FOUR RESIDUAL PRODUCTS OF ACEPHATE : THEIR FORMATION IN WATER AND IN MICE, AND THEIR ANTI-ACETYLCHOLINESTERASE ACTIVITIES IN-VITRO

by

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Four Residual Products of Acephate: Their formation in Water and in Mice, and their anti-acetylcholinesterase activities in vitro.

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1. ABSTRACT

Previous studies on the residual profile of acephate have centred mostly on the formation of methamidophos only. This study was undertaken to determine the presence of three other residual products, in addition to methamidophos, in water and livers of mice and to test their toxicological significance through their reaction with RBC AChE of mice, in vitro .

Acephate (O,S-dimethylacetyl phosphoramidothiolate) was incubated in glass-stoppered Erlenmeyer flasks for 7 days at 37 C and pH of 7.2, 8.1 and 8.6 respectively. The hydrolytic products detected between 0.5 and 7 days were : O,S-dimethyl phosphoramidothiolate (II), O,S-dimethyl phosphorothiolate (III) and S-methylacetyl phosphoramidothiolate (V).

The concentration of acephate dropped by 51% after 7 days at pH 7.2. This was accompanied by a corresponding formation of (II), (III) and (V) which accounted for 2%, 27% and 10% of the original acephate respectively, after 7 days.

At pH 8.1, acephate concentration decreased by 55% after 7 days. This was accompanied by a corresponding presence of (II), (III) and (V) accounting for 4%, 23% and 6% respectively of the initial acephate after 7 days.

At pH 8.6, the same downward trend continues with acephate concentration dropping by 64% after 7 days. Accompanying this drop, (II), (III) and (V) rose to 3%, 23% and 4% of the initial acephate.

Acephate was also administered to mature male mice by stomach tubing. The following metabolites were found in the livers from 0.5 to 30 hours after treatments : (II), (III) and O-methylacetyl phosphoramidothiolate (IV)

Within 0.5 hours after acephate administration, the liver concentration of (II) rose to about 1% of the applied acephate. After 30 hours, it had gone down to less than 0.1%. (III) was detected in the livers of treated mice 7 hours after acephate administration at a concentration of 0.05% of administered acephate. After 24 hours, it was non-detectable.

(IV),undetected in the pH studies, was present in the livers of treated mice 4 and 7 hours after acephate administration, but it could not be detected after 24 and 30 hours.

The distribution of acephate in mice was studied using ^{14}C acephate. At 0.5 hours after the administration of ^{14}C acephate, 60% of the applied radioactivity was detected in the intestinal tract. After 7 hours, the level had gone down to 7%. Coupled to this decrease, the radioactivity present in the rest of the animal (carcass) increased from 15% to 62% between 0.5 and 7 hours respectively.

Of the four residual products of acephate detected in this study, only (II), (I_{50} of 5×10^{-5}M) exhibited anti-acetylcholinesterase activity greater than acephate (I_{50} of 5×10^{-4} M). (III), (IV) and (V) did not inhibit acetylcholinesterase at concentrations of up to 0.26 M.

Therefore considerations of terminal residues in relation to the insecticidal action of acephate should devolve on the parent compound itself, and (II).

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

2. INTRODUCTION

Acephate (Fig. 1-I) is an organophosphorus insecticide (OP) with several excellent residual properties (Magee 1974). A comprehensive account of its genesis as a commercial insecticide would not be complete without reference to methamidophos (Fig.1-II).

Methamidophos was first synthesized independently by Lorenz at Bayer in 1964 and by Magee at Chevron Chemical in 1967. The insecticide is marketed under the trade name of Monitor^{R} by the Chevron Chemical Company and the Chemagro division of Mobay Chemical Corporation in the United States. In Europe, it was given the trade name Tamaron^{R} by Farbenfabriken Bayer (Magee 1974). Although an excellent broad-spectrum insecticide, it is of high mammalian toxicity (oral LD_{50}, rat : 20mg/kg; dermal LD_{50}, rabbit : 118mg/kg). Attempts to combine its desirable insecticidal activity with a low mammalian toxicity led to the synthesis of acephate by the N-acetylation of methamidophos in 1968 (Magee 1974). Acephate has been marketed since 1972 by Chevron under the trade name Orthene^{R}.

Unlike methamidophos, acephate combines low acute mammalian toxicity with insecticidal properties comparable with those of methamidophos (oral LD_{50}, rat : 945mg/kg; mouse : 361 mg/kg; LC_{50},96 h, bluegill: 2050ppm; dermal LD_{50}, rabbit > 2,000 mg/kg

(Spencer 1982)).

Acephate has been used in controlling forest and agricultural insect pests.

Bioassays have shown that it is highly toxic (LD_{50} :28.6 ug/g) to the first instar larvae of Mexican bean beetle Epilachna varivestis after application with a Potter spray tower. This renders it more toxic than malathion (LD_{50} : 36.5ug/g), one of the recommended insecticides for control of these beetles,and suggests a potential use for acephate in such situations (McClanahan,1981).

At the rate of 57g/ha,acephate was effective in controlling the climbing cutworm Agrotis badinodis, an impor tant pest in grape growing vineyards (Marmor et al.,1981) ,and the pale western cutworm Agrotis orthogonia, an important pest on cereal crops when applied at the rate of 56g/ha (DePew,1980).

Acephate is rapidly absorbed and translocated and has a short residual life in pine seedlings.Pales weevils, Hylobius pales,feeding on loblolly pine seedlings, are controlled effectively following root treatment with acephate (LD_{50} : 1.96ug/g), indicating that it is systemic(Werner,1974).

It has been effective in controlling the Douglas-fir tussock moth Orgyia pseudotsugata in British Columbia when applied by fixed-wing aircraft at a rate of 1.12 Kg/ha (Hard,1978). Its activity has been found to be equal to that of fenitrothion and better than that of Phoxim^{R} in the control of the western spruce budworm, Choristoneura fumiferana (Nigam et

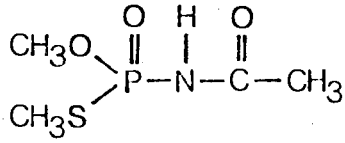
Figure 1 : Acephate and Four Major Metabolites --

(II) O,S-dimethyl phosphoramidothiolate.

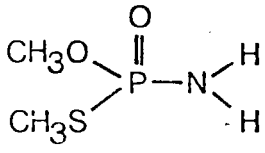
(III) O,S-dimethyl phosphorothiolate.

(IV) S-methyl acetyl phosphoramidothiolate.

(V) O-methyl acetyl phosphoramidothiolate.



ACEPHATE. (I)



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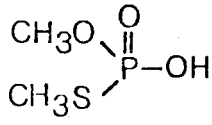

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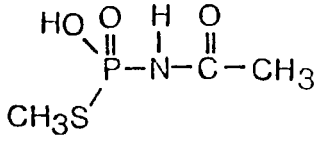

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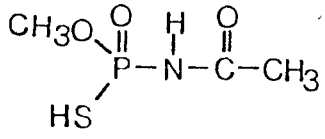


(V)

al.,1973).

Acephate has also been used successfully against other forest insects,such as the gypsy moth, Lymantria dispar,the elm spanworm,Ennomos subsignarius,the elm leaf beetle,Pyrrhalta luteola,and a looper,Lambdina fiscellaria fiscellaria (Neisess et al.,1976).

Despite its good efficacy in controlling many insect pests acephate has only minimal effects on non-target components of the ecosystem. No serious adverse effects have been detected on aquatic insects, bird populations and small mammals as a result of operational sprays of acephate (Shea,1978). Similar results have been obtained when soil microorganisms are monitored after soil treatment with acephate (Focht et al.,1974).

Despite such evident selectivity,details of its mode of action still remain uncertain.

In resistance,rapid inactivation,through transformation of insecticides,in vivo by insects is one of the translational results of gene amplification and consequent insecticide resistance (Hodgoson et al.,1974; Long and Dawid, 1980; Miyata and Hayashida, 1981; Plapp, 1976; Schimke, 1982; Schimke, 1980; Suksayretrup and Plapp, 1977; Yu and Terriere,1979). In insects,these degradative reactions for OP's,are catalysed by a vast array of enzymes which include NADPH-dependent microsomal oxidases (MFO), glutathionedependent (GSH) alkyl and aryl transferases, aliesterases and cholinesterases (ChE) (Nakatsugawa and Dahm, 1965; Nakatsugawa and Morelli, 1976;

Suksayretrup and Plapp, 1977). Conversely,it is logical to expect that a poor conformational fit between an insecticide and xenometabolic enzymes can be the basis of insecticide susceptibility.

Compared to most OP's,acephate is a weak inhibitor of housefly acetylcholinesterase (AChE) in vitro O'Brien, 1967; Rojakovick and March, 1972). Nevertheless,it possesses lethal inhibitory properties in vivo ,suggesting that it is enzymatically activated to biologically more active chemical species(Rojakovick and March, 1972). But in vitro,acephate is also a poor substrate for the common biochemically critical xenometabolic enzymes,including the NADPH-dependent parathion oxidase, GSH-dependent alkyl transferase and aliesterase;a property shared by methamidophos (Suksayretrup and Plapp, 1977).

Acephate may therefore exert its high insecticidal activity through chemical stability or resistance to detoxifying enzyme-mediated transformations and, consequently,remain active for longer periods.

In general,inhibition of AChE is cited as the primary biochemical lesion responsible for the toxicity of OP insecticides in vertebrates. In insects too,inhibition of AChE does occur,but the chain of events leading to death has not been established (O'Brien, 1967; Pichon, 1974). Despite this inadequacy in the understanding of OP toxicology in insects,a few empirical relationships are valid.

Firstly,the anti-AChE activity of OP's in vitro tends to be proportional to their insecticidal activity (O'Brien,1967).

Secondly,a direct relationship exists between the ease of alkaline hydrolysis of OP's,estimated by their Hammett's σ constants,and their anti-AChE activity. The more stable an OP is to alkaline hydrolysis,the lower is its σ value which in turn implies a low positive charge on the phosphorus atom as well as a low anti-AChE activity (Aldridge and Davison, 1952; Fukuto, 1976; Fukuto and Metcalf, 1956; Metcalf and Fukuto, 1964; Pullman and Valdemoro, 1960). However,such interrelations between OP toxicity, ease of alkaline hydrolysis,magnitude of Hammett's σ values and AChE inhibition do not always hold. Some factors,such as the differing rates of cuticular penetration,detoxification and transport to the site of action have been shown to account for such situations (Murdock and Hopkins,1968). According to O'Brien (1967),OP's with I_{50} of greater than 10^{-4}M, under specified conditions, are broadly classified as weak AChE inhibitors. These critical conditions include the specific activity of the catalytic protein preparation,the concentration of AChE in relation to that of its substrate, duration of incubation of enzyme with inhibitor and the temperature condition existing during the assays. As a term denoting the molar concentration of inhibitor at which 50% of total enzyme activity is lost,I_{50} is biologically,statistically,and kinetically complex and subject to wide numerical variations (Dixon and Webb, 1964; Fest and

Schmidt, 1973; Witter, 1963). It can nevertheless be a useful index in roughly categorizing the inhibitory potencies of OP's on AChE,provided the experimental conditions, under which it is determined,are specified.

Acephate combines weak *in vitro* inhibition of housefly AChE (I_{50} > 10^{-2}M) with high toxicity (LD_{50} 1.8 ug/g) when applied topically to the abdomen of these insects (Rojakovick and March,1972; Hussain and Oloffs,1981). Such discrepancy between *in-vitro* and *in-vivo* activities suggests that acephate undergoes metabolic activation in susceptible organisms. The long delay between exposure to acephate and the onset of toxic symptoms in susceptible insects, lends some credence to this line of thinking. Thus knock-down takes 10 hours to occur in houseflies treated at the LD_{90} level as compared with 4 hours for those treated with methamidophos at comparable dosages (Rojakovick and March,1972;Khasawinah *et al.*, 1978).

Aging of ACHE has also been postulated to explain, in part,the high insecticidal efficacy of acephate (Quistad *et al.*,1970). This irreversible inhibition of AChE,coupled with its stability *in vivo* could compensate,in part, for its weak anti-AChE properties. In some susceptible organisms,conversion to methamidophos is the major first step in acephate metabolism (Bull,1979;Hussain and Oloffs,1981;Kao and Fukuto,1977; Szeto,1978). This reaction has been considered by some authors to be the metabolic activation accounting for the high insecticidal activity of acephate. This applies, especially when

the low affinity of methamidophos for some insects' xenometabolic enzymes, and its higher activity relative to acephate,are considered (Khasawinah et al.,1978; Rojakovick and March,1972).

But Eto et al.,(1977) have postulated that a chemical species metabolically formed from methamidophos is the active principle of the latter and acephate. The assumed active principle,the sulphoxide of methamidophos,however,has not been isolated and identified, due perhaps,to an instability in the peroxyacid medium used by Eto and his co-workers. It may however be more stable in a more physiological environment such as the ganglia of insects.

Also,the significant differences observed in the anti-AChE activity of acephate between susceptible and relatively tolerant organisms (Mohamad,1982),could also be a reflection of their target site physiology.

Phosphoramidothiolates possess an electrophilic phosphoryl group. This electrophilic character is enhanced by the poor $p\pi$-$d\pi$ overlap in the P-S bond. The higher electronegativity of sulphur compared to phosphorus leads to a low electron density on the latter thereby increasing its electrophilic nature and consequently,its ability to discharge a leaving group in preference for AChE (Eto et al.,1977;Eto,1974;Murdock and Hopkins,1968). This thiolo effect,however,is not translated directly into anti-AChE potency because it is always antagonized by the electron donating ability of the nitrogen atom. This

deactivation of the electrophilic reactivity of the phosphoryl group by the nitrogen atom may explain the susceptibility of phosphoramidothiolates to sulphoxidations (Eto *et al.*,1977;Eto,1974).

The insecticidal effectiveness of acephate would therefore most likely reside in those groups which tend to enhance the electrophilic character of its phosphorus atom. In this respect,the alkylthiol and the N-alkyl moieties would be deemed as structurally crucial.

Any activated toxic species is most likely to arise by a chemical transformation of the phosphoryl and alkylthiol,or,the nitrogen and carboxyalkyl moieties.

It is obvious so far that many factors operate in concert to bring about the observed insecticidal activity of acephate. Before a relative scale of importance can be assigned to each factor,it would be relevant to undertake a study of the residual products of acephate *in vivo* and *in vitro*. The objectives of this study were:

(i) To study the formation of (II), (III), (IV) and (V) (Fig.1) by hydrolysis in buffered alkaline water.

(ii) To determine the presence of these products as acephate metabolites in livers of mice.

(iii) To determine the effects of these on erythrocyte AChE of mice.

3. MATERIALS AND METHODS

3.1. Chemicals

The acephate and methamidophos used in this study were both of analytical grade with purities of 99.3% and 99.6% respectively. O,S-dimethyl phosphorothiolate (III), S-methylacetyl phosphoramidothiolate (IV) and O-methylacetyl phosphoramidothiolate (V) were all of a purified grade, containing 90% of active ingredient. (III) was obtained as the sodium salt while (IV) and (V) were obtained as their ammonium salts. Acephate and these four residual compounds were supplied by the Chevron Chemical Company,Richmond,California. The 0.1M tris-HCl buffer used for the experiment was made up with analar grades of Tris base and dilute hydrochloric acid as described in Dawson *et al* (1969). Since acephate has a pH-depressing property,the observed pH of the buffer after the dissolution of acephate was brought up to the desired value using additional drops of 0.1M tris base. Final pH values were checked with a pH meter (pH METER 26, Radiometer Copenhagen). Throughout the 7-day incubation period,no significant changes in the pH of the various solutions were observed.

3.2. Incubation of Acephate with Tris-HCl buffer at 37 C

Aqueous solutions of acephate, 80ml per sample, with an average concentration each of 1085ug/ml were incubated in triplicate at pH 7.2, 8.1 and 8.6 in 0.1M tris buffer at 37 C in 250ml Erlenmeyer flasks. At intervals of 0, 0.5, 1.5, 3, and 7 days, aliquots were taken from each flask for the analysis of acephate and the hydrolytic products.

3.3. Metabolism of Acephate by Mice

3.3.1. Materials

The acephate used in this study was of a technical grade , with a purity of 97%, obtained from the Chevron Chemical Company. The ^{14}C-acephate was obtained, with a specific activity of 4.4mCi/mM and a purity of greater than 98% from the New England Nuclear Inc.(NEN),Boston,Massachusetts. The Protosol^{R} tissue and gel solubilizer, and the Scintiverse II scintillation cocktail,both of analytical grades, were obtained from NEN and Fisher Scientific Company,Pittsburg,Pennsylvania, respectively. The mice were 60 day old CD-1 Swiss males with weights ranging from 33g to 44g, obtained from the University of British Columbia Animal Care Center. The rest of the chemicals used in this study are all of ACS approved grades, except when otherwise

specified.

3.3.2. Metabolic Studies with non-radioactive Acephate

The standard aqueous solution of acephate, with a concentration of 12.03mg/ml, was administered to the mice by direct stomach injection tubes to levels of 0.1ml per 10g of mice. The final concentration administered to the mice, 120.03mg/kg, was therefore equivalent to one-third of their LD_{50} value (361mg/kg). Controls were injected also by stomach tubes with distilled water to a concentration of 0.1ml per 10g of mice.

At intervals of 0.5, 4, 7.25, 24, and 30 hours respectively, three acephate-treated mice are killed by decapitation together with a water-treated control and their livers excised for the analysis of acephate and (II), (III), (IV) and (V).

3.3.3. Metabolic Studies with (S-methyl) ^{14}C Acephate

The aqueous solution of acephate standard, with a concentration of 200ug/ml was prepared to a specific activity of 0.24uCi/ug of acephate. This was administered to the mice by stomach tubing, at a ratio of0.1ml per 10g, to a concentration of 2ug/g of body weight or approximately 1/180 of their LD_{50} value for acephate (361mg/Kg). Control mice were administered

distilled water at the ratio of 0.1ml per 10g.

At the intervals of 0.5 and 7.25 hours after acephate administration, two mice were killed , by decapitation together with a water-treated control and their livers excised for the analysis of acephate and (II), (III), (IV) and (V) using both microautoradiography and liquid scintillation counting. In addition, aliquots of the intestinal tracts and and the remaining carcass, with weights ranging from 100mg to 400mg were solubilized in protosol^{R}, in preparation for liquid scintillation counting (LSC). The faecal samples, all weighing less than 50mg were also solubilized in protosol^{R} inside glass collection vials. The urine samples, containing no particulate matter were mixed with scintillation cocktail for LSC without any further solubilization.

3.4. Anti-AChE Studies

3.4.1. Materials

The acephate used for this study was of a technical grade (97%) while analytical grade methamidophos (99.6%) was employed. (III), (IV) and (V) were all of a purified 90% grade. These five compounds were all obtained from the Chevron Chemical Company. They were all dissolved in 0.1M phosphate buffer, pH 7.6 prepared from analar grades disodium hydrogen phosphate

(Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4) as described by Gomori *et al.*, 1955. Owing to the pH depressing action of acephate, it was dissolved in pH 8.0 buffer and the final pH adjusted to 7.6 with appropriate drops of 0.1M Na_2HPO_4 and a pH meter (pH METER 26, Radiometer Copenhagen). The aceylthiocholine iodide (ACth), quinidine sulphate and dithio bis nitrobenzoic acid (DTNB) used for this study were all of analytical grades obtained from the Sigma Chemical Company, St. Louis, Missouri. The 60 day old CD-1 Swiss mice (males) were obtained from the University of British Columbia Animal Care Center. All the solutions prepared for this study were made up with the 0.1M phosphate buffer, pH 7.6.

3.4.2. Extraction and Determination of AChE Activity

The method of Ellman *et al.* (1961) as described by Hussain and Oloffs (1979) was employed for this study. 0.05ml of blood, obtained by decapitation using two mice per sample was washed by centrifugation at 1630g for 10 minutes in 9ml of 0.1M phosphate buffer, pH 7.6 (buffer). The supernatant was decanted and the erythrocyte pellets further washed with 4ml of buffer, by centrifugation at 1630g for another 10 minutes. The supernatant was again discarded and the erythrocyte pellets finally suspended in a total of 2ml of buffer.

0.6ml of this suspension was diluted to 9ml with buffer before slurrying with 0.9mg of Saponin powder (obtained from the

Sigma Chemical Company,St.Louis,Missouri) to lyse the erythrocyte cells. To 3ml of the lysed erythrocytes' suspension in buffer was added 25ul of 0.1% quinidine sulphate to inhibit the non-specific esterases.

The AChE preparation was then incubated for 10 minutes at 37 C in a metabolic shaker. The relevant volume (from 2.5ul to 250ul) of a buffer solution of the test inhibitors acephate, (II), (III), (IV) and (V) with concentrations of 1.0M, 1.0M, 1.05M, 1.02M and 1.43M respectively was then added. This was followed by a further 10 minute incubation at 37 C in a metabolic shaker. After the incubation, 50ul of a 0.075M solution of the synthetic substrate, ACth was added and followed up with 50ul of a 0.01M solution of the chromogenic reagent, DTNB.

The reference enzyme activity was determined by incubating the lysed suspension of erythrocytes with the substrate analogue to the exclusion of the test inhibitors. The reference cuvette blank consisted of all the reagents in the test samples except the substitution of the substrates with buffer.

For the spectrophotometric determination of AChE activity, silica cuvettes, with 10mm path lenghts were used. The cuvette compartments were kept at a temperature of 37 C while inside the spectrophotometer chamber ,using a water bath.

The yellow anion of DTNB,the presence of which is an index of hydrolysis by AChE, was monitored at 412nm in a Cary-14 spectrophotometer operating under the following parameters :

Attenuation 0.1-0.2; Chart recorder speed 0.5inches/minute.

3.5. Analytical Procedures

3.5.1. Extraction and Clean-up

3.5.1.1. Water

Acephate and Methamidophos (II) : 1ml volume from each sample was made up to an azeotropic mixture with 15ml of acetonitrile (nanograde), flash evaporated at room temperature (20 C) to dryness and picked up with appropriate volume of glass-distilled acetone for analysis by gas-liquid chromatography (GLC).

