STUDIES ON THE ACTIVITY AND METABOLISM OF THE INSECTICIDE ACEPHATE IN A PEST INSECT, *CHORISTONEURA OCCIDENTALIS*, AND IN OTHER NON-TARGET ORGANISMS

by

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Studies on the activity and metabolism of the insecticide acephate, in a pest insect, Choristoneura occidentalis, and in other non-target organisms

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ABSTRACT

Aspects of the activity and metabolism of acephate, an organophosphorus insecticide, were studied in non-diapausing, laboratory-bred larvae of the western spruce budworm Choristoneura occidentalis Freeman, larvae of the Mediterranean flour moth Anagasta kuehniella (Zeller), and in male Wistar rats. Inhibition of human erythrocyte acetylcholinesterase (AChE) *in vitro* was investigated also.

Topically applied acephate was twice as toxic to last-instar larvae of western spruce budworm (LD_{50} 23.2 μg/g) as to last-instar larvae of Mediterranean flour moth (LD_{50} 48.3 μg/g). However, it was less toxic to budworm larvae than methamidophos (LD_{50} 7.5 μg/g) or paraoxon (LD_{50} 1.34 μg/g). These results were supported by a series of *in-vitro* experiments in which anti-AChE activities were paraoxon > methamidophos > acephate. Methamidophos was confirmed to be a metabolite of acephate.

The inhibition of AChE by acephate was progressive for 30 to 60 minutes. Maximum inhibition of AChE from insects was reached faster than maximum inhibition of AChE from rats and man. From 10 to 33% of AChE were inhibited immediately, depending on the enzyme source.

Treating the insects with sublethal doses of acephate produced substantial and irreversible depression of AChE

activities, but after similar doses in rats, the brain and erythrocyte AChE and serum cholinesterase activities had returned to 79 to 93% of the control levels after 72 hours.

Acephate was metabolized quantitatively to methamidophos in the insects and in rat livers for up to 12 hours after treatments with low doses. Thereafter, both compounds appeared to be transformed rapidly into innocuous substances and eliminated.

Incubation of acephate with microsomal preparations from rat livers and midguts of budworm larvae prior to or during incubation with AChE did not affect the anti-AChE activity of acephate. Methamidophos and acephate could not be shown to synergize or antagonize each other's anti-AChE activities *in vitro*.

In organisms treated with acephate, no substance could be discovered with greater anti-AChE, or insecticidal, activity than methamidophos; nor could any indirect evidence be found in support of any hypothetical substance that might arise from either acephate or methamidophos and be the toxic principle of these two insecticides.

A diapause-free colony of western spruce budworm was established and maintained, with a generation time of 38 to 40 days. Females deposited an average of 307 eggs; the survival was about 91%.

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1. INTRODUCTION

Pest control is necessary for the production of adequate food and fibre, which are vital to the well-being of mankind. For centuries, man has supplemented the inadequacy of inherent regulatory mechanisms with other pest control practices, such as biological, cultural, physical, and mechanical controls. Only recently, synthetic insecticides have provided effective, fast control. Now the first line of defence against many devastating insect pests, they have minimized catastrophic crop damage, preserved valuable forests and parklands from destruction, and protected households against damaging insects. Moreover, synthetic insecticides have been credited with saving millions of lives through control of disease-carrying pests. Thus, pesticides have been of great benefit to man, and continue to play a major part in crop production and human health.

Chlorinated hydrocarbons were among the first synthetic organic insecticides to be used widely against many species of insect pests. Forest pests like the spruce budworm, gypsy moth, and tussock moth against which man had been comparatively helpless, became susceptible to treatment. DDT proved to be effective in spray programmes and was generally considered to be suitable for use in forest insect control.

Soon, however, the success of chlorinated hydrocarbon insecticides was reduced by the development of resistance by insect populations. In addition, persistent residues of the parent compounds and their metabolites were detected more and more frequently in non-target locations, e.g., in air, natural water bodies, soil, and in the food and tissues of invertebrates, fish, birds, mammals, and man. Chemical persistence and resultant environmental contamination provoked concern that continued widespread use of such pesticides would cause adverse long-term effects on the environment and on human health. Thus, despite its effectiveness and successes, the use of DDT to control forest pests remained controversial (Harwood 1975). Finally, DDT and other chlorinated hydrocarbons were taken out of common use during the 1960's. High priority was given to searching for environmentally less hazardous insecticides, especially for the control of spruce budworm.

Since many organophosphorus (OP) and carbamate insecticides are very effective, yet relatively non-persistent, their toxicity, metabolism, and selectivity have been actively investigated. As a result, several insecticides with reduced environmental impact have been used. These include malathion, dimethoate, fenitrothion, carbaryl, acephate and its metabolite, methamidophos.

