate of decomposition reactions at low temperatures.

In the feeding experiments with rats no peak corresponding to ethoprop was detected in the analysis of blood samples taken after the initial dose. However, ethoprop peaks were obtained for samples from rats fed a second dose of ethoprop 24hrs. after the first dose. The results are shown in Table II.

The results show that relatively small amounts of ethoprop were extractable, mainly in the 30ng levels. In all five rats treated ethoprop levels in the blood rose in the first two to four hours after the administration of a second dose and then fell to virtually a constant level over the period (24 hours) of the experiment. However, in rat #2, levels detected remained high compared to the others, and rose unexpectedly to 4.9×10^4ng and 8.7×10^3ng over a

Table II.   Amounts of Ethoprop Extracted from Blood of Rats Treated
with Ethoprop.

Time (hr.) After 2nd Dose	Ng. Ethoprop Extracted per ml of Blood				
	Rat #1	Rat #2	Rat #3	Rat #4	Rat #5
1	31.4	70.5	33.3	30.9	56.4
2	49.9	80.2	46.7	40.9	73.8
4	57.4	58.1	*	31.8	30.9
6	33.3	40.7	48.3	*	42.9
8	34.1	4.90×10^4	36.7	38.3	33.3
10	36.2	8.7×10^3	37.2	*	25.7
12	*	-	37.0	23.8	23.8
24	*	-	40.0	20.0	23.8

* peak corresponding to ethoprop was not observed.

- samples could not be obtained because the animal died.

period of 8-10hrs. respectively. It was not surprising, then, that this particular rat did not survive after 10hr. The reasons why this rat showed this kind of behavior could not be explained satisfactorily. It may be due to the inability of this rat to metabolize the insecticide rapidly as the others. Ethoprop in the blood thus rose to the high levels detected. Contamination of food and water could be ruled out since particular efforts were made to ensure that this sort of thing would not occur. Ethoprop peaks corresponding to 12 and 24 hours in rat #1, 4 hours in rat #3 and 6 hours in rate #4 were not observed. The reason for this could not be explained since similar peaks showed up in the other rats. On the whole it appeared that ethoprop persisted for some time (24 hrs after the administration of a second dose) in the blood of the surviving rats. However, the low concentrations detected in the blood of these surviving rats indicated that such levels of ethoprop posed no visible harmful effects on them. This was borne out by the fact that none of these rats showed any visible sign of ill-health, and they continued to live even after the end of the experiments.

The detection of ethoprop in rat blood even after six hours of administration may seem to contradict the findings of Menzer and Iqbal [3] that ethoprop was completely metabolized in six hours. But it should be emphasized that in this study ethoprop could not be detected after an initial dose; it was only after giving the rats a second dose 24 hours after the first that ethoprop could be detected in the blood. The non-detection of ethoprop after an initial dose would

not be surprising, if the insecticide was metabolized completely within six hours as expressed by Menzer and Iqbal. Menzer and Iqbal administered only one dose, and also used isotopic methods, which may be more sensitive than the method used in this study. The persistence of the insecticide in the blood as observed in this study may be due to the fact that ethoprop might have been absorbed slowly in the alimentary canal into the blood, and coupled with a second booster dose, concentrations rose to levels that could easily be detected by the method used. On the other hand, levels of ethoprop in blood after a single dose might have been too low to be detected especially if metabolism was so rapid as reported by Menzer and Iqbal.

In the analysis of urine extracts, no ethoprop was detected. This was in consonance with findings of Iqbal and Menzer [3]. In their study with radiolabelled ethoprop they did not detect any ethoprop in the urine extracts. Only metabolites of ethoprop were observed and these were identified to be methyl propyl sulfide, methyl propyl sulfoxide and methyl propyl sulfone. The last two metabolites were believed to be oxidation products of methyl propyl sulfide.

B. Cholinesterase Inhibition Studies

Results of the in vitro studies with rat, bovine and human blood are presented in Tables III, IV, and V respectively.

In all the in vitro studies, it could be observed that inhibition at 0°C was high initially, and then dropped off or became nearly stable, irrespective of the source of the enzyme. This initial high inhibition was unexpected, especially for the rat and cow blood

Table III. Average Percent Inhibition of Cholinesterase by Ethoprop
in Rat Blood after Various Incubation Periods at 0°C,
37°C and Room Temperature.