Hydrolysis Products (III), (IV) and (V) : 1ml of each sample was made up to azeotropic mixture with 15ml of acetonitrile, flash evaporated at room temperature and picked up in 1ml electronic grade methanol for separation and clean-up by thin-layer chromatography (TLC).

3.5.1.2. Mouse Liver

The procedure employed here is an adaptation of the method described by Szeto *et al.*,(1982). Whole livers, with weights ranging from 1.6g to 3.12g were extracted four times in a Polytron^{R} PT20 homogenizer with a total volume of 200ml of a 1:1 mixture of acetonitrile and methanol. Ten grammes of granular anhydrous sodium sulphate (analytical grade) were added during the first homogenization, to facilitate extraction. The extracts were filtered through glass wool and granular sodium sulphate into 500ml round bottom flasks. The resulting extracts were concentrated to dryness at room temperature in a flash evaporator. They were subsequently redissolved in 50ml of methanol and transferred to a 1cm(i.d.) capillary column packed with a 5cm layer of a 2:5 w/w mixture of Nuchar C-190N charcoal (Matheson Coleman and Bell, East Rutherford,New Jersey) and Whatman CF-11 cellulose powder (Whatman Limited,Kent,England), sandwiched between two 2cm layers of granular anhydrous sodium sulphate. Acephate and compounds (II) to (V) were then eluted with 100ml of methanol. Each eluate was measured and divided into two aliquots. One was evaporated as described and picked up in acetone for GLC analysis of acephate and methamidophos. The other aliquot was reserved for separation and further clean-up by TLC.

For the ^{14}C-acephate treated mice, the whole eluates were taken down to dryness in a flash evaporator at room temperature

before finally redissolving in 0.5ml of methanol for seperation and autoradiography, using a 2-dimensional TLC.

3.5.2. TLC and Autoradiography

Silica Gel 60G was slurried in twice its weight of glass-distilled water and coated, to a thickness of 0.25mm on 20cm x 20cm glass plates, using a Desaga^{R} TLC spreader. The coated plates were heated for 5 hours at an oven temperature of 120 C and subsequently stored in a desiccator at room temperature. A 50ul portion of each methanolic sample was spotted at 3.2cm above the lower edge of the plate using a Microcaps disposable lambda pipette. The mobile phase consisted of 200ml of acetonitrile : methanol : water (7 : 2 : 1 by volume). For the 2-dimensional TLC, the second run was carried out with a mobile phase of diethyl ether : 2-propanol (8 : 2 by volume). Plates were developed until the solvent front had moved a distance of 15cm. Table 8 shows the Rf values of acephate and compounds (II) to (V) in various mobile phases.

For the chromatogram containing non-radioactive samples (water,mouse liver), appropriate sections of the silica gel on the plates were scraped off after a one-dimensional run into capillary columns (1cm i.d.) fitted with fritted glass filters. Since (III) has an Rf value lying between that of (IV) and (V), fractions of it were co-scraped with (IV) while the remainder went with (V). The distinct GLC retention time of (III) in

relation to (IV) and (V), after alkylation precludes any ambiguities in its flame photometric detector (FPD) identification.

Each silica gel scraping was subsequently eluted with 35ml of methanol into 50ml round bottom flasks, flash evaporated to dryness at room temperature and picked up in 2ml of acetone and three drops of 2M HCl for subsequent alkylation.

The ^{14}C containing chromatograms were dried , after a 2-dimensional run, by placing them inside a fume hood with a weak air draught. The dried chromatograms were placed in Kodak film holders with background enhancer screens and closely clasped with NS-2T high silver density X-ray film for 7 days at -70 C. At the end of exposure, the films were developed , with standard Kodak^{R} solution. Each chromatogram spot corresponding to a sensitized grain on the film was then suctioned off, with Eppendorf^{R} micropipettes fitted with a base of silanized glass wool,into glass scintillation vials.

Glass-distilled water (0.5ml) was added to each silica gel scraping and its corresponding silanized glass wool, and the system heated at 60 C for 2 hours. After cooling, 10ml of Scintiverse II scintillation cocktail was added. Phase separation was prevented by shaking with a vortex mixer in preparation for the LSC.

3.5.3. Alkylation of (III), (IV) and (V)

These three compounds, compared to acephate and methamidophos, are sufficiently polar that they are not vaporized under the high temperature conditions normally encountered during GLC (Uden and Henderson, 1977). To make them hydrophobic enough, and therefore amenable, for GLC analysis the amyl carbene of diazopentane was used for the formation of their derivatives.

To prepare diazopentane,2.3g of potassium hydroxide was dissolved in 2.3ml of glass-distilled water and the solution cooled at -10 C in a freezer for 30 minutes. Anhydrous diethyl ether (25ml) was added and the mixture cooled for another 30 minutes, also at -10 C. N-amyl-N'-nitro-N-nitrosoguanidine (2.1g) obtained from Aldrich Chemical Company Milwaukee,Wisconsin, was subsequently added slowly over a period of 2 minutes, with moderate shaking. The generated diazopentane dissolved in the upper ether layer; the solution was decanted into aluminium-wrapped test tubes with teflon-lined stoppers and stored in the freezer at a temperature of -10 C for periods not exceeding one hour. These procedures are as described by Bradway et al.,(1977); Lores and Bradway, (1977); Shafik et al., (1973).

Derivative formation with (III), (IV) and (V) present in the acidified extracts was carried out by adding successive drops of ethereal diazopentane to their solution in a 25ml round

bottom flask, with moderate shaking, until a persistent yellow colour was maintained, which indicates saturation with diazopentane. The reaction mixture was kept at room temperature for 24 hours to ensure completion after which appropriate dilutions were made with glass-distilled acetone for GLC analysis. Excess diazopentane was destroyed by adding a few drops of a (1:99 v/v) mixture of formic acid and benzene until the discharge of the yellow colour. In all cases, care was taken not to add an excess of this reagent, since it creates interference on detector sensitivities, in general.

3.5.4. Determination of ^{14}C present in the Intestinal tracts, Urine, Faeces and Carcass

This was determined by solubilizing aliquots of them, ranging from less than 50mg for faecal samples, to as much as 400mg for carcass and alimentary samples, for 18 hours in 2ml of the 0.5M quarternary ammonium hydroxide solution, protosol^{R}. The temperature was kept at 60 C throughout the digestion period. The solubilized materials were cooled to room temperature and 0.3ml of 30% hydrogen peroxide was added as a decolorizer. The glass vials were loosely capped and incubated at 60 C for an additional 30 minutes . A subsequent cooling down to room temperature was followed by the addition of 10ml of Scintiverse II scintillation cocktail. Phase mixing was achieved for LSC by shaking the mixture with a vortex mixer. Urine samples, when

present, were mixed directly with Scintiverse II cocktail using a vortex mixer, in preparation for LSC.

3.5.5. GLC Analysis

3.5.5.1. Acephate and Methamidophos

The conditions employed are as described by Szeto et al. , (1982).

A Tracor^{R}-222 gas chromatograph, equipped with FPD, was used. A 36cm x 3mm (I.D) U-shaped Pyrex glass column packed with 1% carbowax TPA 20M on chromosorb HPW,100-200 mesh, was used under the following parameters :
The column temperature was programmed from 145 C to 185 C at a rate of 29 C per minute. Temperatures for the inlet, detectors 1 and 2 were 195 C, 165 C and 160 C respectively. Carrier gas flows for nitrogen, hydrogen and air were 70ml/min., 180ml/min., and 110ml/min. respectively for detectors 1 and 2.

Detector response was calibrated before and after sample analysis with authentic reference grade standards (0.6ug/ml, 0.12ug/ml and 0.06ug/ml for acephate; 0.3ug/ml, 0.06ug/ml and 0.03ug/ml for methamidophos), in glass-distilled acetone. These were prepared from stock solutions of acephate (60ug/ml) and methamidophos (30ug/ml) for each set of analysis.

The retention time of acephate and methamidophos under these conditions were 1.05 and 0.47 minutes respectively. Acephate and methamidophos are classified as non-detectable if 8ul of the undiluted extract gives no chart recorder response. The limits of quantification were 4ppb for acephate and 2ppb for methamidophos. These figures are equivalent to 4% recorder's response. Detector responses below these levels are reported as trace and computed as one-half of the appropriate quantification limits.

Quantification was done by comparisons of average peak heights of reference standards , injected before and after analysis, with those due to the samples. For the standard solutions, each peak height average consisted of four different injections. For the samples, they were obtained from two injections.

3.5.5.1.1. Recovery Studies

Recovery studies were carried out on acephate and methamidophos in water and livers of mice.

The water was fortified to levels of 0.2ppm, 0.4ppm and 0.1ppm, 0.2ppm with acephate and methamidophos respectively. Liver samples were spiked with acephate at 1ppm, 0.4ppm and with methamidophos at 0.5ppm, 0.2ppm. The water and liver samples were extracted as described. The percentage recoveries for the two compounds ranged from 98% to 100%.

3.5.5.2. Amyl Derivatives of (III), (IV) and (V)

A Tracor^{R} MT 220 gas chromatograph equipped with an FPD detector was used for the analysis of these chemical species.

A 180cm x 5mm U-shaped Pyrex^{R} glass column, packed with 3.5% diethyl glycol succinate (DEGS), 80-100 mesh, on supelcoport was employed. For operational use, column temperatures were programmed from 165 C to 180 C at a rate of 25 C per minute, with a final holding time of 4 minutes. Detector and inlet temperatures were 155 C and 170 C respectively. Carrier gas flows for nitrogen, hydrogen and air were 80ml/min., 180ml/min. and 110ml/min. respectively.

Detector responses were calibrated before and after sample analysis with authentic reference grade amyl derivative standards of (III), (IV) and (V) in glass-distilled acetone prepared, as described, just prior to analysis. For amyl derivatives of (III), (IV) and (V) the respective concentrations were 0.8ug/ml, 0.2ug/ml; 4ug/ml, 1.7ug/ml, 0.4ug/ml; 4ug/ml,1ug/ml and 0.4ug/ml. The retention times for these amyl derivatives were : 2.4 minutes for (III) and 3 minutes for (IV) and (V).

The derivatives are regarded as non-detectable if 8ul of the undiluted extract gave no distinct and measurable chart recorder response. The limits of quantification were 30ppb, 68ppb and 40ppb for compounds (III), (IV) and (V), respectively

, equivalent to 4% of recorder's response. Detector responses below these levels were classified as trace and computed as one-half of the appropriate quantification limits. Quantification was carried out by comparison of average peak heights of authentic amyl derivative standards injected before and after analysis, with the samples. For the standards, averages were obtained from four injections while for the samples, they were got from two injections.

3.5.5.2.1. Recovery Studies

Recovery studies were carried out with water and mouse liver,fortified to levels of 0.2ppm, 0.4ppm; 0.4ppm, 0.8ppm; and 0.8ppm, 1.7ppm with (III), (IV) and (V) respectively. Samples were extracted, converted to amyl derivatives and analysed, as described earlier. Recoveries for (III), (IV) and (V) were 91%, 89% and 90% respectively,in livers of mice. In water samples, recoveries ranged from 99% to 100%.

3.5.6. LSC

The protosol-solubilized ^{14}C-containing samples from the carcass, intestinal tract and faecal matter were thoroughly mixed with 10ml of Scintiverse II scintillation cocktail, using a vortex mixer. For the liver samples, silica gel scrapings from their chromatograms and the corresponding silanized glass wool

containing the relevant radioactive residues were also mixed with 10ml of Scintiverse II cocktail by shaking with a vortex mixer.

The samples, with fluors, and solubilizers where applicable were all contained in glass scintillation vials with teflon-lined stoppers and dark adapted for 3 hours to correct for chemoluminescence. For operational use, the upper and lower discriminator windows were set respectively at 0-655nm and 397-655nm. Samples were counted for 5 minutes or, until the accumulation of 10,000 counts.

3.5.6.1. Recovery Studies

Recovery studies were carried out on silica gel chromatograms seperately spiked with 1.5ug, 5.1ug, 5.3ug, 6.0ug, 6.25ug, 6.5ug, 12.5ug and 14.5ug of S-methyl ^{14}C-acephate. The silica gel scrapings and their corresponding silanized glass wool were extracted and counted, as described. Recoveries obtained were : 84.3%, 99%, 98.5%, 98.2%, 99.1%, 97.3%, 98% and 103.4% respectively.

Confirmatory studies were also carried out ,using GLC, to determine the concentrations of ^{14}C-acephate and methamidophos in the liver samples. The percent standard deviation between the two analytical techniques,GLC and LSC, was ±19% and ±9.5% for acephate and methamidophos, respectively. On the average, LSC gave higher values than GLC.

4. RESULTS AND DISCUSSION

4.1. Hydrolysis of Acephate in Water

The results are shown in Tables 1 to 5 and Figures 2 to 17. The decline of acephate concentrations was biphasic in each case : it was rapid for 1.5 days in water of pH 7.2 and 8.6; but at pH 8.1, the phase of rapid acephate breakdown lasted somewhat longer. For the remainder of the experiment, the rates of acephate hydrolysis was much lower. Quantitatively, acephate hydrolysis increased with pH. This agrees substantially with the nucleophile-dependent hydrolysis of pentavalent phosphorus compounds having one $p\pi$-$d\pi$ and four σ bonds (Hudson, 1965).

Several authors have confirmed the validity of this hydrolytic behaviour for most organophosphorus pesticides, including acephate (Eto, 1974; Fest and Schmidt, 1973; Gomaa and Faust, 1972; Heath, 1956; Quistad et al , 1970).

The biphasic nature of acephate hydrolysis follows a general pattern characteristic, but by no means diagnostic, of SN2 reactions displaying pseudo first order kinetics (Barrow, 1973; Heath, 1956; Jencks, 1969; Moore and Pearson, 1981; Piszkiewicz, 1977). Thus, transformation of the data in Figure 2, shown in Figure 9, reveal that, despite a paucity of data points, it displays first order kinetic characteristics. This

agrees well with the trend reported by Ouistad et al. (1970) for the related compound, O-ethyl,S-methyl phosphoramidothiolate at high pH. A similar kinetic trend is also followed by the hydrolysis of acephate in unbuffered pond water carried out by Oloffs and Yee (1981). Some of their results, shown in figure 10, reveal that despite a wide range of environmental variables associated with the experiments, first order kinetic trends are still followed. Therefore, in terms of kinetic behaviour, a direct parallel exists between the results of this experiment and the fate of acephate in natural waters.

4.1.1. Formation of (II), (III), (IV) and (V) in Water

The results are shown in Tables 1 to 5 Figures 3 to 8.

The formation of (II) increased linearly with increasing pH for up to 1.5 days(Fig.3). At 3 and 7 days after incubation however, the highest formation of (II) occured at pH 8.1. Since water generally acts as an anionoid reagent with organophosphorus compounds in alkaline solutions(Heath, 1956), this higher content of (II) at pH 8.1 compared to pH 8.6 probably reflects a higher secondary tranformation rate at the latter pH. The highest concentration of (II) making up 4.3% of initial acephate after 7 days at pH 8.1 agrees closely with the 4.5% reported earlier by Szeto et al., (1979).

Coupled to acephate hydrolysis, (III) was detected at the three pH levels between 0.5 and 7 days as quantitatively, the

Table 1 : Concentrations of Acephate and Its Hydrolysis Products II, III, IV and V [1/] , in ug/ml [2/] , after Incubation of Acephate for 0.5 days in Water at 37°C and pH 7.2, 8.1 and 8.6 .

pH	Initial Acephate	Acephate	(II)	(III)	(IV)	(V)	Total, in % of Initial Acephate
7.2	1083	953	0.34	86	0.0	29	99
7.2	1083	917	0.32	98	0.0	28	96
7.2	1050	933	0.32	98	0.0	22	100
$\bar{X} \pm$ S.D.	1072 ± 19.1	934 ± 18	0.33 ± 0.01	94 ± 6.9	---	26 ± 3.8	98 ± 1.9
8.1	1050	877	1.6	173	0.0	7.0	101
8.1	1067	877	1.6	201	0.0	6.0	102
8.1	1067	900	1.7	206	0.0	7.0	104
$\bar{X} \pm$ S.D.	1061 ± 9.8	883 ± 11.7	1.6 ± 0.06	193 ± 17.8	---	7.0 ± 0.6	102 ± 1.5
8.6	1117	767	1.7	376	0.0	38	106
8.6	1083	747	1.6	360	0.0	36	106
8.6	1167	747	1.6	359	0.0	36	98
$\bar{X} \pm$ S.D.	1122 ± 42.3	754 ± 11.5	1.6 ± 0.06	365 ± 9.5	---	37 ± 1.2	103 ± 4.6

1/ see Figure 1.

2/ Concentrations of (II) to (V) in Acephate Equivalents, i.e., corrected for changed molecular weights.

Table 2 : Concentrations of Acephate and Its Hydrolysis Products II, III, IV and V [1/], in ug/ml [2/], after Incubation of Acephate for 1.5 days in Water at 37°C and pH 7.2, 8.1 and 8.6 .

pH	Initial Acephate	Acephate	(II)	(III)	(IV)	(V)	Total, in % of Initial Acephate.
7.2	1083	663	5.5	452	0.0	2.7	104
7.2	1083	615	5.6	464	0.0	5.4	101
7.2	1050	655	5.6	518	0.0	8.1	113
$\bar{X} \pm$ S.D.	1072 ± 19.1	644 ± 25.7	5.6 ± 0.06	478 ± 35.2	—	5.4 ± 2.7	106 ± 6.2
8.1	1050	645	20.3	539	0.0	13.0	116
8.1	1067	625	20.1	538	0.0	12.1	112
8.1	1067	642	20.0	585	0.0	10.3	118
$\bar{X} \pm$ S.D.	1061 ± 9.8	637 ± 10.8	20.1 ± 0.2	554 ± 26.9	—	11.8 ± 1.4	115 ± 3.1
8.6	1117	413	20.8	346	0.0	31.6	73
8.6	1083	413	21.4	406	0.0	32.4	81
8.6	1167	422	20.8	407	0.0	33.1	76
$\bar{X} \pm$ S.D.	1122 ± 42.3	416 ± 5.2	21.0 ± 0.3	386 ± 35.0	—	32.4 ± 0.8	77 ± 4.0

1/ see Figure 1.

2/ Concentrations of (II) to (V) in Acephate Equivalents, i.e., corrected for changed molecular weights.

Table 3 : Concentrations of Acephate and Its Hydrolysis Products II, III, IV and V [1/], in ug/ml [2/], after Incubation of Acephate for 3 days in Water at 37°C and pH 7.2, 8.1 and 8.6 .

pH	Initial Acephate	Acephate	(II)	(III)	(IV)	(V)	Total, in % of Initial Acephate .
7.2	1083	612	11.5	563	0.0	0.0	110
7.2	1083	595	11.7	526	0.0	0.0	105
7.2	1050	625	11.5	548	0.0	0.0	113
$\bar{X} \pm$ S.D.	1072 ± 19.1	611 ± 15.0	11.6 ± 0.1	546 ± 18.6	—	—	109 ± 4.0
8.1	1050	530	36.3	371	0.0	0.0	89
8.1	1067	468	36.2	388	0.0	0.0	84
8.1	1067	448	37.2	363	0.0	0.0	79
$\bar{X} \pm$ S.D.	1061 ± 9.8	482 ± 42.8	36.5 ± 0.5	374 ± 12.8	—	—	84 ± 5.0
8.6	1117	449	29.7	480	0.0	0.0	86
8.6	1083	411	29.7	470	0.0	0.0	84
8.6	1167	413	28.9	458	0.0	0.0	77
$\bar{X} \pm$ S.D.	1122 ± 42.3	424 ± 21.4	29.4 ± 0.5	469 ± 11.0	—	—	82 ± 4.7

1/ see Figure 1.

2/ Concentrations of (II) to (V) in Acephate Equivalents, i.e., corrected for changed molecular weights.

Table 4 : Concentrations of Acephate and Its Hydrolysis Products II, III, IV and V [1/], in ug/ml [2/], after Incubation of Acephate for 7 days in Water at 37°C and pH 7.2, 8.1 and 8.6 .

pH	Initial Acephate	Acephate	(II)	(III)	(IV)	(V)	Total, in % of Initial Acephate .
7.2	1083	540	22.7	281	0.0	99	87
7.2	1083	523	23.4	284	0.0	108	87
7.2	1050	513	25.0	297	0.0	111	90
$\bar{X} \pm$ S.D.	1072 ± 19.1	525 ± 13.7	23.7 ± 1.2	287 ± 8.5	—	106 ± 6.2	88 ± 1.7
8.1	1050	456	47.9	249	0.0	53	77
8.1	1067	494	43.8	240	0.0	76	80
8.1	1067	493	44.6	238	0.0	74	80
$\bar{X} \pm$ S.D.	1061 ± 9.8	481 ± 21.7	45 ± 2.2	242 ± 5.9	—	68 ± 12.7	79 ± 1.7
8.6	1117	394	31.4	258	0.0	51	66
8.6	1083	405	34.1	260	0.0	40	68
8.6	1167	423	31.4	270	0.0	32	65
$\bar{X} \pm$ S.D.	1122 ± 42.3	407 ± 14.6	32.3 ± 1.6	263 ± 6.4	—	41 ± 9.5	66 ± 1.5

1/ see Figure 1.

2/ Concentrations of (II) to (V) in Acephate Equivalents, i.e., corrected for changed molecular weights.

Table 5 : Acephate and Its Hydrolysis Products II, III, IV and V [1/] in Water Samples of Various pH, Incubated at 37°C for Various times after Application of Acephate to the Water .

Time, in Days.	pH	Acephate % [2/]	(II) % [3/]	(III) % [3/]	(IV) % [3/]	(V) % [3/]	Total, in % of Initial Acephate .
0.0	7.2	100	0.0	0.0	0.0	0.0	100
0.0	8.1	100	0.0	0.0	0.0	0.0	100
0.0	8.6	100	0.0	0.0	0.0	0.0	100
0.5	7.2	87	0.03	9.0	0.0	2.0	98
0.5	8.1	83	0.2	18	0.0	0.7	102
0.5	8.6	67	0.1	33	0.0	3.0	103
1.5	7.2	60	0.5	45	0.0	0.5	106
1.5	8.1	60	2.0	52	0.0	1.0	115
1.5	8.6	37	2.0	34	0.0	3.0	77
3	7.2	57	1.0	51	0.0	0.0	110
3	8.1	45	3.0	35	0.0	0.0	84
3	8.6	38	3.0	42	0.0	0.0	82
7	7.2	49	2.0	27	0.0	10	88
7	8.1	45	4.0	23	0.0	6.0	79
7	8.6	36	3.0	23	0.0	4.0	66

1/ see Figure 1.