An ideal insecticide should be highly toxic to a pest insect, but innocuous to other forms of life with which it comes into contact (Winteringham and Barnes 1955). It should show its

maximum physiological selectivity against only one or a few insect species. Although insecticides of such specificity may not exist, acephate, an OP insecticide with high insecticidal activity and low mammalian toxicity, perhaps comes close to it. As a promising candidate in forest and agricultural pest control programmes, it has generated investigations to understand its selectivity. It is now widely used for insect control, but the molecular basis of its selectivity is still not well understood.

Information concerning the fate of a pesticide after application is essential for its safe and effective use. It is important, for instance to know whether a compound will persist or be detoxified. Persistency may provide prolonged control, but rapid detoxification may be even more desirable to avoid accumulation. Of particular significance in this respect is metabolic transformation of a pesticide by biological systems. The importance of this becomes apparent when one considers these facts: that the toxicity of an insecticide can be decreased or intensified upon conversion to a metabolite; that the duration of its toxic action is related to the rate and manner in which it is metabolized; that the rate of elimination from the body depends on the physico-chemical properties of its metabolic products; and that its transport to a site of action can be limited by the rate at which it is metabolized and the character of the metabolic products (Menzie 1969). Therefore, it is important to know the pathways of metabolism as well as the degree of accumulation of metabolic products in tissues. From a

practical standpoint, the identification and quantification of residue levels of pesticides and their metabolites are necessary in order to evaluate fully the effects of pest control programmes that involve pesticides.

It is the objective of this research to investigate aspects of the mechanism of selectivity of acephate by studying the toxicity and metabolism in a target insect species and other non-target species. To accomplish this, laboratory cultures of the insects had to be established, to ensure the supply as needed.

2. LITERATURE REVIEW

2.1. The Western Spruce Budworm

The problem of infestations and outbreaks of spruce budworms, *Choristoneura* spp., in the coniferous forests of North America has long been recognized. Species in this genus are amongst the most destructive forest insect pests (McKnight 1968), defoliating millions of hectares of conifer forests in Canada and the United States. One of this group is the western spruce budworm, *C. occidentalis* Freeman (Freeman 1967). It is very widely distributed and the most destructive defoliator in western North America, ranging from interior southern British Columbia to Washington, Oregon, California, Montana, Idaho, Colorado, and New Mexico (Carolin and Honing 1972; Stehr 1967). Its principal host is the Douglas-fir, but it also attacks true firs including grand-, white-, and subalpine-firs; and blue, Englemann, and white spruces (Carolin and Honing 1972; McKnight 1968).

2.1.1. Life-history, Infestation, and Outbreaks

The life-history of western spruce budworm under field conditions was described by Carolin and Honing (1972) and McKnight (1968). A complete cycle from egg to adult normally requires 12 months. However, a small proportion of some populations may undergo a second diapause and, therefore, require 24 months to complete their development.

Oviposition usually takes place in midsummer. The eggs are laid in masses of 25 to 40 eggs on the needles of host trees. The eggs hatch in about 10 days. The newly hatched larvae do not feed, but seek suitable sites where they spin hibernacula, molt to second instar, and remain dormant over winter. They occur most densely on the foliage and bark of branches of the lower crown, and on the mid-crown bole of the host trees. The second-instar larvae emerge from their hibernacula in early May of the following season, or soon after maximum daily temperatures reach 16°C. These larvae move to the foliage where they tunnel into old needles and feed. About the time when the buds start to swell, the growing larvae of third and fourth instars leave the old needles, and bore into and feed on the developing needles within the buds. As the new shoots unfurl, the larvae spin loose webs between the needles and tips. The flowers of true-firs are also an important food source for the larvae. The fourth, fifth, and sixth (last) instars are found within the webs, where they feed on the new foliage until it is

destroyed before moving to establish new feeding sites on older foliage. The larvae become full-grown in 30 to 40 days after attacking the buds. They pupate at their feeding sites by July, and the moths emerge after about 10 days and start the new cycle. In the two-year-cycle, the third instar larvae cease their activity in July of their first feeding season, and spin second hibernacula. The larvae emerge the following May, continue feeding, and complete their development. Cool temperatures apparently are responsible for the selection and maintainance of populations with a second diapause.

Apart from mining the needles, attacking the developing buds, and webbing and destroying the new foliage (McGugan 1954), the insect also harms natural regeneration by attacking and destroying the young cones of its principal host trees, thus reducing the seed drop (Dewey 1970). Epidemic population levels cause loss of growth, topkilling, and, in some cases, the death of the trees (Keen 1952). The importance of this insect as a pest in the forest industry has led to enormous efforts and expenditure for its study and control.