Incubation Period	Percent Inhibition (Average)		
	0°C	37°C	Room Temperature
1 hr.	40.5	36.6	6.9
6 hr.	30.2	55.5	14.3
24 hr.	22.6	90.8	36.9 (18 hr.)
7 days	12.7	90.6	69.4

Table IV. Average Percent Inhibition of Bovine Blood Cholinesterase
Activity by Ethoprop after Various Incubation Periods at
0°C, 37°C and Room Temperature.

Incubation Period	Percent Inhibition (Average)		
	0°C	37°C	Room Temperature
1 hr.	16.5	18.2	7.3
6 hr.	8.6	22.0	16.4
24 hr.	7.3	78.6	56.2
7 days	6.7	96.6	87.1

Table V. Average Percent Inhibition of Human Blood Cholinesterase
Activity by Ethoprop after Various Incubation Periods at
0°C, 37°C and Room Temperature.

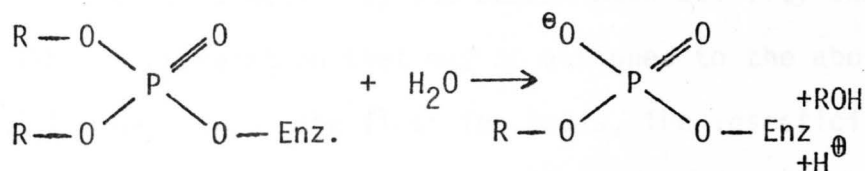| Incubation Period	Percent Inhibition (Average)		
	0°C	37°C	Room Temperature
1 hr.	12.7	16.3	15.2
6 hr.	6.8	25.4	17.1
24 hr.	5.4	56.7	30.9
7 days	4.1	87.8	76.3

where the 0°C values were higher than those at room temperature. No satisfactory explanation could be given for this. As time progressed, inhibition became less and less which would suggest that the enzyme was somehow protected from the inhibitor at low temperature.

In all the three cases (i.e., rat, cow and human) studied, inhibition increased at high temperatures with time. This may be due to increased interaction of ethoprop with cholinesterase which may be supported by the observation that as contact time between insecticide and enzyme increased, inhibition became more and more pronounced, rising to 90% or near 90% in rat, cow and human blood. The above observations were in conformity with the observation that organophosphate-cholinesterase complex formation was progressive and temperature dependent [24]. Schematic representation of the mechanism of reaction of organophosphate with acetylcholine esterase is given below. It is believed that complex formation involves hydroxyl residue of serine and some acid and basic side chains of some amino acid residues in the enzyme molecule [24].
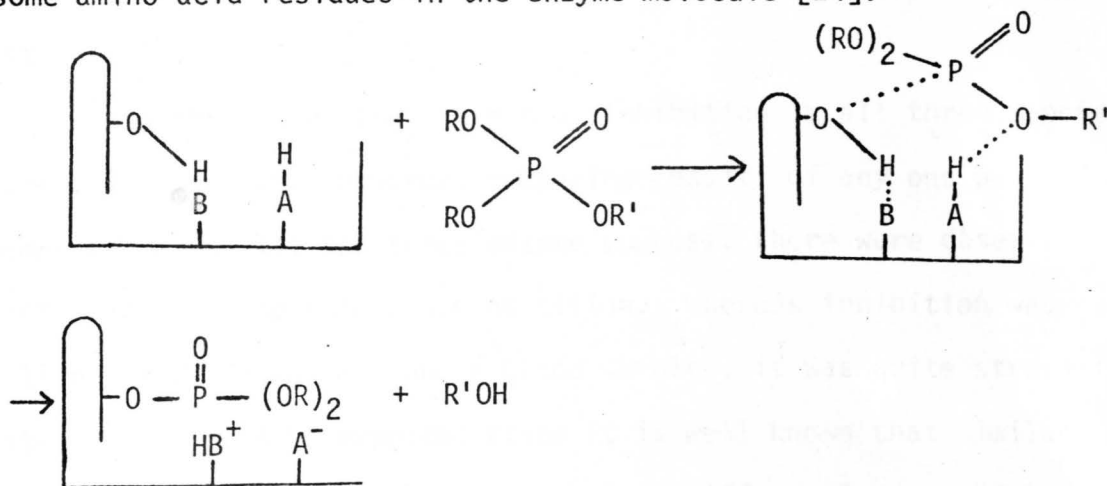
The insecticide first forms an intermediate complex with