2/ Means of initial concentration of Acephate.

3/ Means, in % of Acephate equivalents, i.e., corrected for changed molecular weights.

Figure 2 : Hydrolytic breakdown of Acephate at three elevated pH.

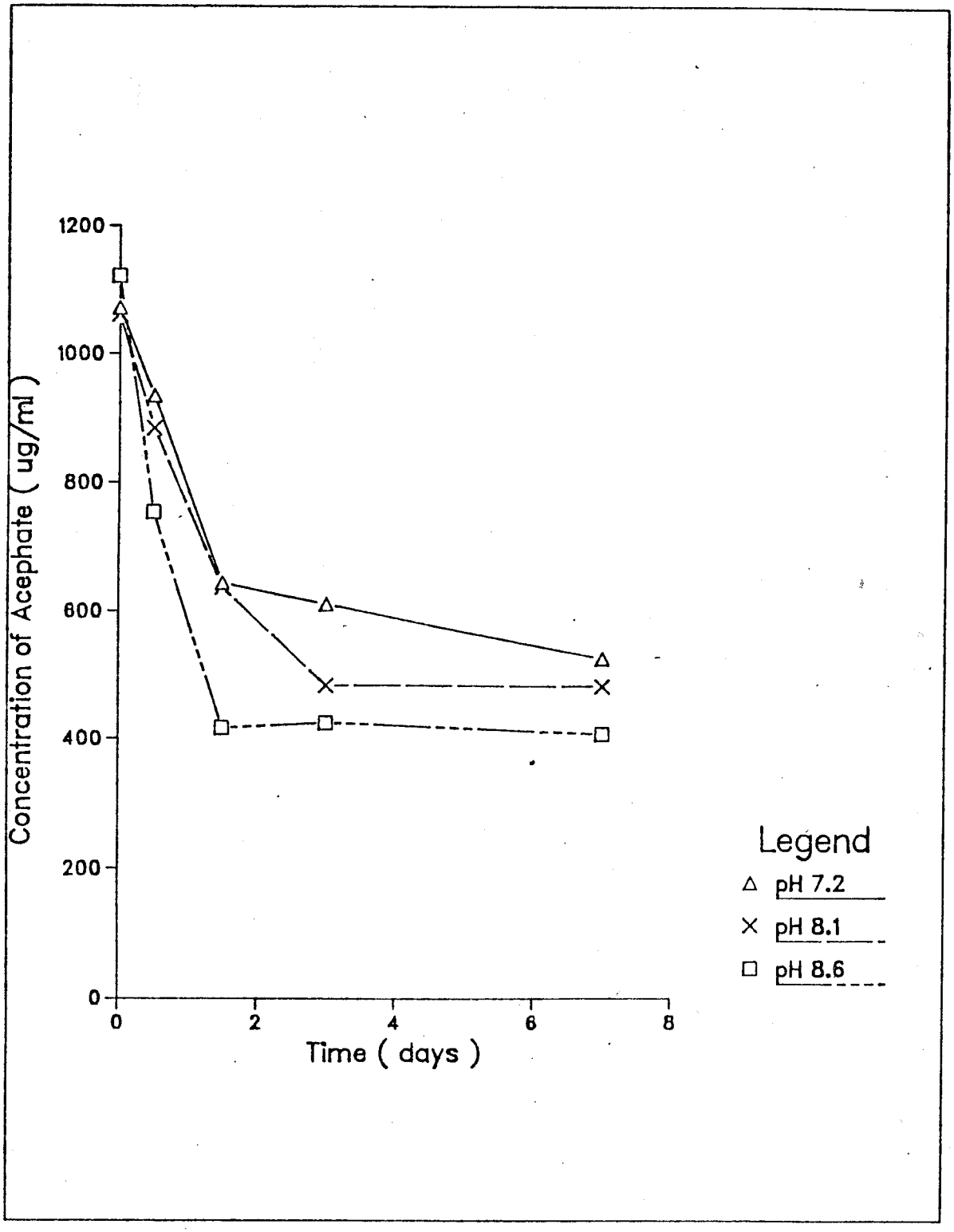

1200
1000
800
600
400
200
0
Concentration of Acephate (ug/ml)
0
2
4
6
8
Time (days)
Legend
pH 7.2
pH 8.1
pH 8.6

Figure 3 : Hydrolytic formation of (II) from acephate .

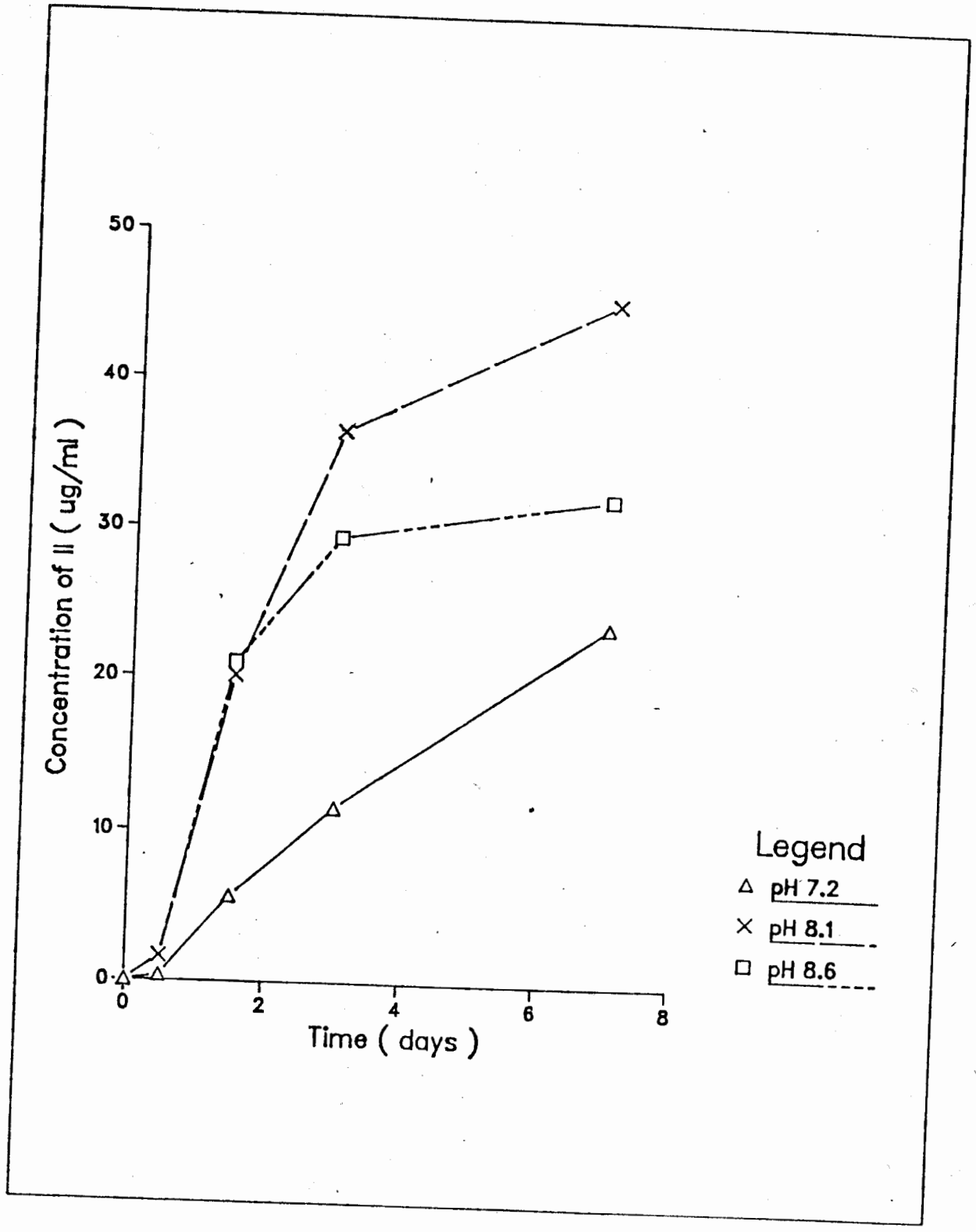

50
40
30
20
10
0
Concentration of II (ug/ml)
0
2
4
6
8
Time (days)
Legend
pH 7.2
pH 8.1
pH 8.6

Figure 4 : Hydrolytic formation of (III) from acephate .

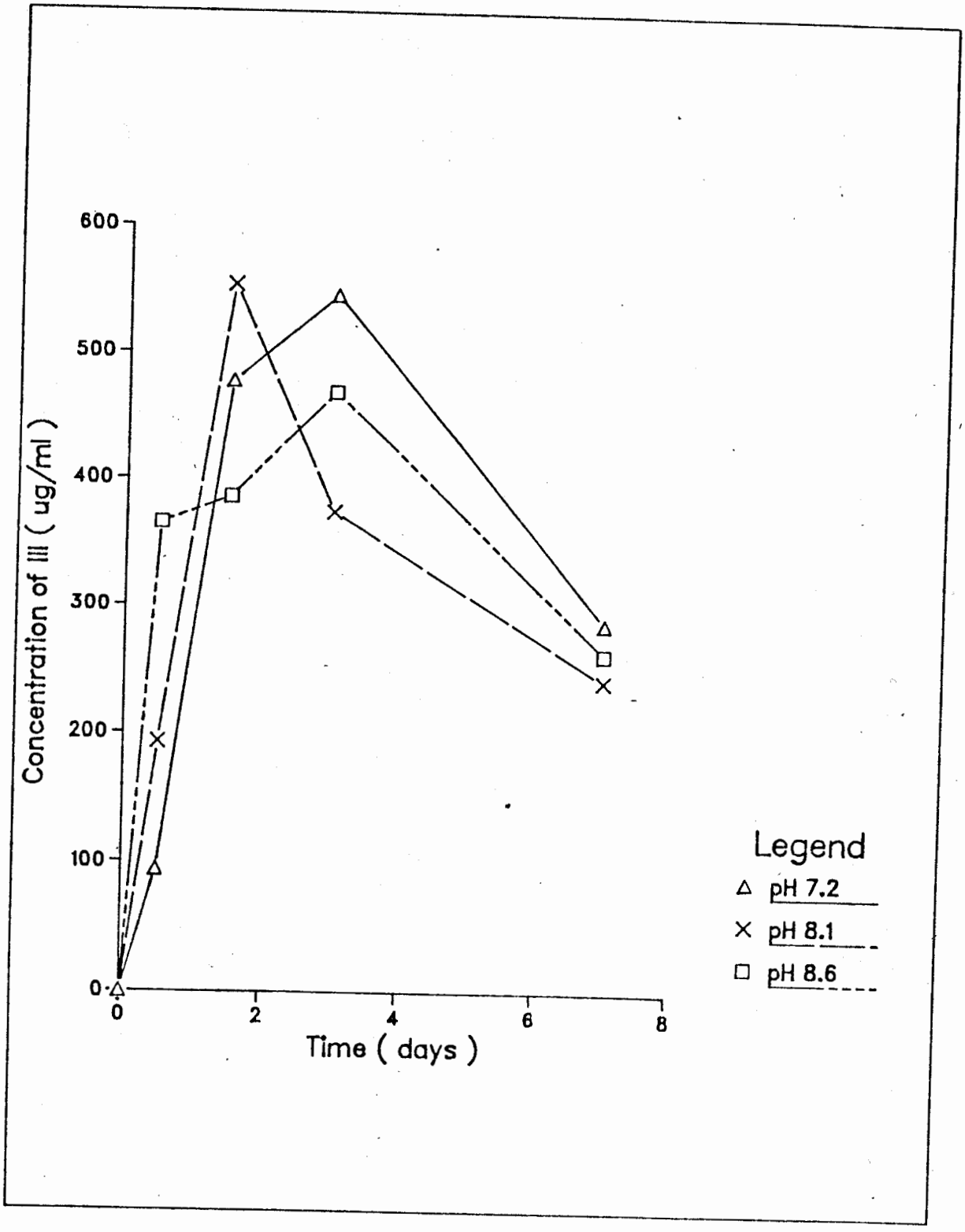

600
500
400
300
200
100
0
Concentration of III (ug/ml)
0
2
4
6
8
Time (days)
Legend
pH 7.2
pH 8.1
pH 8.6

Figure 5 : Hydrolytic formation of (V) from acephate .

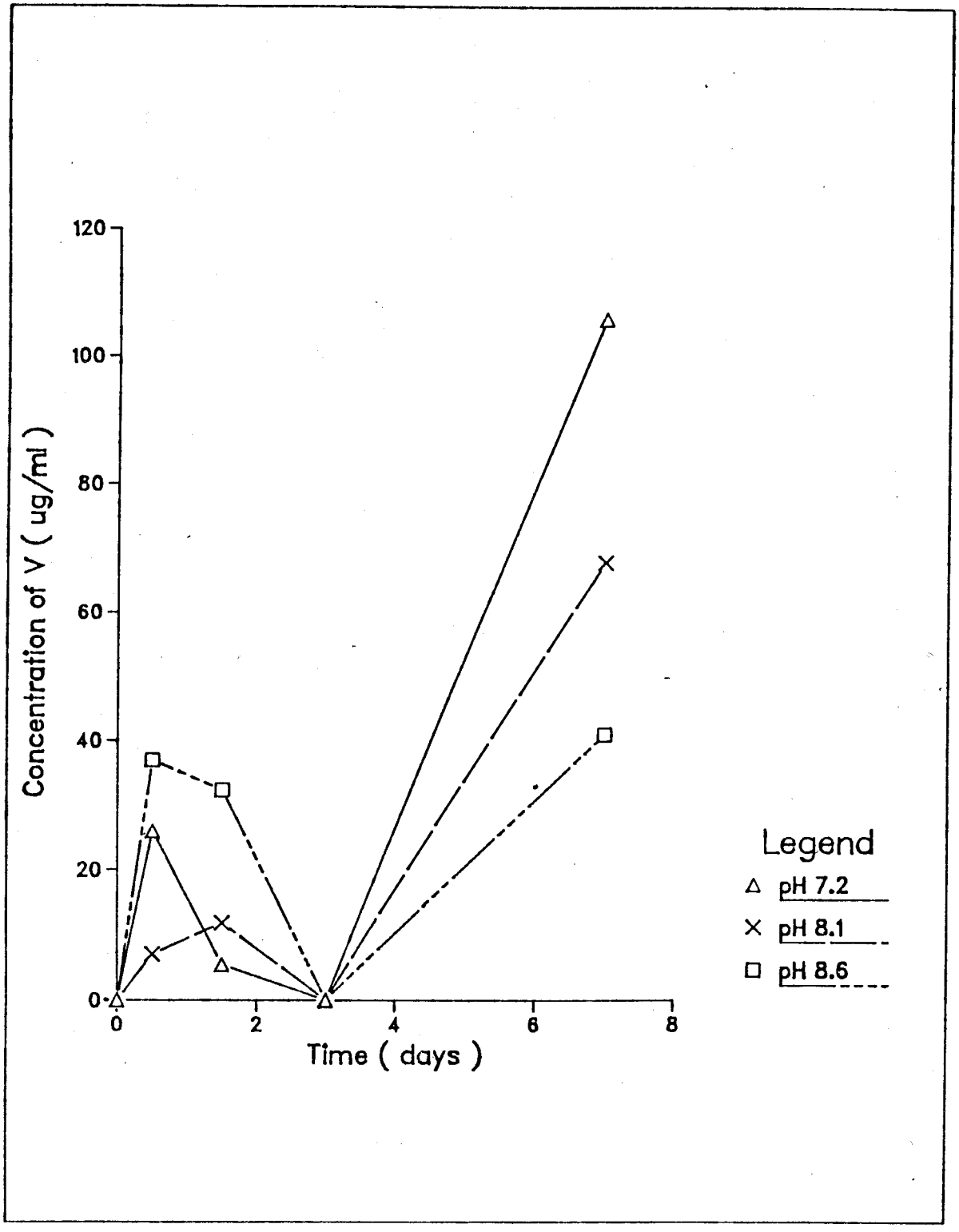

120
100
80
60
40
20
0
Concentration of V (ug/ml)
0
2
4
6
8
Time (days)
Legend
pH 7.2
pH 8.1
pH 8.6

Figure 6 : Residual products of acephate hydrolysis at pH 7.2 .

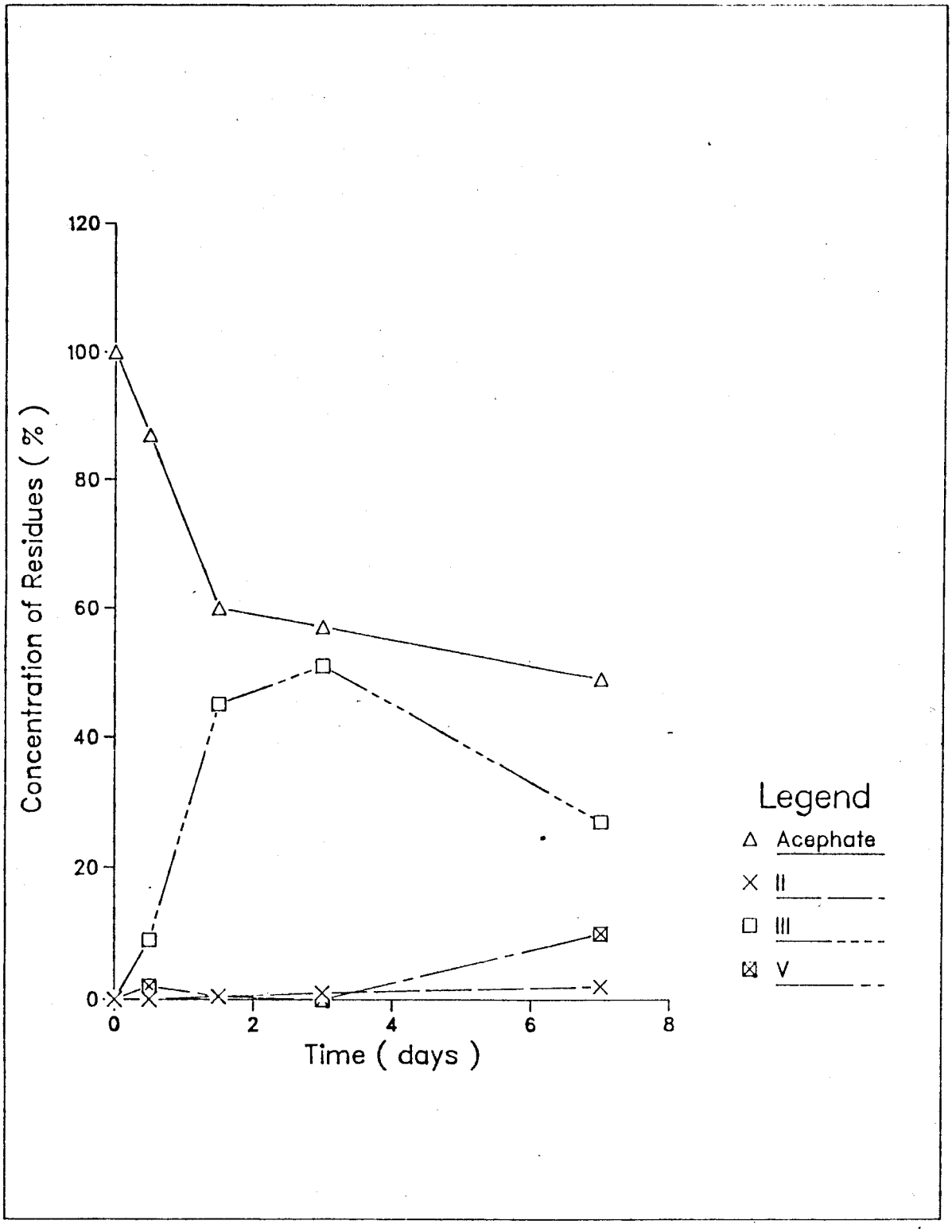

120
100
80
60
40
20
0
Concentration of Residues (%)
0
2
4
6
8
Time (days)
Legend
Acephate
II
III
V

Figure 7 : Residual products of acephate hydrolysis at pH 8.1 .

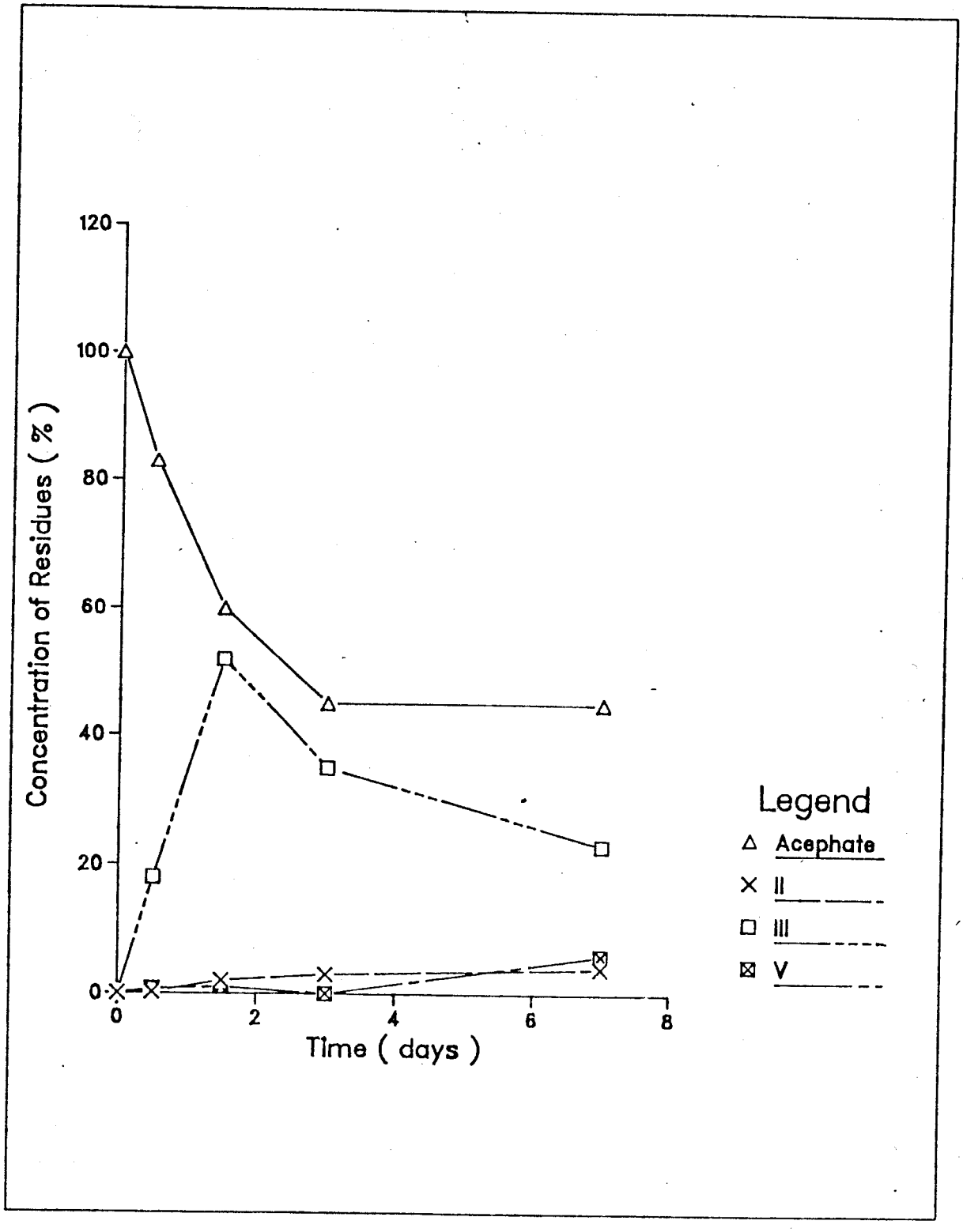

120
100
80
60
40
20
0
0
2
4
6
8
Concentration of Residues (%)
Time (days)
Legend
Acephate
II
III
V

Figure 8 : Residual products of acephate hydrolysis at pH 8.6 .

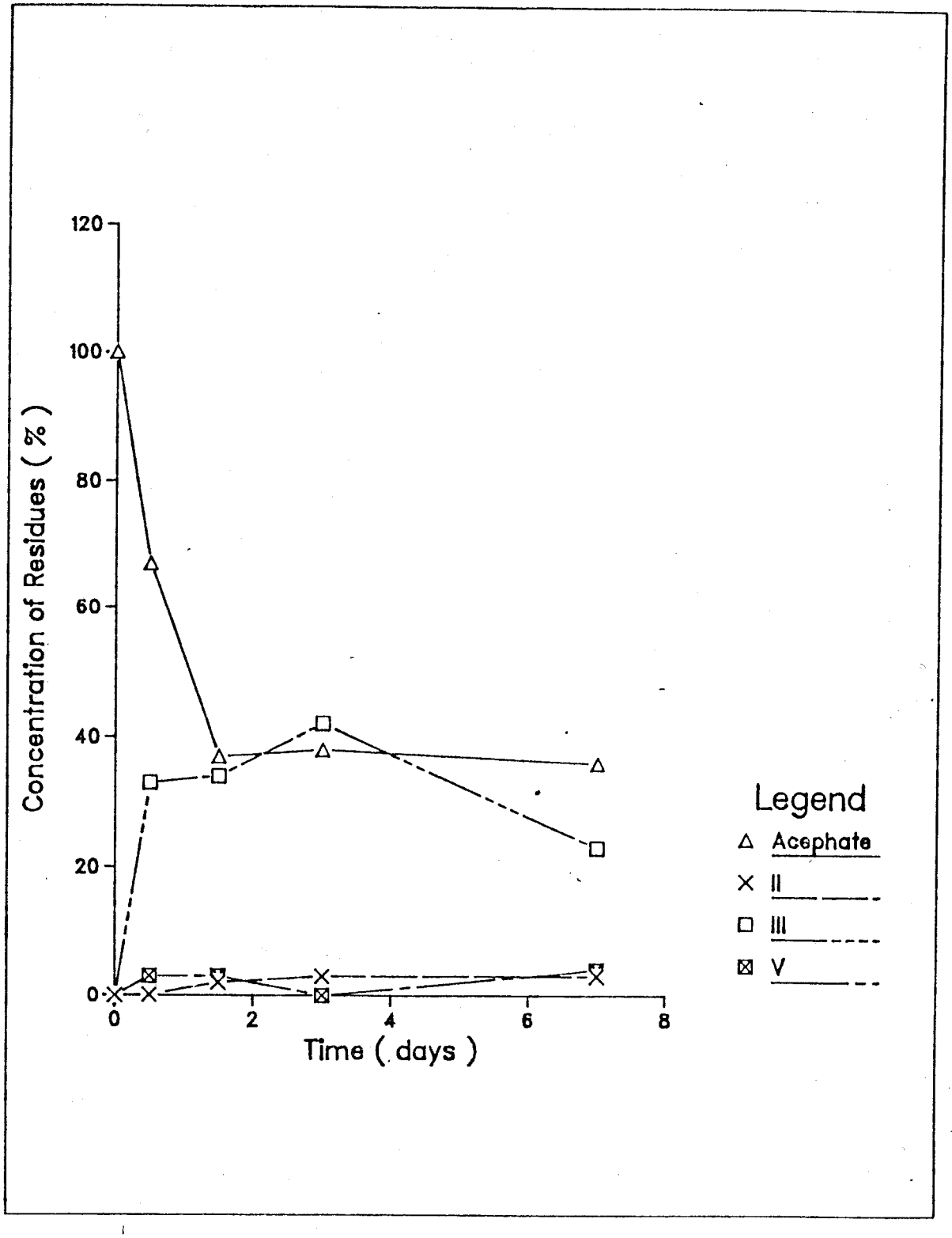

120
100
80
60
40
20
0
Concentration of Residues (%)
0
2
4
6
8
Time (days)
Legend
Acephate
II
III
V

Figure 9 : First-order kinetic plot showing the rates of acephate hydrolysis in water at three elevated pH.

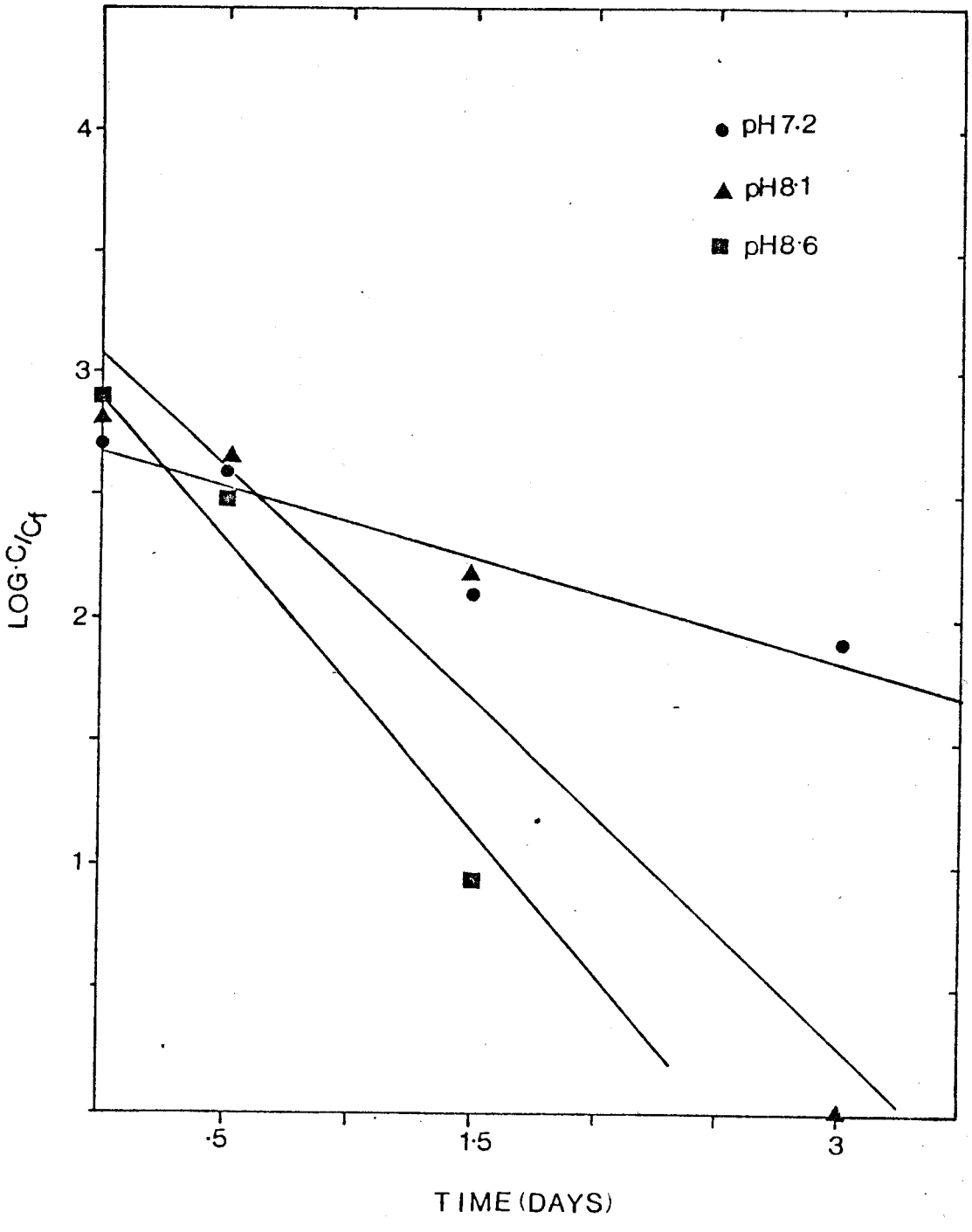
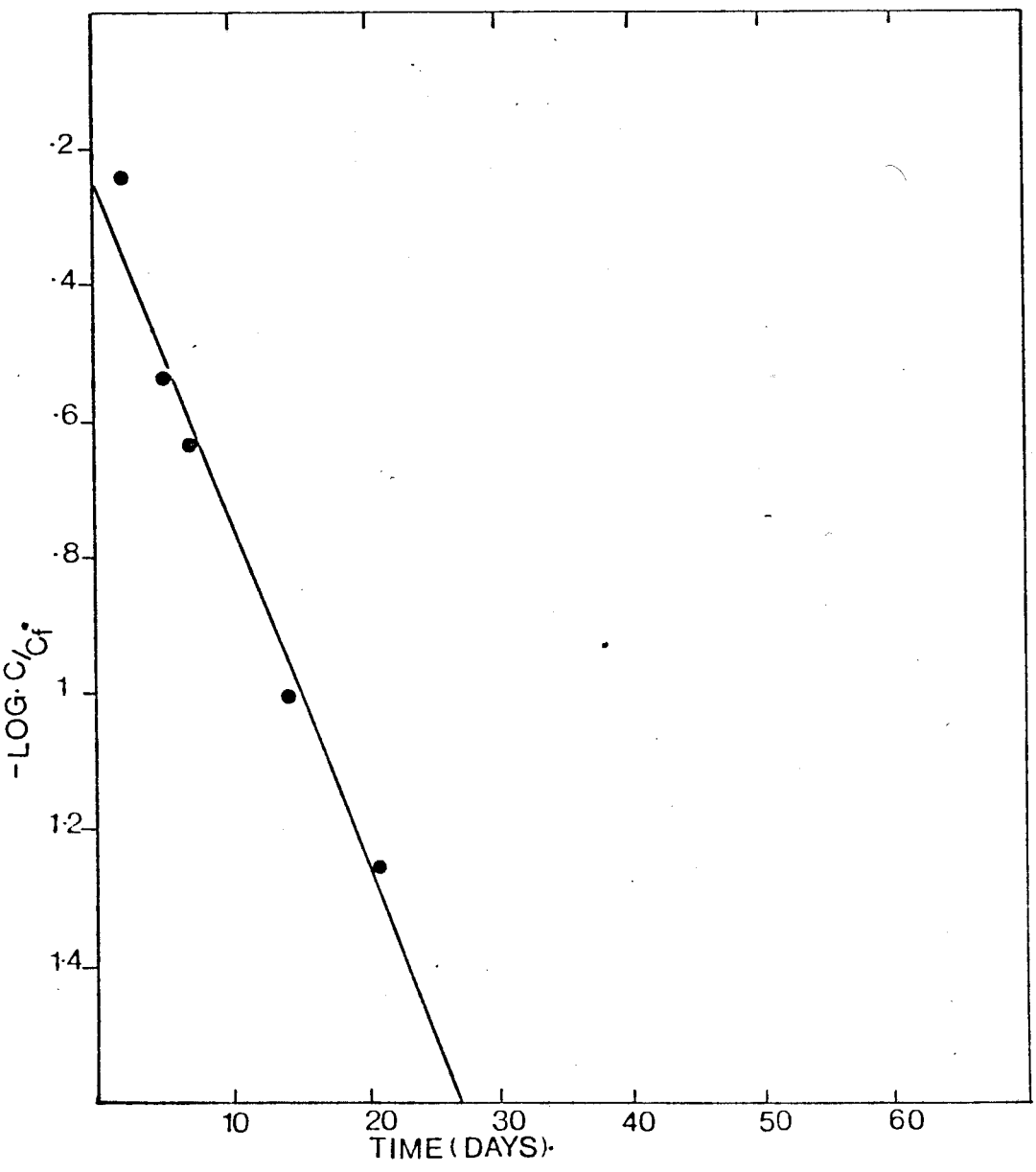

pH 7·2
pH 8·1
pH 8·6
LOG·C/Cf
4
3
2
1
·5
1·5
3
TIME (DAYS)

Figure 10 : First-order kinetic plot showing the decrease of acephate with time in the water of a pond.

(Courtesy of Oloffs and Yee, 1981).


·2
·4
·6
·8
1
1·2
1·4
-LOG·C/Cf·
10
20
30
40
50
60
TIME (DAYS)·

highest product (Tables 1 to 5 and Figures 6, 7 and 8).The formation of (III) was positively correlated with increasing pH after 0.5 days of incubation showing that water is acting as an anionoid reagent, to degrees depending on the magnitude of pH (Fig.4). This pH-dependent correlation however breaks down after longer incubation due, probably, to secondary nucleophilic transformations of (III) (Fahmy et al., 1972).

(IV) was not detected at any pH during the 7 day incubation period. Since mechanistically preferred leaving groups in the SN2 hydrolysis of OP's with one $p\pi$-$d\pi$ bond tend to have low pka values and form strong acids, the alkoxy moiety of acephate with its high pka and weak attraction for protons does not meet such requirements (Eto, 1974; Hudson, 1965; Quistad et al., 1970).

(V) was detected at the three pH levels after 0.5, 1.5 and 7 days respectively. It was not detected at all after 3 days. Tables 1 to 5 and Figures 6 to 8 and 11 to 17 shows these results. This zero value after 3 days is not satisfactorily explained by invoking non degradative secondary transformation for one reason : the classical Pummerer rearrangement by thiolo compounds requires an acidic environment which is not provided by the anionoid water (Eto et al., 1977; Szmant, 1971). Since GC-MS spectra confirmed the analytical validity of the techniques used for detecting (V), (Fig.11 to 17) the total residue recoveries after 3 days at pH 7.2 (Table 3) could be ascribed to a statistical over-estimation possible in residue analysis (Horwitz, 1979). Therefore the less than total

Figure 11 : Replot of GC-MS of the Amyl-Derivative of (V) showing the Total Ion Current (T.I.C.) and the Single Ion Current (S.I.C.).

GC Conditions for Figures 11 to 22 :

Initial Temperature : 80 C

Final Temperature : 220 C

Programme Rate : 10 C/minute

Retention Time (T_R) for (V) : 9.2 minutes.

Column : DB-1

length : 15mm

Internal diameter : 0.2mm

Coating thickness : 0.25μ

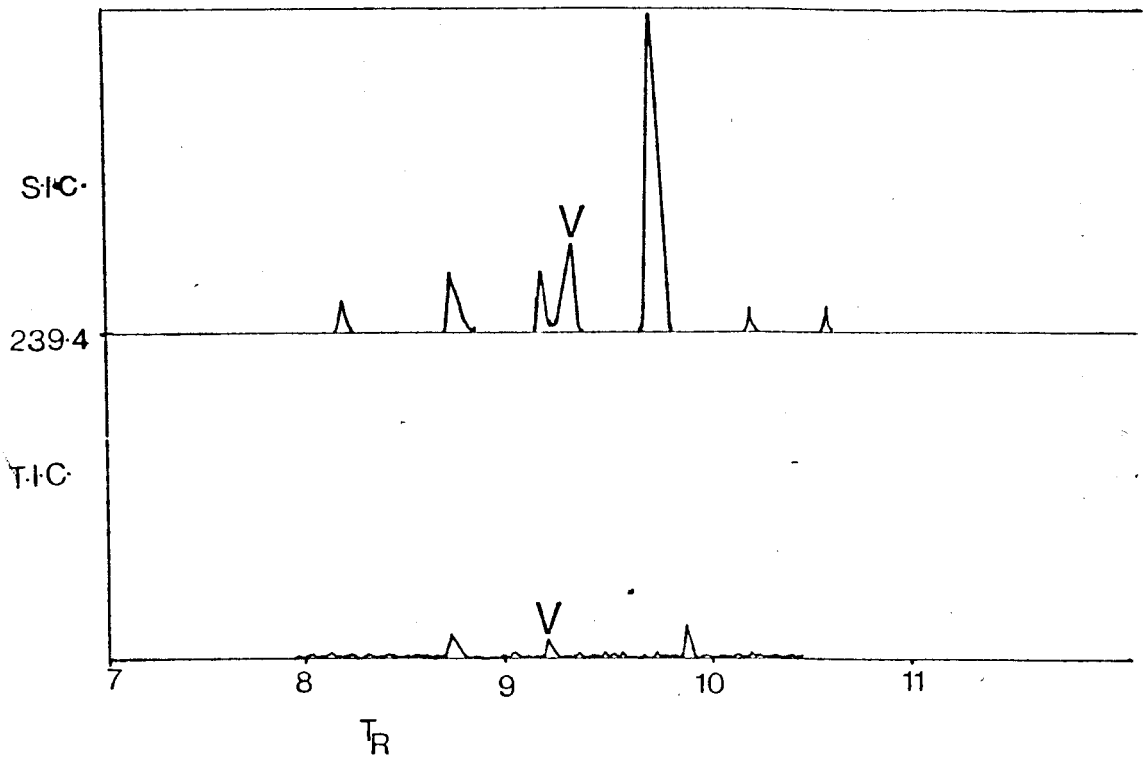

S·I·C·
239·4
T·I·C·
V
V
7
8
9
10
11
T_R

Figure 12 : Replot of MS of (V) (from GC-MS).

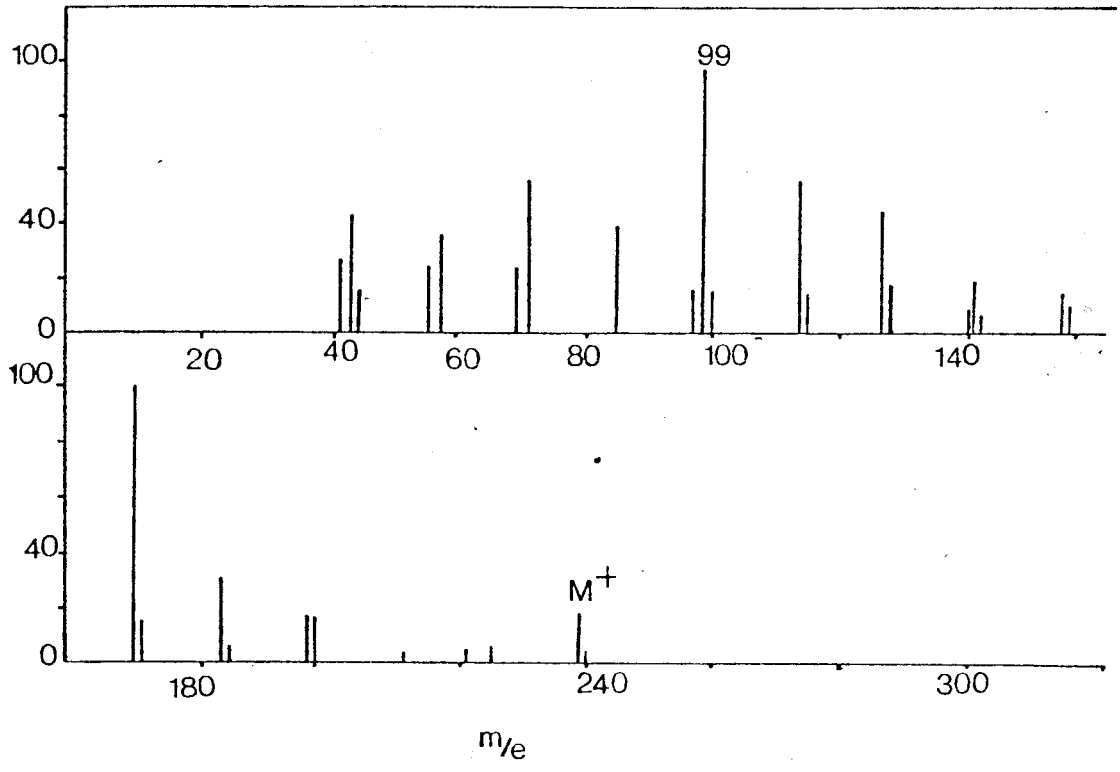

100
40
0
99
20
40
60
80
100
140
100
40
0
M+
180
240
300
m/e

Figure 13 : Replot of the GC-MS of Acephate Sample at pH of 7.2, after 7 days.