these side groups and then there is breakdown of this complex to give a phosphorylated enzyme which is inactive. It is believed that in this inactive form, with some organophosphorus insecticides, the enzyme can be reactivated by treatment with nucleophilic agents like certain oximes. However, with time the inhibited cholinesterases change gradually into a non-reactivatable form, a process referred to as aging. The aging process is generally accepted to be due to de-alkylation of the dialkoxyphosphinyl enzyme as illustrated below:

$$R-O \underset{R-O}{\overset{\displaystyle O}{\diagdown}}\!\!P\!\!\overset{\displaystyle O}{\diagup} O-Enz. \;+\; H_2O \longrightarrow \;\; {}^{\ominus}O\underset{R-O}{\overset{\displaystyle O}{\diagdown}}\!\!P\!\!\overset{\displaystyle O}{\diagup} O-Enz \quad \begin{array}{l}+ROH\\ {}^{\oplus}\\ +H^{\oplus}\end{array}$$

After the dealkylation, the phosphorylated enzyme becomes stable and resists attack by reactivating agents [24]. It would not therefore be surprising that ethoprop inhibition of blood cholinesterase increased with time in all three animal blood samples studied. It was, however, surprising that the inhibition at room temperature for cow and rat blood were initially quite low, an observation we could not explain.

On the whole, the pattern of inhibition in all three species were quite similar. However, comparing results of any one particular temperature for all the three enzyme sources, there were observed differences in the extent of inhibition. Whereas inhibition was relatively weak in cow and human blood samples, it was quite strong in rats. This would be expected since it is well known that similar enzymes from different sources may behave differently towards the

same external factors such as inhibitors. Even enzymes in the same organism but from different tissues are known to exhibit different characteristics.

In the in vivo experiments with rats, the results (Tables VII, VIII, IX) indicated a rapid increase in inhibition in the first few hours following insecticide administration, reaching a maximum of 97.6% in six hours. From there it fell off gradually, reaching 8.3% after a week. By the second week activity became normal. The interpretation that may be assigned to the above observation is that during the first few hours, the insecticide was being absorbed into the blood stream and the detoxication had not set in yet, or even if it had, it had not reached the maximum capacity yet. Thus ethoprop could exhibit its inhibitory effect to the maximum within those initial hours. As time went on, detoxication became very significant and thus the inhibition decreased. Incidentally, Iqbal and Menzer [3] reported that rats metabolized ethoprop completely within six hours which is coincident with the time of maximum inhibition in this study.

Despite the fact that ethoprop may be metabolized completely within six hours, results in Table VII, indicated that the effect of ethoprop on blood cholinesterase of rats was quite appreciable, and that the effect persisted for a considerable length of time. This may mean that even though the insecticide may not be present after the first six hours, it took some time for the enzymes to be regenerated in the blood back to the normal level. This is not

Table VII. Average Percent Inhibition of Blood Cholinesterase in
Rats Treated with Ethoprop.

Time (hrs.)	Percent Inhibition (Average)
1	77.8
2	88.2
6	97.6
12	88.4
24	81.4
30	78.6
48	75.0
96	57.0
120	51.4
144	39.2
168	8.3
336	0.0

Table VIII.   Average Percent Inhibition of Blood Cholinesterase in
Rats fed with Various Doses of Ethoprop - 2hrs. after
Dosing.

Dosage Mg ethoprop/kg body weight	Average Percent Inhibition
20	49.1
25	64.3
30	80.0
35	74.4

Table IX.   Average Percent Inhibition of Blood Cholinesterase in
Rats Fed Various Doses of Ethoprop - 6hrs. after
Dosing.

Dosage Mg. ethoprop/kg body weight	Average Percent Inhibition
20	95.2
25	92.8
30	97.8
35	89.7

surprising since most organophosphorus insecticides are known to be irreversible inhibitors, and therefore until ethoprop was detoxicated, the enzyme would be inactive. Even though the inhibitory effect of ethoprop remained for quite a while, the rats did not show any visible harmful effects, except for the initial restlessness and erratic movement immediately after the administration of the insecticide which may be attributed mainly to the initial wearing off of the anesthetic effect of the ether used.