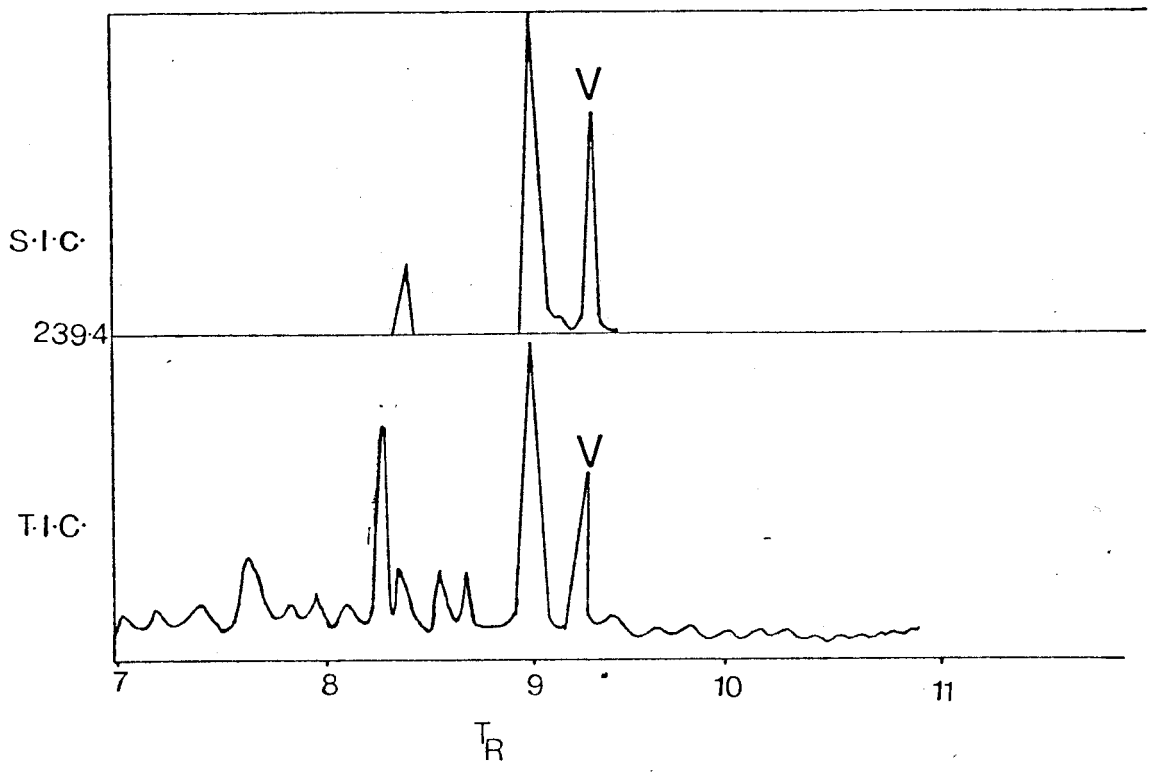

S·I·C·
239·4
V
T·I·C·
V
7
8
9
10
11
T_R

Figure 14 : Replot of MS of Acephate Sample at a pH of 7.2, after 7 days (from GC-MS).

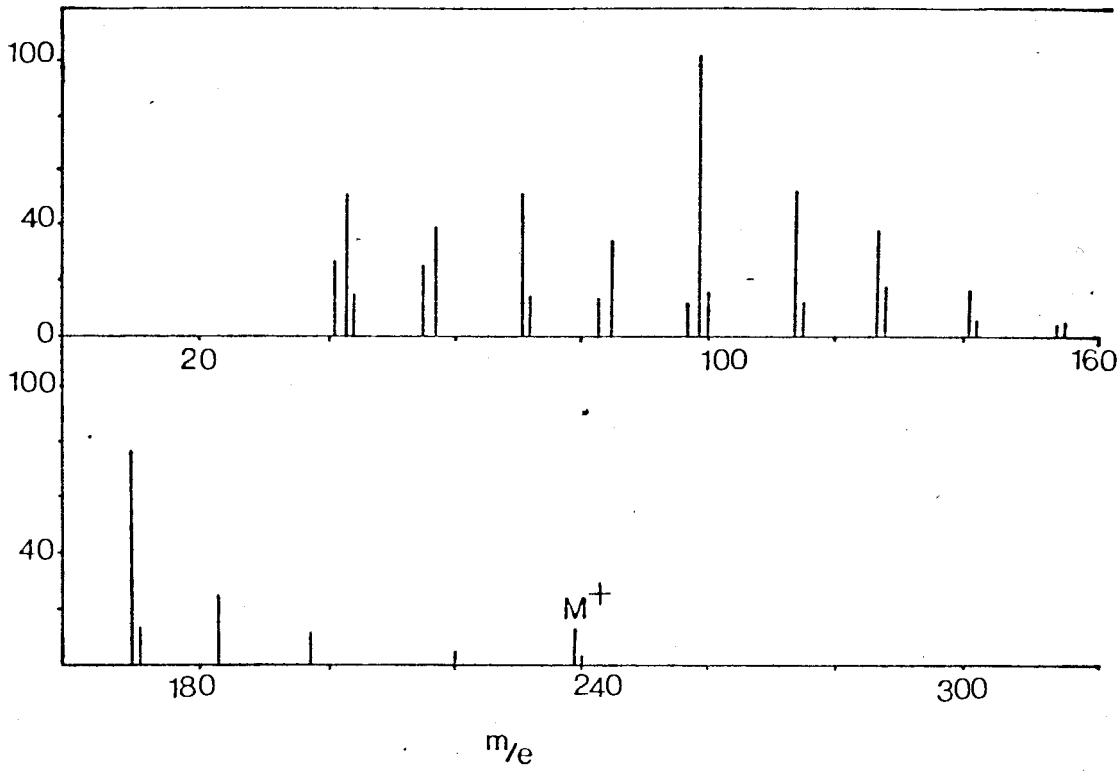

100
40
0
20
100
160
100
40
M+
180
240
300
m/e

Figure 15 : Replot of GC-MS of Acephate sample at pH 8.1, after 7 days.

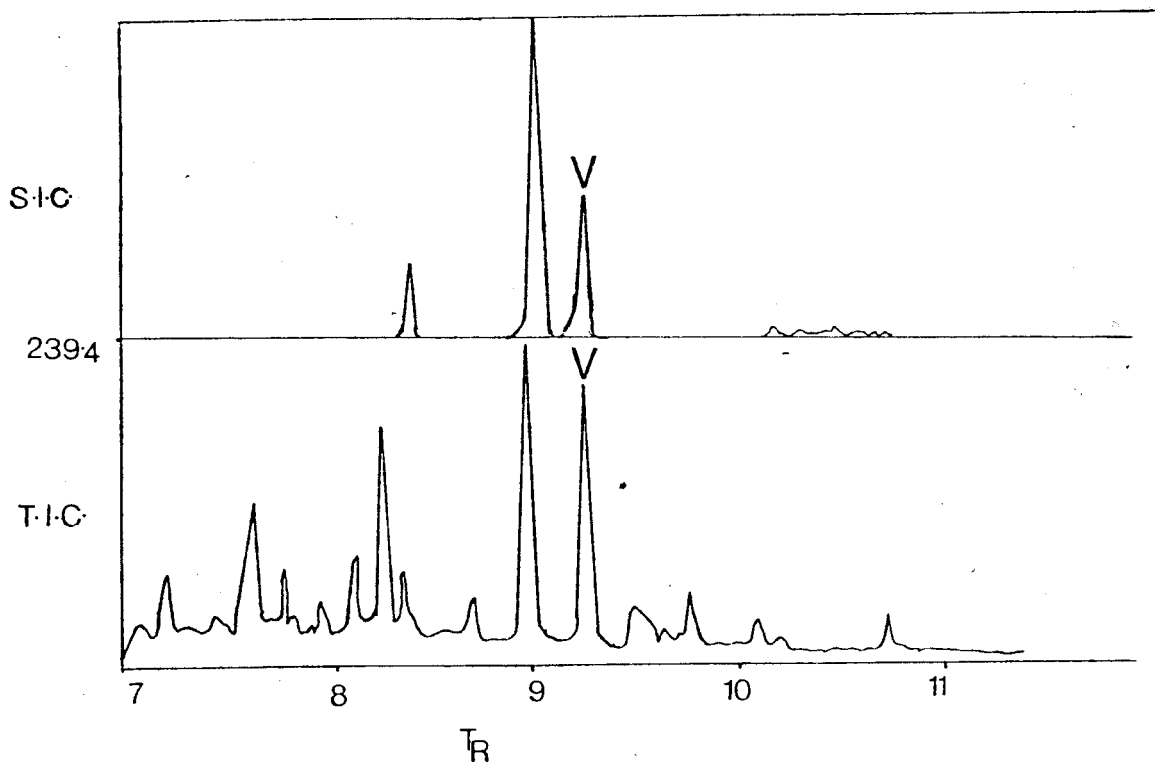

S·I·C·
239·4
T·I·C·
V
V
7
8
9
10
11
TR

Figure 16 : Replot of MS of Acephate Sample at a pH of 8.1, after 7 days (from GC-MS).

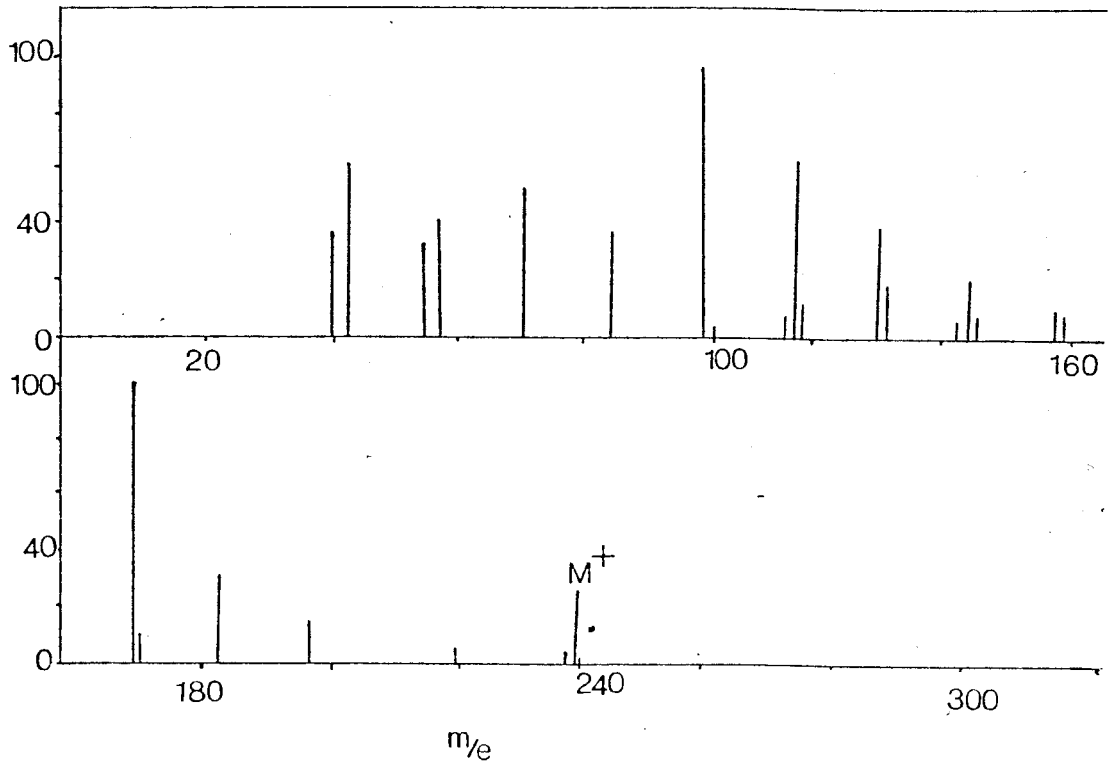

100
40
0
20
100
160
100
40
0
M+
180
240
300
m/e

Figure 17 : Replot of GC-MS of Acephate Sample at a pH of 8.6, after 7 days.

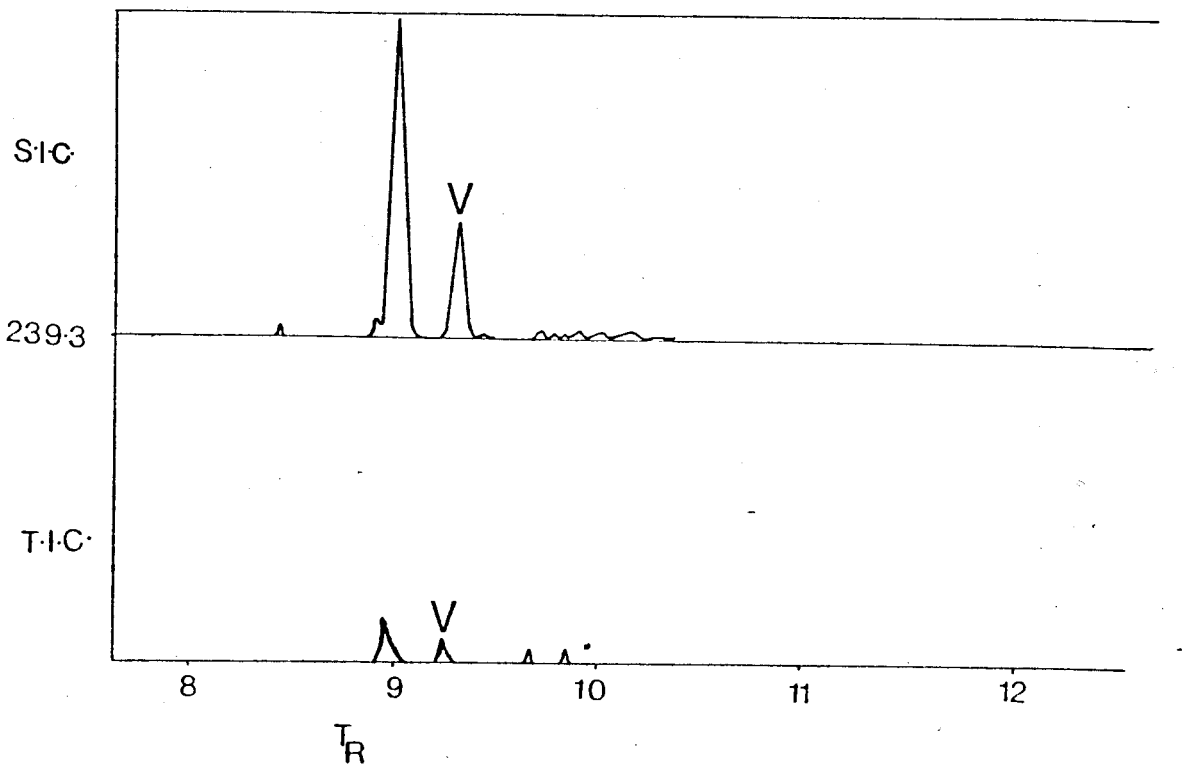

S·I·C·
239·3
V
T·I·C·
V
8
9
10
11
12
TR

Figure 18 : Replot of MS of Acephate Sample at a pH of 8.6, after 7 days (from GC-MS).

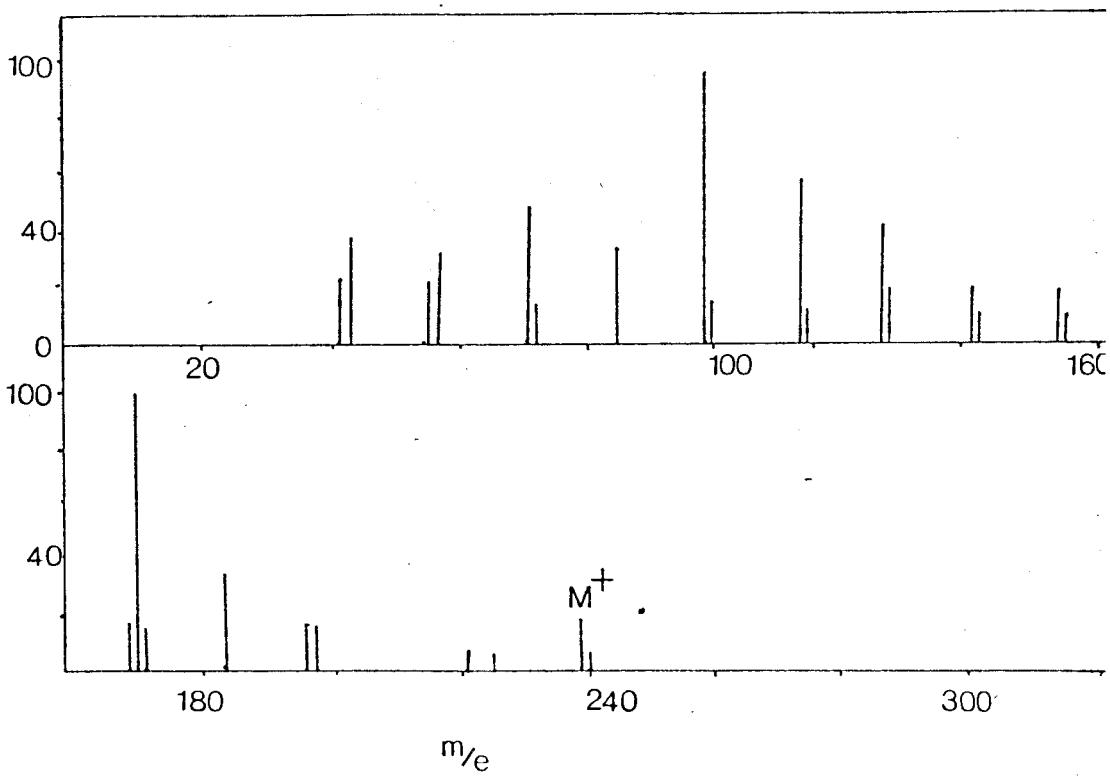

100
40
0
20
100
160
100
40
M+
180
240
300
m/e

Figure 19 : Replot of GC-MS of Acephate Sample at pH 8.1, after 1.5 days.

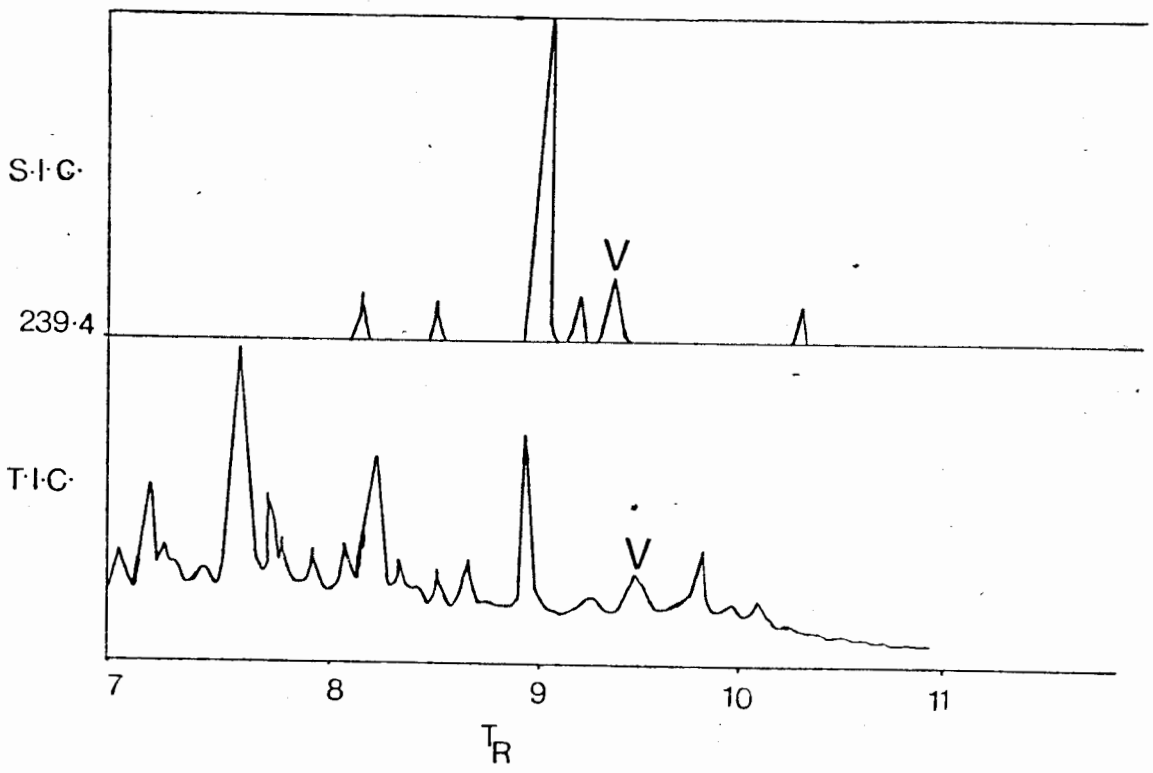

S·I·C·
239·4
V
T·I·C·
V
7
8
9
10
11
TR

Figure 20 : Replot of MS of Acephate Sample at pH 8.1, after 1.5 days (from GC-MS).

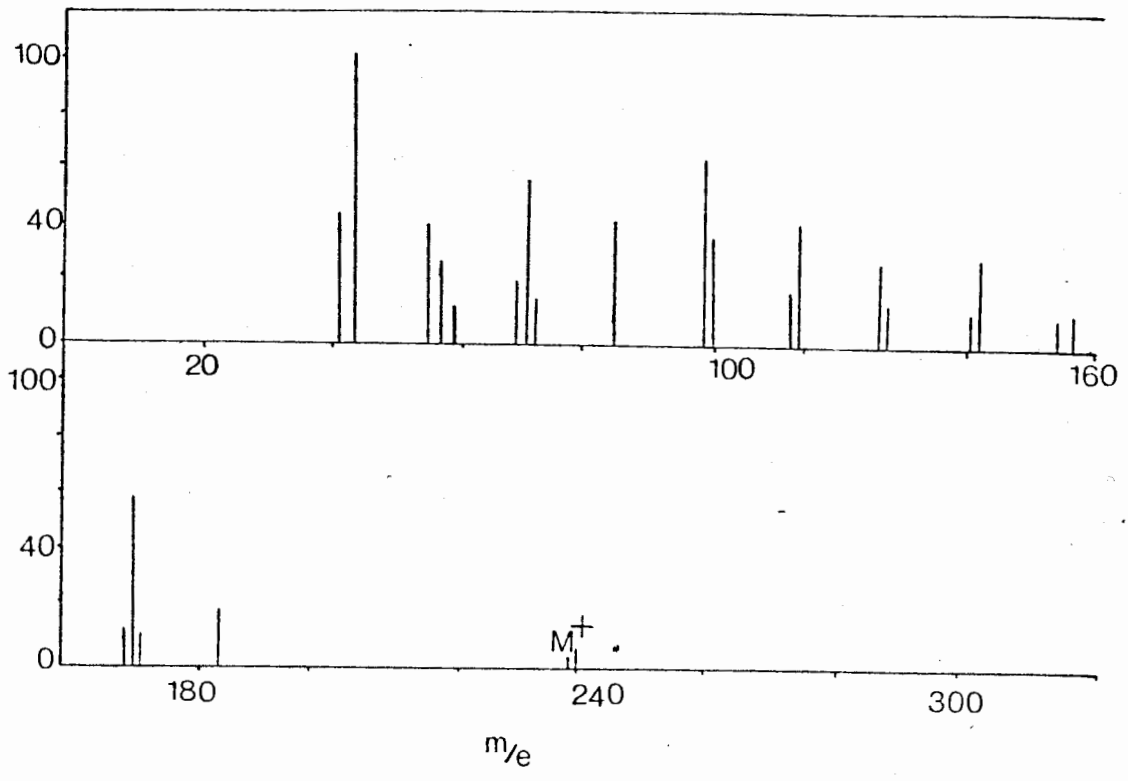

100
40
0
20
100
160
100
40
0
M+
180
240
300
m/e

Figure 21 : Replot of GC-MS of Acephate Sample at pH 8.1, after 3 days.

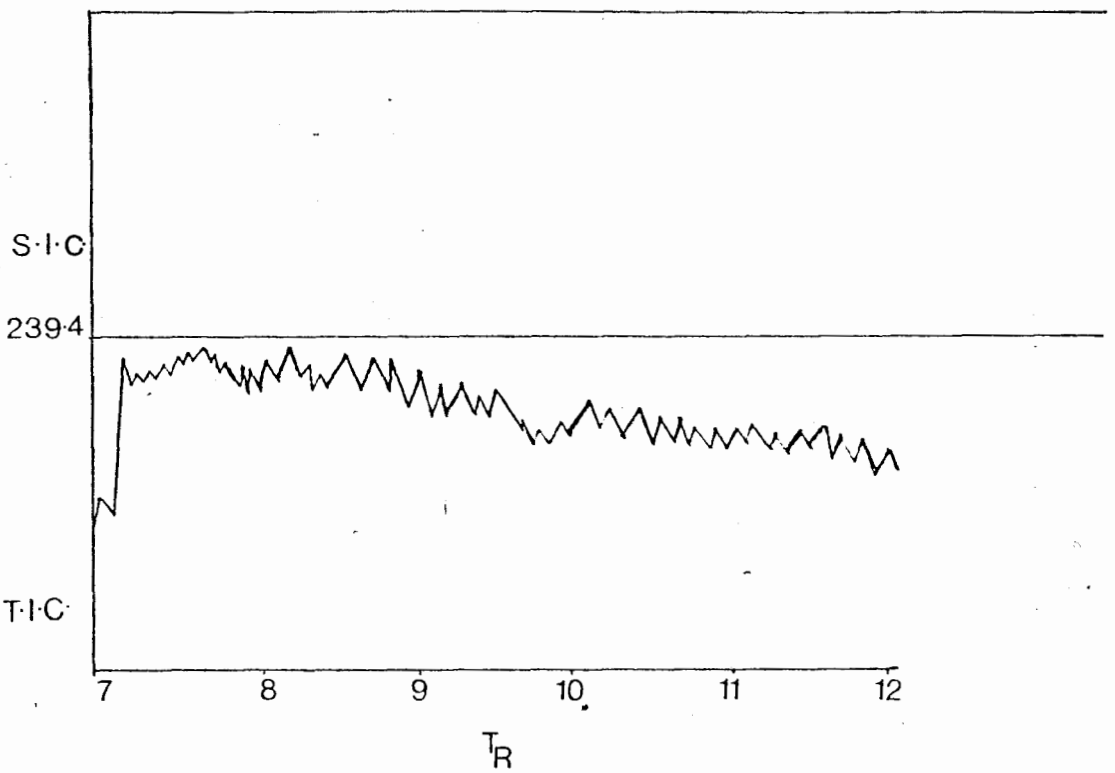
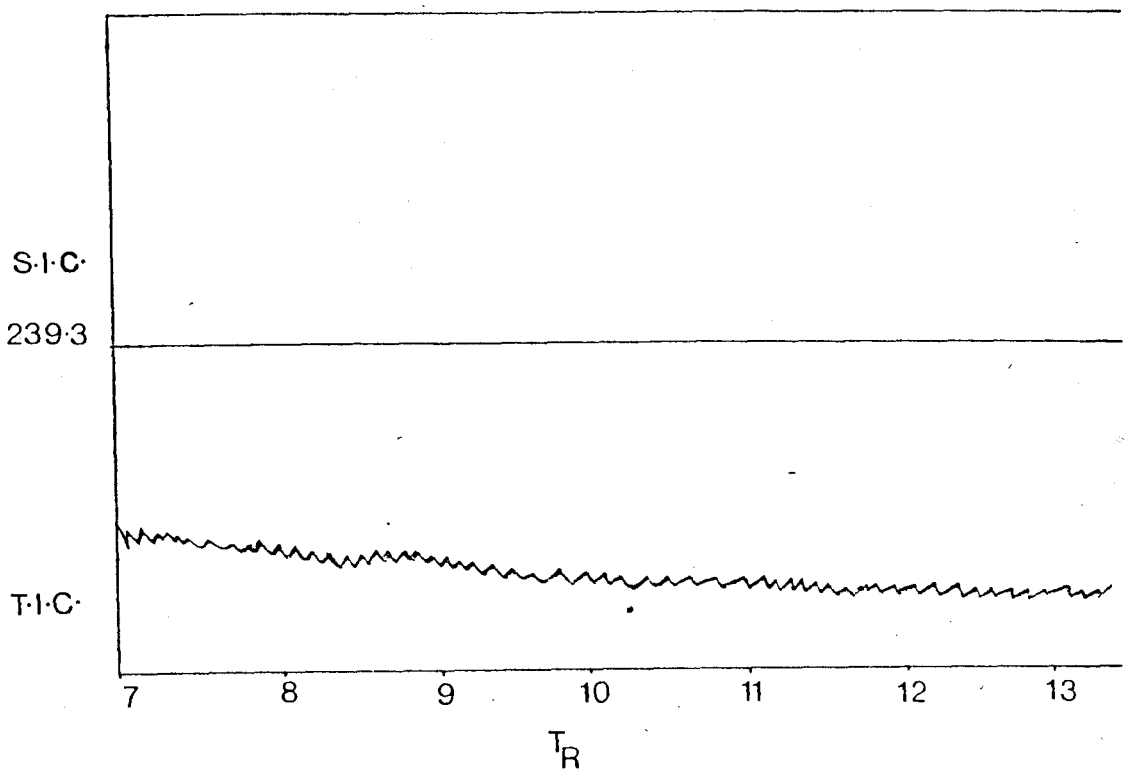

S·I·C
239·4
T·I·C
7
8
9
10
11
12
T_R

Figure 22 : Replot of GC-MS of Distilled Water Blank at pH 8.1, after 7 days.


S·I·C·
239·3
T·I·C·
7
8
9
10
11
12
13
T_R

recoveries at pH 8.1 and 8.6 (Table 3) after 3 days coupled with the possibility of analytical error at pH 7.2 suggest that the non-detectability of (V) was due to further hydrolytic breakdown.

These findings are noteworthy with regards to the information they provide on the chemistry of phosphoramidothiolates, in general.

According to Fahmy et al.(1972), Gerrard and Hamer (1967), Sanborn and Fukuto (1972) and Quistad et al.(1970), P-O and P-S bond cleavage are the most mechanistically favoured hydrolytic reaction of phosphoramidothiolates in alkaline media. The strong electron donating ability of the nitrogen atom relative to the phosphoryl group would seem to prevent any hydroxyl attack on the P-N bond (Eto, 1974; Eto et al. 1977; Quistad et al. 1970). This inductive effect along the P-N bond arises primarily as a result of the electromeric donation of the 2p electrons on nitrogen to form $p\pi$-$d\pi$ bonding with the vacant 3d orbital of the phosphorus atom (Eto,1974).

In this study with acephate, a substituted phosphoramidothiolate, the absence of (IV) and the relatively small levels of (V) seem to dispute the P-O and P-S bonds as the most kinetically-favoured sites for nucleophilic attack. This apparent contradiction between accepted theories and the results of this hydrolytic study is resolved when note is taken that N-substitution on phosphoramidothiolates , as in acephate, introduces a new feature to their chemical reactions with

nucleophilic reagents (Bellet and Fukuto, 1972).

The carbonyl group of the N-acetyl moiety in acephate has electron-withdrawing properties owing to the strong electronegativity of the oxygen atom. The very high levels of (III) detected in this study (Fig.4), therefore suggests that a polarization of the P-N bond takes place, in a direction away from the 3d orbital of phosphorus, due to the electrophilic character of this N-substituted carbonyl group.

The formation of (V) in preference to (IV) is also consistent with the lower pka value of the alkylthiol compared with, the alkylalcohol moiety (Quistad et al.1970).

Since (II), (III) and (V) are still pentavalent phosphorus compounds,each having one $p\pi$-$d\pi$ and four σ bonds, they are still susceptible to SN2-type hydrolysis in anionoic reagents (Hudson, 1965). These secondary transformations are probably responsible for the lower product recoveries after 7 days as opposed to the almost total recoveries before then(Figs.6 to 8 and Table 5).

4.2. Metabolic Studies in Mice Using Acephate

The results of this study are summarized in tables 6 and 7 and Figures 23 to 24b.

The first metabolite detected in the liver of acephatetreated mice within 30 minutes after oral administration This trend has been reported by other workers including Larson (1975). Since the basic rationale of xenometabolism in mammalian

Table 6. Metabolism of Acephate in Mouse Liver : Determination of Residues [1/], expressed in ppm of Acephate Equivalents [2/], including Means and Standard Deviations [3/].

Time, in Hours .	Weight of Mouse (g).	Weight of Liver (g).	Acephate	(II)	(III)	(IV)	(V)
0.5	44	2.9	28.8	1.6	0.0	0.0	0.0
0.5	33	2.6	19.5	1.8	0.0	0.0	0.0
0.5	35	2.4	19.2	1.5	0.0	0.0	0.0
$\bar{X} \pm$ S.D.	37 ± 5.9	2.6 ± 0.3	22.5 ± 5.5	1.6 ± 0.15	0.0	0.0	0.0
4	43	2.9	28.5	2.8	0.0	2.6	0.0
4	42	2.9	9.3	0.93	0.0	3.1	0.0
4	41	2.4	13.8	1.3	0.0	3.6	0.0
$\bar{X} \pm$ S.D.	42 ± 1.0	2.7 ± 0.3	17.2 ± 10	1.7 ± 1.0	0.0	3.1 ± 0.5	0.0
7.25	41	2.0	3.8	0.56	0.01	9.5	0.0
7.25	40	2.7	2.5	0.35	0.13	14.5	0.0
7.25	33	2.3	3.9	0.56	0.11	7.3	0.0
$\bar{X} \pm$ S.D.	38 ± 4.4	2.3 ± 0.4	3.4 ± 0.8	0.49 ± 0.12	0.08 ± 0.06	10.4 ± 3.7	0.0
24	38	2.1	0.35	0.13	0.0	0.0	0.0
24	41	3.0	0.19	0.09	0.0	0.0	0.0
24	41	2.1	0.36	0.08	0.0	0.0	0.0
$\bar{X} \pm$ S.D.	40 ± 1.7	2.4 ± 0.5	0.3 ± 0.1	0.1 ± 0.03	0.0	0.0	0.0
30	35	1.6	0.14	0.05	0.0	0.0	0.0
30	37	1.7	0.1	0.23	0.0	0.0	0.0
30	36	1.9	0.14	0.03	0.0	0.0	0.0
$\bar{X} \pm$ S.D.	36 ± 1.0	1.7 ± 0.2	0.13 ± 0.02	0.1 ± 0.11	0.0	0.0	0.0

1/ Acephate and Compounds (II) to (V) as shown in Figure 1.

2/ Concentrations corrected for changed molecular weights.

3/ No residues detected in controls.

Table 7a. Metabolism of Acephate in Mouse Liver : Recovery of Acephate and Its Residues [1/], expressed as % of Orally Administered Acephate [2/].

Time, in Hours .	Weight of Mouse (g).	Weight of Liver (g)	Acephate	(II)	(III)	(IV)	(V)
0.5	37	2.6	18.8	1.4	0.0	0.0	0.0
4	42	2.7	14.3	1.3	0.0	2.5	0.0
7.25	38	2.3	2.9	0.4	0.07	8.7	0.0
24	40	2.4	0.25	0.08	0.0	0.0	0.0
30	36	1.7	0.13	0.1	0.0	0.0	0.0

1/ Acephate and Compounds (II) to (V) as shown in Figure 1.

2/ Concentrations of (II) to (V) have been corrected for changed molecular weights.

Table 7b. Metabolism of Acephate in Mouse Liver : Recovery of Acephate and Its Residues 1/, expressed as % 2/ of Initial Acephate at 0.5 Hours.

Time, in Hours.	Weight of Mouse (g)	Weight of Liver (g)	Acephate	(II)	(III)	(IV)	(V)
0.5	37	2.6	100	7.1	0.0	0.0	0.0
4	42	2.7	76.4	7.6	0.0	13.8	0.0
7.25	38	2.3	15.1	2.2	0.4	46.2	0.0
24	40	2.4	1.3	0.4	0.0	0.0	0.0
30	36	1.7	0.6	0.4	0.0	0.0	0.0

1/ Compounds (II) to (V) as shown in Figure 1.

2/ Corrected for changed molecular weights.

Figure 23 : Acephate Residues in Livers of Mice following an Oral Application of 120ug/g of Acephate.

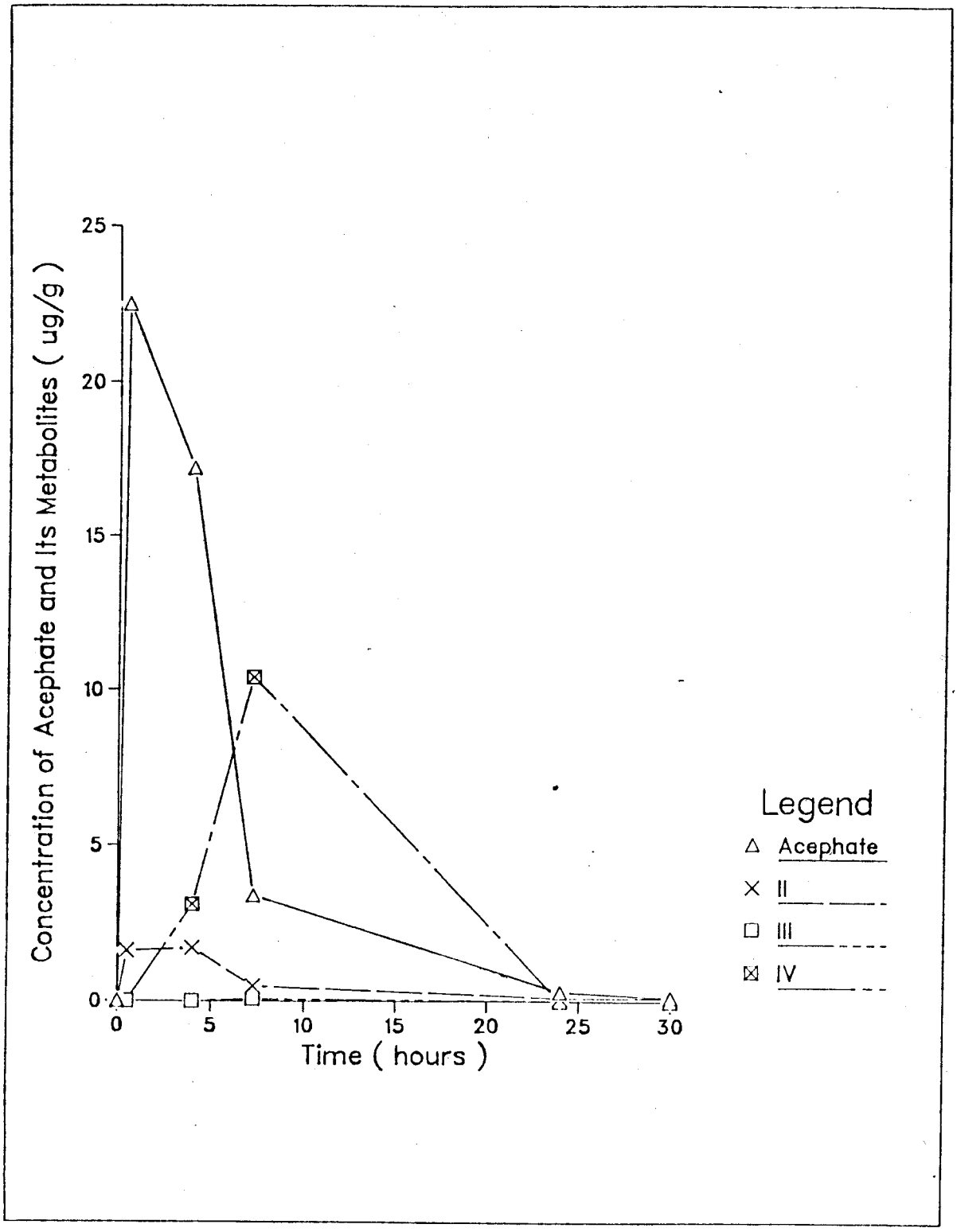

Concentration of Acephate and Its Metabolites (ug/g)
25
20
15
10
5
0
0
5
10
15
20
25
30
Time (hours)
Legend
Acephate
II
III
IV

Figure 24a : Acephate Residues in Livers of Mice, in % of Acephate Administered (see Figure 23).

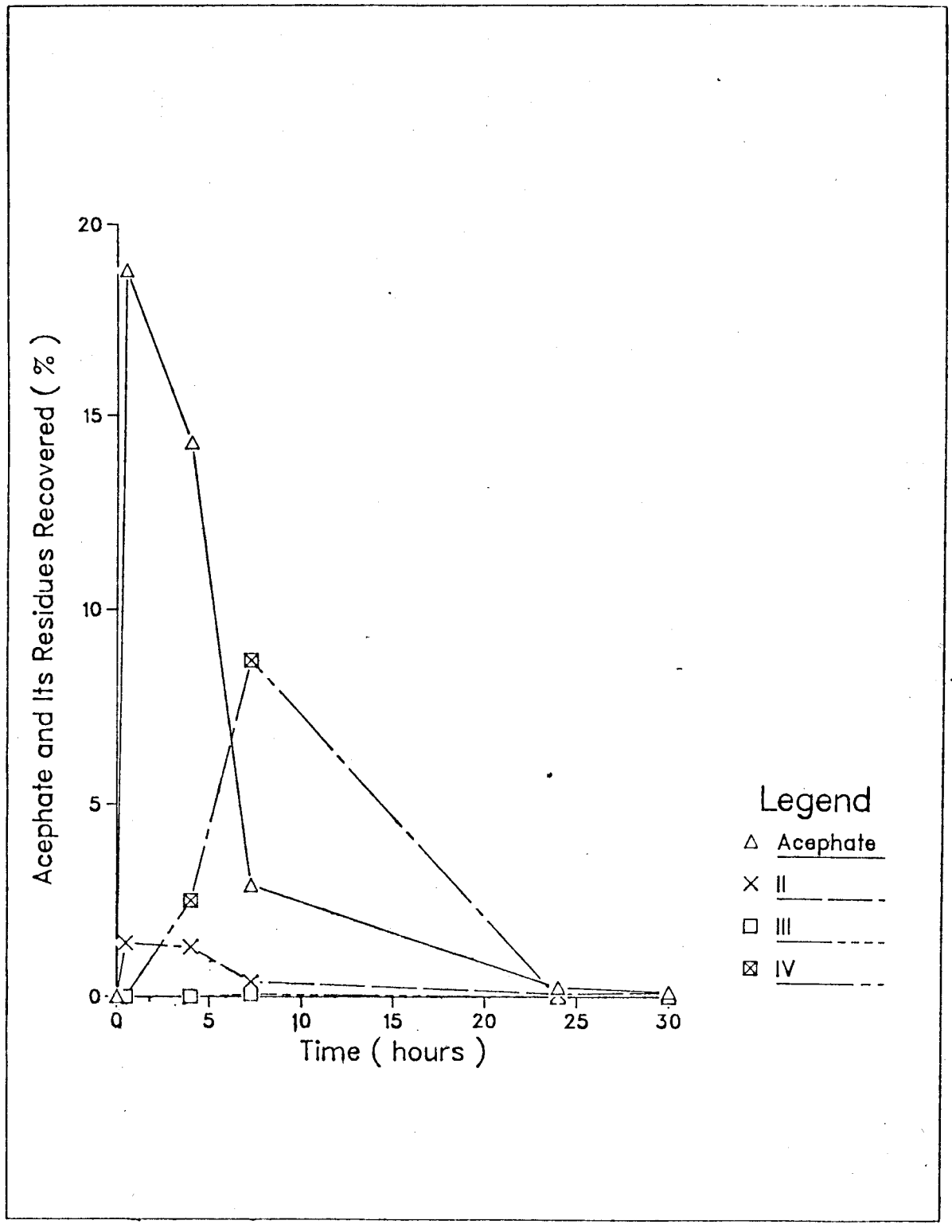

Acephate and Its Residues Recovered (%)
20
15
10
5
0
0
5
10
15
20
25
30
Time (hours)
Legend
Acephate
II
III
IV

Figure 24b : Acephate Residues in Livers of Mice, in % of Acephate found by Analysis in the Livers 0.5 h after Oral Application (see Figure 23).