Tables VIII and IX contain data giving the percent inhibition of blood cholinesterase of rats fed with various doses of ethoprop and sampled at two and six hours after dosing. Except for the initial increase in inhibition with increased dosage in the two hour run, it could be said that there was very little correlation between the dosage and the extent of inhibition, notably for the six hour run. Attempts to use higher doses than 35mg/kg were not successful because of their lethal effects.

CONCLUSION

From the results of this study, the following conclusions can be made. The method of extraction using 1:9 acetone-hexane mixture was efficient and time did not affect the in vitro levels of ethoprop in blood of rats greatly. Ethoprop could not be determined in blood and urine of rats after administration of a single dose. A second dose of the same concentration twenty-four hours after the first however, resulted in detectable amounts in the blood. This may be due to the reduced ability of the rats to detoxify ethoprop with the administration of the second dose. Even though the levels of ethoprop detected were very low, it was quite important that these low levels in the blood could be detected by the method used.

The study also indicated that blood cholinesterase of rats, cows, and humans was inhibited in vitro by ethoprop, and that in in vivo conditions inhibition persisted for as long as one week in the case of rats, after a single dose of ethoprop. Whether ethoprop also binds to receptor sites of post-synaptic nerves was not investigated.

REFERENCES

1. Mobil Oil Corporation, Technical Bulletin - 4/15/80.

2. R.E. Menzer, Z.M. Iqbal and G.R. Boyd, J. Agric. Food Chem., 19, 351 (1971).

3. Z.M. Iqbal, R.E. Menzer, Biochem. Pharmacol., 21, 1569 (1972).

4. J.A. Bunt, "Effect and Mode of Action of the Nematicide Ethoprophos," Med. Fac. Landbouw Rijksuniv. Gent., 44/1, 1979.

5. C.M. Heald, R.L. Self, Plant Dis. Rep., 51, 1035 (1967).

6. S.H. Kerr, J. Econ. Entomol., 61, 523 (1968).

7. J.H. O'Bannon, A.L. Taylor, Plant Dis. Rep., 51, 995 (1967).

8. A.L. Taylor, J.H. O'Bannon, Plant Dis. Rep., 52, 218 (1968).

9. W. Birchfield, Plant Dis. Rep., 52, 786 (1968).

10. W.J. Mistric, Jr., F.D. Smith, J. Econ. Entomol., 62, 712 (1969).

11. W. Birchfield, W.J. Martin, Plant Dis. Rep., 52, 127 (1968).

12. H.L. Rhoades, Plant Dis. Rep., 53, 728 (1969).

13. A.J. Huvor, Mobil Chemical Co. Report, (1967).

14. J.H. Smelt, M. Leistra, S. Voerman, Pest. Sci., 8(2), 147 (1977).

15. N. Ahmad, D.D. Walgenback, G.R. Sulter, Bull. Enviro. Contam., (1980).

16. M. Leistra, Soil Sci., 128, 303 (1979).

17. B.B. Brodie, J. Nematol., 3, 292 (1971).

18. W.A. Rohde, et al, USDA & Ed. Adm. ARR-S-2 (1979); P.B. Udy, Proc. 28th, N.Z. Weed & Pest Control Confab., 115-8 (1975).

19. A.J. Overman, Soil & Crop Sci. Soc. of Fla., Proceedings, 34, 197 (1974).

20. R.J. Argauer, J. Feldmaster, J. Agr. Food Chem., 26 (1), 42 (1978).

21. S.R.B. Solly, N.Z.J. Agric. Res., 15 (1): 83-90 (1972).

22. Health Hazard Evaluation Determination Report: No. HE 79-108-647, Mobil Chem. Co., Mt. Pleasant, Tenn; U.S.NTIS, PB80-163; 306; 17 pp - 1979 by Hazard Evaluation and Tech. Asst. Branch, NIOSH, Cinn., OH.

23. E. Epstein, B. Bravado, Phytopathology, 63 (ii): 1411 (1973).

24. M. Eto, "Organophosphorus Pesticides: Organic Acid Biological Chemistry," CRC Press (1974).

25. U.S. Dept. Health, Ed. & Welfare, Food and Drug Administration, Pesticide Analytical Manual, 1, Section 211, 14a.

26. H.O. Michel, J. Lab. Clin. Med., 34, 1564 (1949).