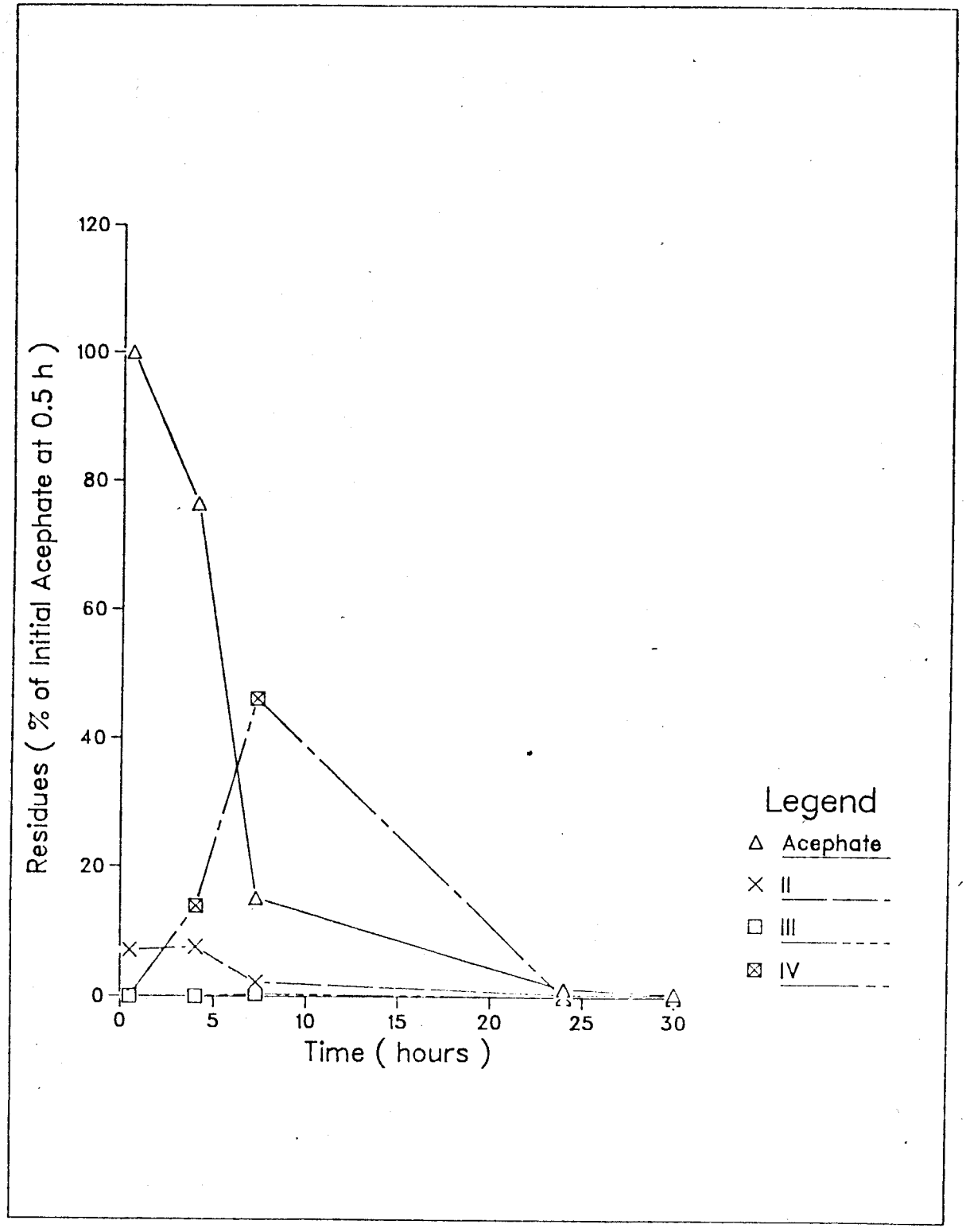

Residues (% of Initial Acephate at 0.5 h)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Time (hours)
Legend
Acephate
II
III
IV

Table 8 : Thin-layer Chromatography of Acephate and Four Major Metabolites [1/] In Various Solvent Systems on Silica Gel-G Plates, Showing Their R_f Values.

Solvent System [2/]	Acephate	(II)	(III)	(IV)	(V)
Acetonitrile : Water (4 : 1)	0.7	0.73	0.55	0.23	0.6
Acetone : Butanol : Water : Ammonia (7 : 2: 0.2: 0.8)	0.32	0.91	N.D.[3/]	N.D.	N.D.
Acetonitrile : Methanol : Water (7 : 2 : 1)	0.63	0.63	0.36	0.27	0.48
Ether : 2-propanol (8 : 2)	0.33	0.45	0.0	0.0	0.13
Acetone : Hexane (2 : 1)	0.23	0.3	0.0	0.0	0.0
Ether : Ethanol (8 : 2)	0.35	0.42	0.07	0.03	0.13
2-propanol : Benzene : Ammonia (6 : 5 : 2)	0.17	0.67	0.12	0.08	0.07

1/ Compounds (II) to (V) as shown in Figure 1.

2/ The ratio of solvents are expressed as volume/volume.

3/ Not determined.

liver cells is to increase the polarity of xenobiotics and render them more amenable to excretion, (Nakatsugawa and Morelli, 1976) It is logical to expect that (II) would be more polar than acephate.

Hussain et al.(1974) observed a general trend towards decreasing polarity with increasing N-alkyl chain length on O,S-dialkyl phosphoramidothiolates. Since such substitutions have the effect of tying up the lone pair of nitrogen electrons involved in $p\pi$-$d\pi$ interaction with the phosphorus atom, it follows that N-acetylation can also lead to a reduced polarity. Larson (1975) using their octanol-water partition coefficients as evidence, reported that (II) is about 1.5 times more polar than acephate. In this study, based on Rf values in the highly polar solvent mixture, acetone : butanol : water : ammonia . (7 : 2 : 0.2 : 0.8 V/V), (II) is more polar than acephate (Table 8).

The detection of (II) in both hydrolysis and metabolic studies suggests a mechanistic parallelism. Since the pH of metabolizing mammalian cells generally lies between 7 and 8, this product similarity with chemical alkaline hydrolysis could , at least, be understood. This empirical parallelism, however, breaks down at 4 and 7.25 hours after the administration of acephate. This is because a metabolite, (IV), undetected in the alkaline water media, became quantitatively significant (Table 6 and Figure 23).

Thus, the concentration of (IV) increased threefold between 4 and 7.25 hours after acephate administration. It was however not detected before or after this time. In contrast, (V), the more kinetically favoured metabolic product, on account of the low pKa value of the alkyl thiol moiety, was not detected Furthermore, (III), the hitherto predominant species in chemical hydrolysis was present in only trace amounts after 7.25 hours.

To explain these results, it must be noted that enzyme-mediated transformations are governed by reaction mechanisms that do not always conform with the mechanistic laws of chemical reactions. In any reaction, whether chemical or biochemical, the electronic composition and arrangement within substrates is a very important factor in the formation of an unstable transition state (Netter, 1969). In enzyme catalysed reaction, the wide variety of electronic arrangements on the enzymes and the multiplicity of spatial orientations arising from it imparts a flexibility to the nature and eventual fate of the enzyme-substrate complex. This mechanistic flexibility can therefore facilitate the formation of, otherwise, energetically-unfavourable products.

Therefore the divergence in degradative pathways between chemical and biochemical systems observed in this study merely restates what is sometimes largely ignored : that enzymes can, and do, influence not only the rate of biochemical reactions but sometimes, as in this study, their directions (Dixon and Webb, 1967; Ling, 1962; Netter, 1969). The limiting factor would be

the presence of enzyme or enzymes with the right structural and biochemical fit for the relevant substrates. A wide range of phosphatases and transferases capable of such O-demethylations have been reported for mammalian liver (Eto, 1974; Fukami and Shishido, 1966; Fukunaga et al.,1969).

It is generally agreed that the metabolism of pesticides in-vivo leads to a production of more polar compounds which are more amenable to excretion (Eto, 1974; Fest and Schmidt, 1973; Nakatsugawa and Morelli, 1976; O'Brien, 1967). Based on their various octanol-water partition coefficients, (II), (III) and (IV) detected in this study are more polar than acephate (Larson, 1975).

4.3. Metabolism of (S-methyl) ^{14}C Acephate in Mice

Substantial agreement exists between the results of this experiment (Tables 9 to 12 and Figures 25 to 28) and the previous one carried out with higher doses of non ^{14}C acephate (Tables 5 to 7 and Figures 23 to 24b).

In the liver, the level of ^{14}C acephate was at 30% of the orally administered value after 0.5 hours. At 7.25 hours after oral treatment, the level was at 17% (Table 10, Figure 27). In contrast, the levels of acephate detected in the non ^{14}C experiment were much smaller (Table 7, Figure 24a). Following the trends as in the previous experiment, radioactive (II), (III) and (IV) were also detected (Table 9). The concentration

Table 9 : Metabolism of Acephate in Mouse Liver after Oral Application of [S-methyl]-^{14}C-Acephate .

Time, in Hours .	Weight of Mouse (g).	Weight of Liver (g).	Residues, in ppm of Acephate Equivalents[1/], Detected as ^{14}C Counts in the Liver[2/]. Acephate	(II)	(III)	(IV)	Unidentified ^{14}C Detected in the Liver, DPM x 10^4.
0.5	37	3.0	1.06	0.23	0.08	0.14	26.3
0.5	44	3.1	0.82	0.14	0.08	0.10	28.7
$\bar{X}$ ± S.D.	40.5 ± 4.9	3.05 ±0.07	0.94 ± 0.17	0.19 ± 0.06	0.08 ± 0	0.12 ± 0.03	27.5 ± 1.7
7.25	41	3.1	0.63	0.07	0.04	0.24	33.8
7.25	43	2.7	0.26	0.04	0.03	0.14	17.5
$\bar{X}$ ± S.D.	42 ± 1.4	2.9 ± 0.28	0.45 ± 0.26	0.06 ± 0.02	0.04 ±0.01	0.19 ± 0.07	25.7 ± 11.5

1/ Concentrations in ug/g corrected for changed molecular weights.

2/ (II), (III) and (IV) as in Figure 1; no residues were detected in untreated controls.

Table 10 : Metabolism of Acephate in Mouse Liver : Acephate and Metabolites [1/] Expressed as % [2/] of Orally Administered [S-methyl] ^{14}C Acephate.

Time, in Hours .	Weight of Mouse (g).	Acephate	(II)	(III)	(IV)	Unknowns
0.5	37	31.9	9.3	3.0	4.6	51.4
0.5	44	27.9	5.8	3.2	4.0	59.2
$\bar{X} \pm$ S.D.	40.5 ± 4.9	29.9 ± 2.8	7.6 ± 2.5	3.1 ± 0.14	4.3 ± 0.42	55.3 ± 5.5
7.25	41	20.1	3.3	1.7	8.1	66.9
7.25	43	13.8	3.2	2.5	8.4	72.2
$\bar{X} \pm$ S.D.	42 ± 1.4	17 ± 4.5	3.3 ± 0.07	2.1 ± 0.6	8.3 ± 0.2	69.6 ± 3.7

1/ referring to compounds (II), (III) and (IV) as in Figure 1.

2/ the percentages have been corrected for changed molecular weights ; no metabolites detected in untreated controls .

Table 11. Metabolism of Acephate in Mouse Liver : Determination of Acephate and its Metabolites [1] in each Liver, expressed as D.P.M. , after Oral Administration of [S-methyl] ^{14}C Acephate.

Time, in Hours .	Weight of Liver (g).	Acephate [2]	(II) [2]	(III) [2]	(IV) [2]	Unknowns [2]
0.5	3.0	16.5	2.8	0.94	2.0	26.3
0.5	3.1	13.3	1.7	0.94	1.5	28.7
$\bar{X} \pm$ S.D.	3.05 ± 0.07	14.9 ± 2.3	2.3 ± 0.8	0.94 ± 0.0	1.8 ± 0.4	27.5 ± 1.7
7.25	3.1	10.2	0.93	0.49	3.6	33.8
7.25	2.7	3.7	0.48	0.32	1.8	17.5
$\bar{X} \pm$ S.D.	2.9 ± 0.28	7.0 ± 4.6	0.7 ± 0.3	0.4 ± 0.1	2.7 ± 1.3	25.7 ± 11.5

1/ Compounds (II), (III) and (IV) as in Figure 1.

2/ DPM x 10^4.

Table 12. Metabolism of [S-methyl] ^{14}C Acephate in Mice : Distribution of Radioactivity Expressed as % of Orally Applied ^{14}C 1/.

Time, in Hours .	Weight of Mouse (g)	^{14}C Administered per Mouse Expressed in D.P.M. x 10^4	Liver (%)	Intestinal Tract . (%)	Faeces (%) x 10^{-3}	Urine (%) x 10^{-5}	Carcass (%).	TOTAL
0.5	37	387	13.2	68.7	0.0	52	8.4	90
0.5	44	460	10.5	51.0	0.0	110	22.5	84
$\bar{X} \pm$ S.D.	40.5 ± 4.9	423.5 ± 51.6	11.9 ± 1.9	59.9 ± 12.5	0 ± 0	81 ± 41	15.5 ± 10	87 ± 4.2
7.25	41	429	11.8	8.0	24.0	7200	56.9	77
7.25	43	450	5.4	5.7	100	260	67.7	79
$\bar{X} \pm$ S.D.	42 ± 1.4	439.5 ± 14.8	8.6 ± 4.5	6.9 ± 1.6	62 ± 53.7	3730 ± 4907	62.3 ± 7.6	78 ± 1.4

1/ no radioactivity detected in non treated control mice.

Figure 25 : Acephate Residues in Livers of Mice following an Oral Application of 2ug/g of [S-methyl] ^{14}C Acephate.

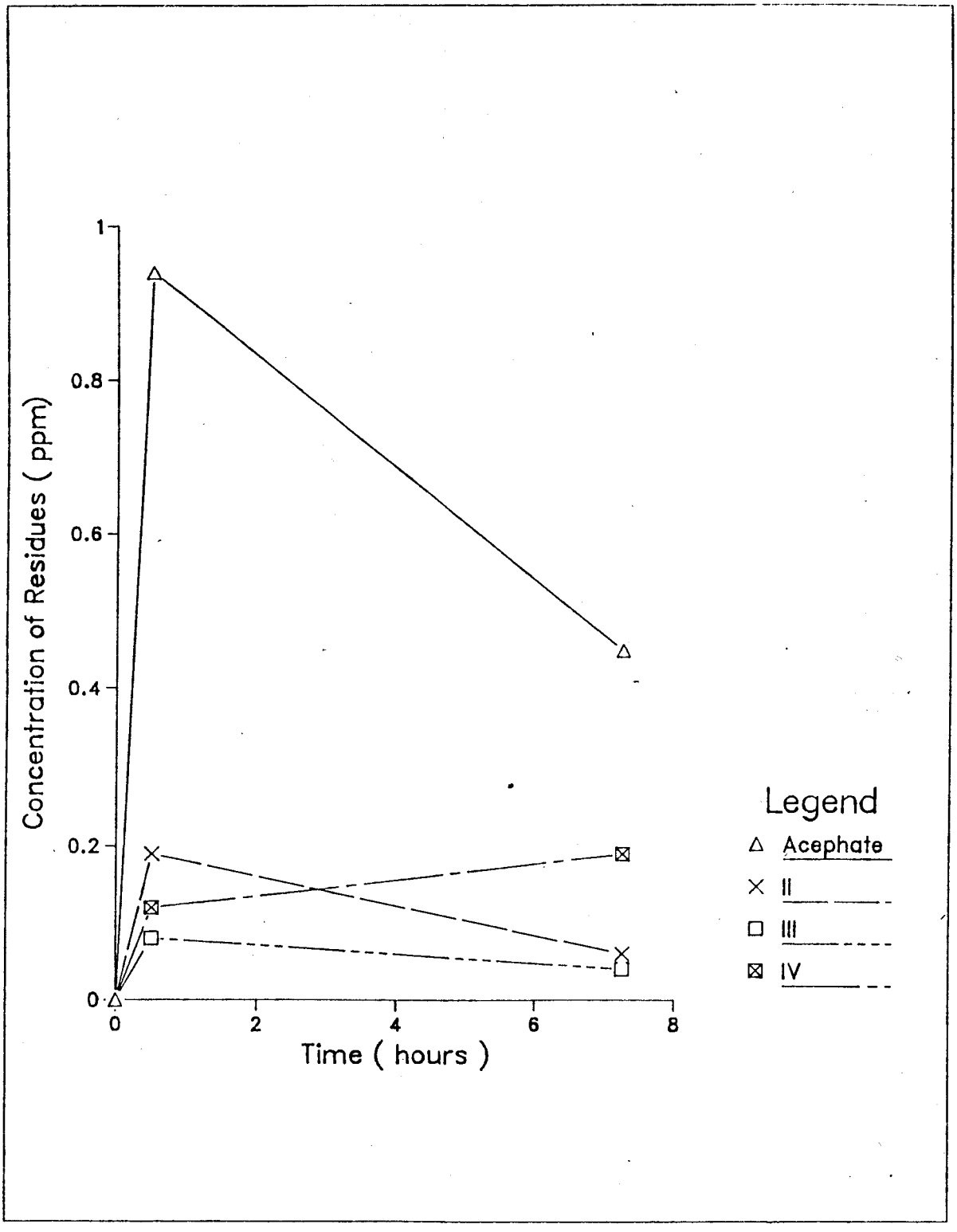

Concentration of Residues (ppm)
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
Time (hours)
Legend
Acephate
II
III
IV

Figure 26 : Acephate Residues in Livers of Mice, in DPM of [S-methyl] ^{14}C Acephate Administered.

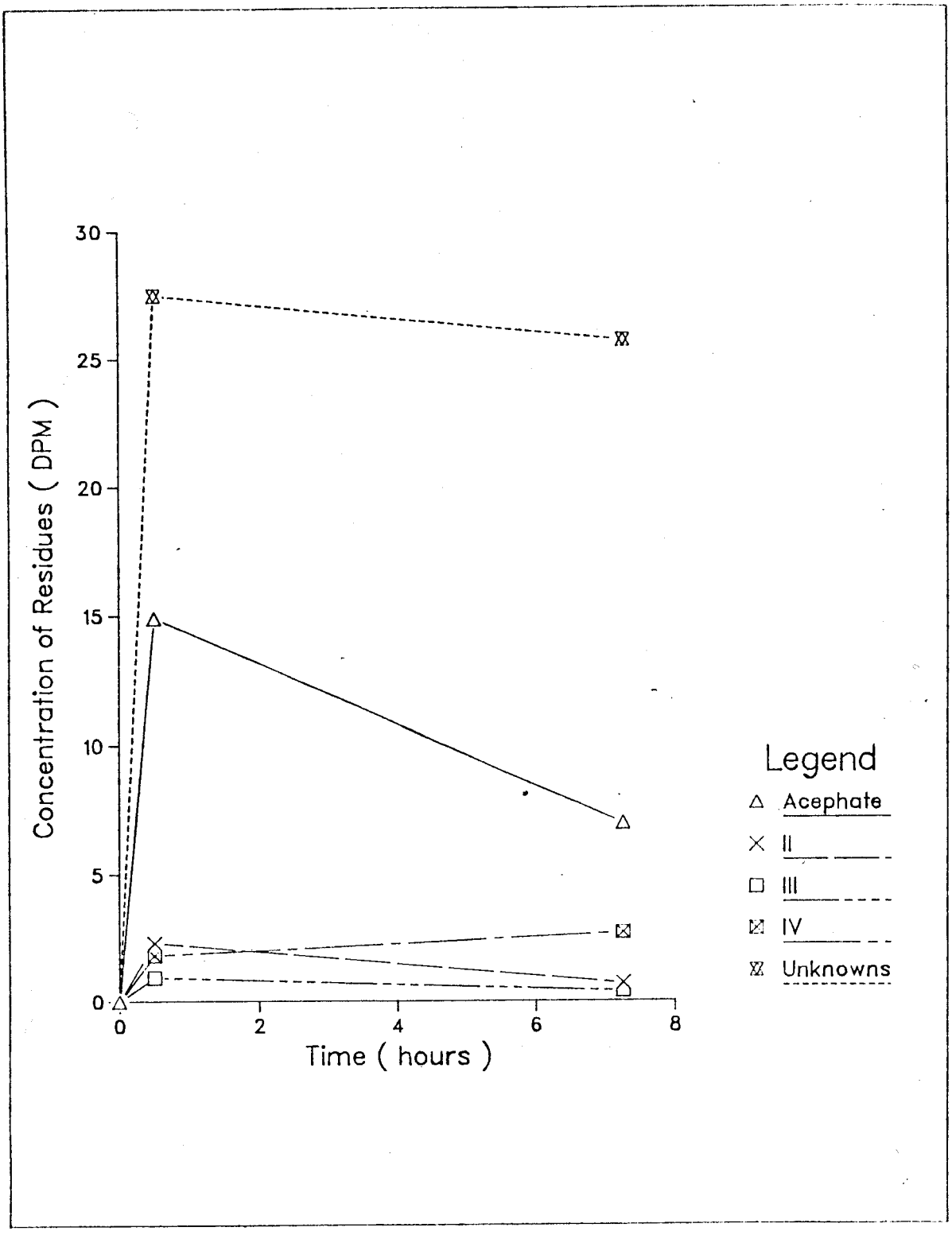

30
25
20
15
10
5
0
Concentration of Residues (DPM)
0
2
4
6
8
Time (hours)
Legend
Acephate
II
III
IV
Unknowns

Figure 27 : Acephate Residues in Livers of Mice, in % of [S-methyl] ^{14}C Acephate Administered (see Figure 25).

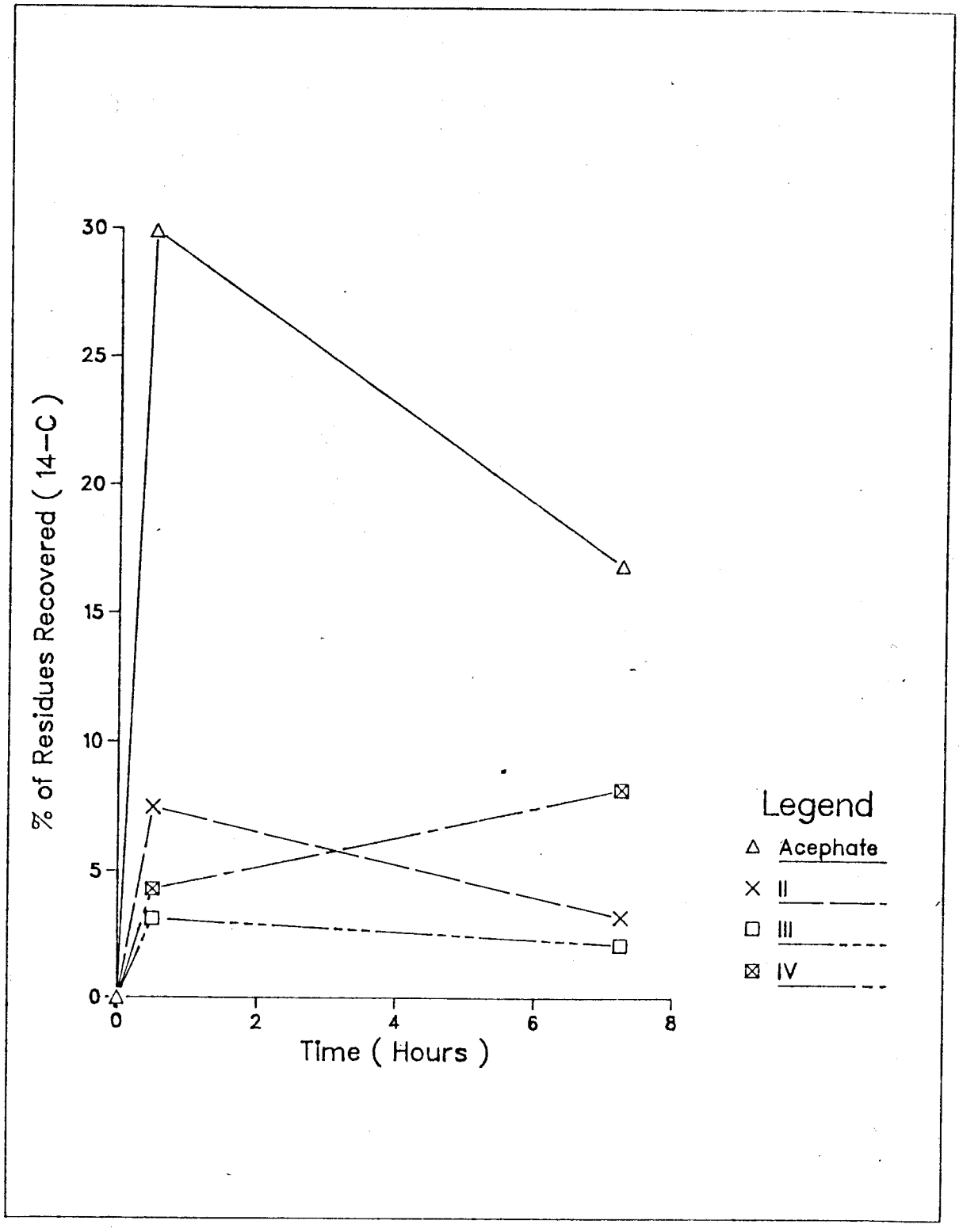
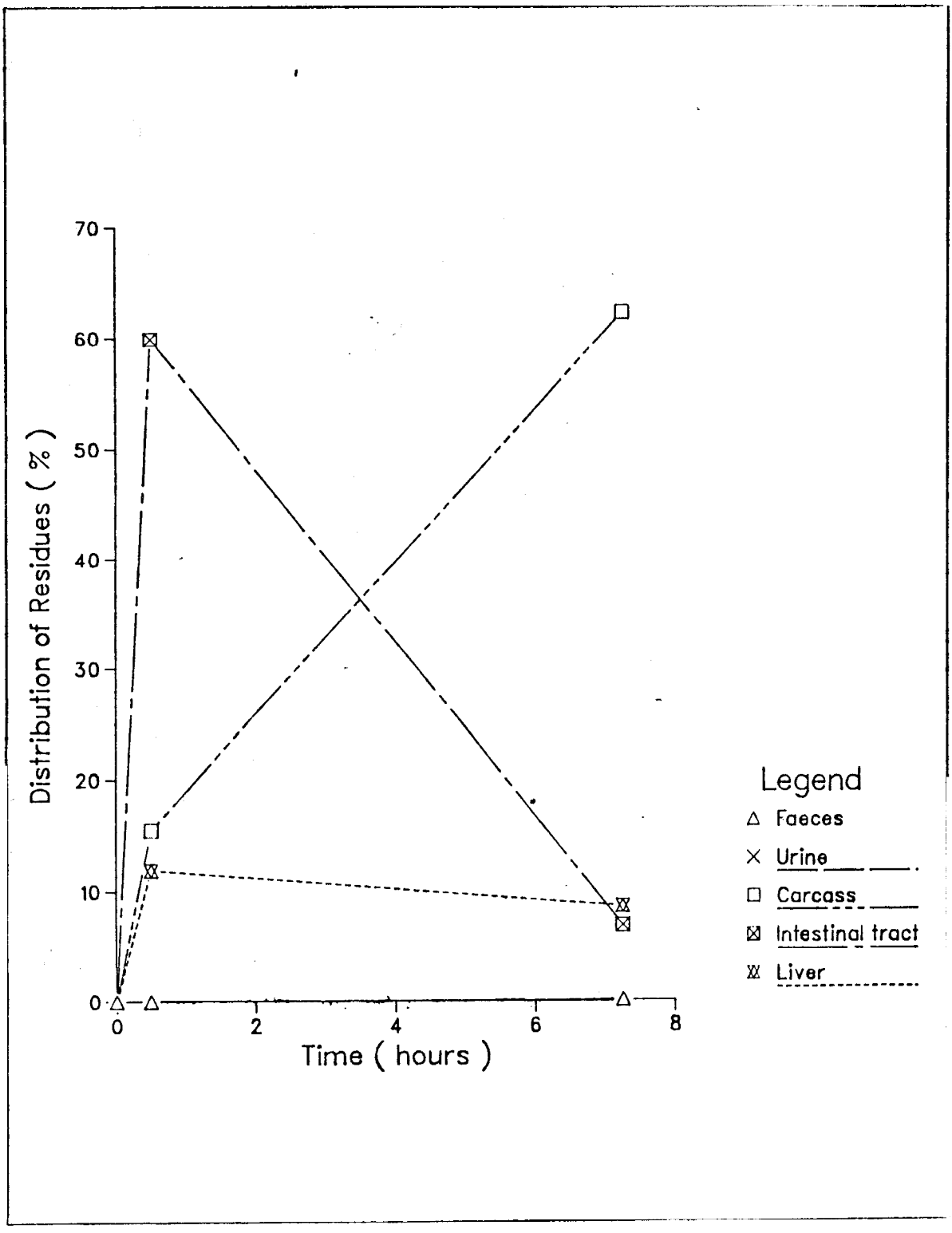

30
25
20
15
10
5
0
% of Residues Recovered (14-C)
0
2
4
6
8
Time (Hours)
Legend
Acephate
II
III
IV

Figure 28 : Distribution of ^{14}C in Mice following an Oral Application, in % of ^{14}C Applied (see Figure 25).


70
60
50
40
30
20
10
0
Distribution of Residues (%)
0
2
4
6
8
Time (hours)
Legend
Faeces
Urine
Carcass
Intestinal tract
Liver

of ^{14}C-(II) in the liver dropped from 8% to 4% of applied ^{14}C acephate between 0.5 and 7.25 hours(Figure 27). levels of ^{14}C-(III) declined from 3% to 2% within the same time. On the other hand, the concentration of ^{14}C-(IV) were higher after 7.25 hours than they were at 0.5 hours after oral administration of ^{14}C acephate (Figure 27).

As in the previous experiment, radioactive (II), (III) and (IV) were not the only metabolites of ^{14}C acephate detected in the liver by the micro-autoradiography studies. Other ^{14}C residues observed were not, however, identified (Table 10).

Compared with the results of the pH studies, the rate of acephate transformation was faster in the metabolic studies with, and without, ^{14}C acephate. The high levels of unidentified radioactivity after 0.5 and 7.25 hours indicates a very fast metabolic change, (Table 10), when compared to the slower process in water where the fate of acephate and the major metabolites was arithmetically accountable for up to 3 days . The implication here is that at 0.5 hours after ^{14}C administration, more than 55% of the methyl thiolate moiety per mole of acephate became involved in a chemical change. At 6.75 hours later, more than 70% underwent a similar fate . This comparatively fast rate of transformation therefore highlights the role of the liver as an important site of rapid xenometabolism in mammalian systems. In the process, it also reveals the kinetic efficiency of its various enzymes with the structural capabilities of metabolizing acephate (Eto,1974;

Fukami and Shishido, 1966; Fukunaga et al. , 1969).

However, an important difference between these two metabolic studies involves the rate of transformation of (II), (III) and (IV) to other secondary metabolites in the livers of the experimental animals. These rates were lower in the studies employing ^{14}C acephate (Tables 7 and 9). Apart from these kinetic differences, most probably due to an isotope effect as well as differing concentrations of orally administered xenobiotic, the lability of the various phosphoryl bonds to enzyme-mediated cleavages remained the same. This is attested to by the fact that (II), (III) and (IV) were detected in both studies.

The systemic distribution of the administered ^{14}C followed a well defined gradient. It was lowest in the liver, intermediate in the carcass and maximal in the alimentary canal after 0.5 hours (Table 12, Figure 28). At 7.25 hours after oral ^{14}Cadministration, the gradient had changed, increasing from the alimentary tract, through the liver, to a maximum level in the carcass (Table 12, Figure 28). These dynamics of ^{14}C migration between tissues agrees with previous models on the movement and distribution of xenobiotics in mammalian systems following oral intake (Brooks, 1976).

Little radioactivity was detected in the urine and none in the faecal matter after 0.5 hours (Table 11,12 and Figure 28). The high water solubility of acephate (Spencer, 1982) probably promotes its dissolution in body water thereby increasing the

ease of absorption through the walls of the alimentary canal. This, could explain the detection of radioactivity in urine as early as 0.5 hours after oral administration of ^{14}C acephate (Brooks, 1976).

The occurence of radioactivity in the faecal matter at 7.25 hours after oral intake (Tables 11, 12 and Figure 28) suggest a possibility of enterohepatic cycling as reported for various mammals by Brooks (1976), Cook (1970) and Wilson and Cook (1970).

About 87% of orally applied radioactivity was recovered after 0.5 hours and 78% after 7.25 hours (Table 12). This suggests that a proportion of the radiolabelled dose was expired as $^{14}CO_2$, indicating loss of the methyl thiolate moiety (Larson, 1975). Since (V) was not detected in the previous metabolic study with non-isotopic acephate, the ^{14}C loss must have arisen from any of the ^{14}C-containing metabolites, including methamidophos.

The loss of this group has been demonstrated in various metabolic studies with mice using S-methyl ^{14}C methamidophos as the xenobiotic (Gray et al.1982; Thompson and Fukuto, 1982). According to Gray et al.(1982), it represents the amount of methamidophos involved in AChE inhibition. The preferential loss of this group in methamidophos was further confirmed in mass spectral and n.m.r. studies with S-methyl ^{13}C methamidophos (Gray et al.1982).

At 0.5 hours after oral administration of ^{14}C acephate 13% of applied radioactivity was lost; at 7.25 hours after, 22% was lost (Table 12). If this loss factor is validly traced to an oxidative loss of the methyl thiolate moiety in methamidophos, then the contribution of the latter in acephate toxicology should be very significant since it is twenty six times more toxic than acephate to mice (Kao and Fukuto, 1977; Spencer, 1982). Metabolic transformation on the ^{14}C methyl thiolate moiety also shares some common features with the oxidative activation on sulphur proposed by Eto et al.,(1977).

However, to postulate any activation of acephate through or to methamidophos, as a mechanism of its biological activity, the toxicological significance of the major metabolites on AChE, at least, must be evaluated.

4.4. Acetylcholinesterase Studies

Tables 13 to 18 show that of the five chemical species tested, only acephate and (II) had anti-AChE activity. (III), (IV) and (V) did not elicit any anti-enzyme activity up to a concentration of 0.36M. No further transformation on these three metabolites in-vivo can be expected to confer sufficient electrophilicity on their phosphorus atom to make them reactive towards the serine hydroxyl moiety of AChE. And no such reactivation is known to have occured in this, or other, study.

Table 13 : Inhibition of RBC [1/] AChE of Mice by Acephate *in vitro* at 37°C.

		ACTIVITY		
Absorbance change per minute. x 10^{-8}	Mean of Absorbance change per minute x 10^{-7}	Rate, in moles/l/min. x 10^{-7}	Activity, in %	Inhibitor Concentration (M) x 10^{-3}
155 120	13.75	10.1	100	0.0
100 105	10.25	7.5	74.26	12.8
65 80	7.25	5.3	52.48	16.0
65 45	5.50	4.0	39.60	19.2
35 45	4.0	2.9	28.71	22.4

1/ Red Blood Cell.

Table 14 : Inhibition of RBC AChE of Mice by Methamidophos in vitro at 37°C.

Absorbance change per minute x 10^{-8}	Mean of Absorbance change per minute x 10^{-7}	ACTIVITY: Rate, in moles/l/min. x 10^{-7}	ACTIVITY: Activity, in %	Inhibitor Concentration (M) x 10^{-3}
185 215	20.00	14.70	100	0.0
135 135	13.50	9.30	67.55	0.8
75 70	7.25	5.33	36.26	1.6
65 70	6.75	4.96	33.74	2.4
65 65	6.5	4.78	32.52	3.2
65 50	5.75	4.23	28.78	4.8

Table 15 : Inhibition of RBC AChE of Mice by (III) in vitro at 37°C.

Absorbance change per minute x 10^{-4}	Mean of Absorbance change per minute x 10^{-4}	ACTIVITY		Inhibitor Concentration (M) x 10^{-3}
		Rate, in moles/l/min. x 10^{-7}	Activity, in %	
165 190	177.5	13.10	100	0.0
195 190	192.5	14.20	108	21.0
180 215	197.5	14.50	111	52.5
225 215	220.0	16.20	124	157.6
150 175	162.5	11.95	91	262.7

Table 16 : Inhibition of RBC AChE of Mice by (IV) in vitro at 37°C.

Absorbance change per minute $\times 10^{-4}$	Mean of Absorbance change per minute $\times 10^{-4}$	ACTIVITY		Inhibitor Concentration (M) $\times 10^{-3}$
		Rate, in moles/l/min. $\times 10^{-7}$	Activity, in %	
155 190	172.5	13.0	100	0.0
195 170	182.5	13.0	100	20.4
155 245	200.0	15.0	115	40.8
165 185	175.0	13.0	100	255.1

Table 17 : Inhibition of RBC AChE by (V) *in vitro* at 37°C.

Absorbance change per minute x 10^{-4}	Mean of Absorbance change per minute x 10^{-4}	ACTIVITY		Inhibitor Concentration (M) x 10^{-3}
		Rate, in moles/l/min. x 10^{-7}	Activity, in %	
155 160	157.5	12.0	100	0.0
190 225	207.5	15.0	125	28.1
205 260	232.5	17.0	142	57.2
195 245	220.0	16.0	133	85.8
240 105	172.5	13.0	108	145.4
135 125	130	9.6	80	363.5

Table 18 : Concentrations of Acephate and Its Metabolites (II), (III), (IV) and (V) [1/] Inhibiting 50% (I_{50}) of Mouse RBC AChE *in vitro*.

Inhibitor	Equation [2/]	r [3/]	I_{50} (M)
Acephate	$-\frac{1}{A} = 766\ [I] + 1.7$	0.87	50×10^{-5}
(II)	$-\frac{1}{A} = 176\ [I] + 2.3$	0.93	5.0×10^{-5}
(III)	$-\frac{1}{A} = 32746\ [I] + 209$	0.34	> 0.26
(IV)	$-\frac{1}{A} = 51033\ [I] + 419$	0.22	> 0.26
(V)	$-\frac{1}{A} = 49124\ [I] + 331$	0.75	> 0.36

1/ See Figure 1.

2/ In the above equation,

A = enzyme activity, expressed as % of uninhibited enzyme;

[I] = concentration of test inhibitor in molar units;

I_{50} = the value of [I] when $\frac{1}{A} = 0.02$.

3/ Correlation Coefficient.

Therefore, considerations of terminal residues of acephate in relation to its insecticidal action should devolve on the parent compound itself, and (II).

Since the chromogenic reagent, DTNB, used for the quantification of AChE activity is very sensitive to free thiol groups, the high enzyme activity observed following incubation with (V) should not be interpreted as being due to an activation (Table 17). Also, differences in AChE titre between the test mice may also have contributed to the comparatively high AChE activity values.

The I_{50}, used for the quantification of the anti-AChE activity of acephate and its metabolites is still a reliable guide, under specified conditions, despite its complex kinetic variables (Boyer, 1967; Dixon and Webb, 1964; Fest and Schmidt, 1973). Even then, numerical departures are to be expected from other published figures (Quistad et al.1970; Kao and Fukuto,1977; Mohammad,1982) especially when the kinetic assumptions made in the equation

$$1/v = A + Km.\,(I)/K\ V\ S$$

are critically analysed (Dixon and Webb, 1964; Netter, 1969). In this equation, v = initial velocity

V = maximum velocity

K = the equilibrium constant of the inhibition

S = substrate concentration

Km = the Michaelis-Menten constant

(I) = inhibitor concentration

A = constant

Firstly, the equation assumes that the enzyme concentration would be constant and infinitely less than that of the substrate. This situation is technically difficult to achieve with enzymes like AChE , having phenomenally high turn-over numbers.

Secondly, the pre-incubation time of enzyme with inhibitor introduces a significant variability to V. This is especially so when the possibility of AChE aging exists, as with acephate (Quistad et al.,(1970)

Thirdly, the equation assumes an all purpose validity for Michaelis-Menten kinetics. Incidentally, some enzyme reactions go through many enzyme-substrate intermediates and side reactions which may be affected by inhibitors in ways that would be difficult to analyse by Michaelis-Menten kinetics (Netter, 1969).

Finally, random errors are likely to arise with the inevitable sorption of the test inhibitors to other erythrocyte proteins and ligands (Boyer, 1967).

Thus, the numerical value of I_{50} for acephate and methamidophos towards erythrocyte AChE, though differing from other published values , still follows the same order of magnitude, with the latter having a stronger anti-enzyme activity than the former.

It would be relevant to attempt a correlation of these in-vitro results to a fixed charge system such as a living cell where there is a greater site-specific association of ions and non-electrolytes. In such a system, no large quantity of free substrates, such as 0.075M for acetylthiocholine, would be thermodynamically feasible (Ning Ling, 1962). According to Ning Ling (1862), the problem of coexistence between enzymes and their substrates in living cells is solved by sequestering the substrates in such a fashion that chances of interacting with enzymes are kept to a minimum.

Theoretically speaking, therefore, the true I_{50} in-vivo with a relatively higher proportion of free enzymes and substrate concentrations that are low and sequestered is expected to be smaller than the case in-vitro . Xenobiotics such as acephate and (II) can therefore manifest a greater lethal bioactivity than their in-vitro determined I_{50}'s would suggest. Acephate would therefore seem to exert its biological activity by three cooperative processes.

Firstly, by its anti-AChE activity which , though characterized as weak from in-vitro experiments, may be very significant in an ordered physiological environment. The aging of AChE by acephate in-vitro, reported by Ouistad et al.(1970) could be a very critical factor in its animal toxicology if such could be proven to occur in-vivo.

Thus, irreversible inhibition of AChE resulting from aging could decrease several-fold its I_{50} to acephate due to the

decreased concentration of free enzyme.

Secondly, by metabolic conversion to methamidophos, a more effective AChE inhibitor. The high tissue persistence of methamidophos has been reported by Khasawinah et al.,(1978). They reasoned that this property would ensure a constant internal concentration for a sufficiently long period of time that would permit the manifestation of its slowly expressed toxicity. Even though the levels of metabolically derived methamidophos are low, their twenty six times greater bioactivity over acephate makes it a likely activation product of acephate in some mammalian systems. Moreover if the 13% to 22% loss of radioactivity balance in the ^{14}C acephate study is validly traced to the alkylthiol moiety of methamidophos, then its role in the biological expression of acephate toxicity would be well established.

A third feasible mechanism of acephate bioactivity would involve an in-vivo sulphoxidation of its metabolically derived methamidophos. As reported by Eto et al. (1977), the sulphoxide of methamidophos is a hundred times more active than its parent compound in its anti-AChE action. Such a highly toxic intermediate would place methamidophos as a key intermediate in the biological activation of acephate. The principal flaw in this postulate is that such an active chemical species has not yet been isolated and characterized. Other workers, including Gray et al.,(1982) have not been able to observe such activations in more physiological environments. Nevertheless,

the preferred loss of the alkylthiol moiety of methamidophos shares some common features with the report of Eto *et al.*,(1977) and points to its important role in the biochemical lesion evinced by methamidophos and, ultimately acephate, in higher animals.

5. CONCLUSION

From the results of this study, the following conclusions can be drawn :

(1) In distilled water, buffered at alkaline pH, the hydrolytic breakdown of acephate proceeds principally through fissure of the P-N bond. Other minor routes include the N-C and P-S bonds.

(2) In mice, to which acephate has been administered by oral application to the stomach, the principal metabolites in the livers were formed by P-O and N-C bond fissures respectively. The latter was the highest 0.5 hours after acephate application while the former became predominant at 4 and 7.25 hours.

(3) Factors that determine the lability of the various bonds in acephate are different for both enzymic and non-enzymic reactions.

(4) Most of acephate residues not detected in the livers of treated mice were distributed in the intestinal tract, carcass, urine and faecal matter.

(5) The loss of radiobalance in the ^{14}C study suggests an oxidative formation of $^{14}CO_2$ from the methyl thiolate moiety of acephate. It may represent the amount of metabolically formed methamidophos involved in AChE inhibition.

(6) Of the four major metabolites of acephate studied, methamidophos has anti-AChE properties higher than acephate in-vitro . The others have no inhibitory action on AChE up to a

concentration of 0.26 M in-vitro .

(7) Considerations of an active principle of acephate should revolve around acephate itself, and methamidophos.

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