Occasional outbreaks of endemic populations to epidemic levels have been recorded. Since 1900, there have been five major outbreaks of the western spruce budworm in southwestern British Columbia alone (Anonymous 1977), and several more in the United States (Johnson and Denton 1975). These outbreaks persisted for several years before subsiding to normal infestations. The reasons for such outbreaks are not fully

understood. It has been speculated that existing natural control mechanisms become insufficient to hold an endemic population in check so that it can erupt and become an epidemic. Climate and age of the trees are believed to be major triggering mechanisms (Greenbank 1963).

2.1.2. Laboratory Rearing

The continuing need for basic research often calls for a large and constant supply of insects and thus for practical mass rearing techniques. Clearly, it is important to have field populations available for specific research projects, but dependence on a wild population produces problems, such as an irregular supply of the proper stages and numbers required, apart from the difficulty and time-consuming effort to collect them. Rearing in the laboratory can circumvent such difficulties.

Many insect species have been reared to order in the laboratory for many years, especially for research on pest problems in forestry. Wellington (1949) described the successful rearing of some phytophagus lepidoptera on an artificial diet, using spruce budworm as one of the test insects. The diet was an agar-base to which were added a suspension of crushed balsam-fir buds, a chemical inhibitor of fungal growth, and autolysed yeast. Successful laboratory rearing of spruce budworm using natural food sources has also been reported. Bergold (1951) and

Stehr (1954) used frozen shoots from balsam-fir, and Heron (1961) used fresh terminal shoots of tamarack, *Larix laricina*. The larvae were reared in Petri-dishes. All these techniques resulted in satisfactory survival and development of the insect, comparable to those of insects reared on fresh fir foliage. However, natural sources of fir foliage are still required as food materials. This supply is seasonal and requires large freezer space for storage in order to maintain continuous insect cultures.

McMorran (1965) described a diet for the spruce budworm, based on one previously developed for rearing cotton bollworms. Wheat embryo was substituted for wheat germ because spruce budworm larvae did not establish on a diet containing wheat germ from which the oil had been removed. The results indicated that this synthetic diet was very suitable for rearing several successive generations. Compared to spruce budworms reared on frozen balsam-fir buds, the synthetic diet-fed insects had a higher survival, developed faster, and were heavier and more fecund. Allen *et al*. (1968) reared a closely related species, *C. pinus*, on the same diet, and reported that these larvae approached field-collected specimens in size. Survival was high enough at 70% to make the technique efficient for mass rearing. Improvement in the rearing technique to a more efficient and relatively simple method was later developed and described by Grisdale (1973). Using McMorran's diet, the larvae were reared in ribbed plastic cups into which the diet had been poured and

then sprayed with an anti-fungal solution of sorbic acid and methyl-*p*-hydroxybenzoate in ethyl alcohol. The cups had the advantage that each second-instar larva could establish its own feeding site between the ribs. With this system, up to 10,000 larvae/week were produced. In all these rearing techniques, the second-instar larvae were placed in cold storage at 0 to 5°C upon emergence for 4 to 8 months to simulate natural diapause.

Although colonies with normal diapause are useful in some work, the ability to produce a non-diapausing colony on artificial diet would certainly give mass rearing an enormous advantage by increasing the number of generations reared in a given period, and ensuring a constant supply.

Harvey (1957) found that some *C. fumiferana* could develop without diapause if the first-instar larvae were exposed to photoperiods of at least 15 h at 22°C. Shorter photoperiods ensured almost universal diapause, and longer photoperiods resulted in progressively greater proportions of non-diapausing insects, approaching 100% in continuous light. The response was reduced by lower temperatures. The author developed a diapause-free strain with a two-month life-cycle after selection through six generations. The insects retained the same general characteristics as the diapausing stock population. The larvae were noticed to leave the hibernacula after its construction, fed readily on frozen balsam-fir buds, and developed normally. Therefore, both temperature and photoperiod appear to be essential to development without diapause.

Lyon *et al.* (1972) reared diapausing and diapause-free western spruce budworm on an artificial diet, modified after McMorran's (1965). The main changes were replacing formaldehyde with potassium sorbate, increasing the agar and reducing the water contents, and substituting a commercially prepared vitamin mixture. The larvae were reared in clear one-pint plastic containers. Paper covers were used to allow some water loss from the diet and to prevent condensation on the container walls. They were held at 23 to 26°C and 33 to 52% RH, with 24 h photoperiod. The authors also noted the critical factor for preventing diapause to be the physical environment presented to the first-instar larvae. The second-instar larvae could be made to diapause or forego diapause, depending on their rearing experience in the first-instar. The newly hatched larvae were confined so that they had immediate access to palatable food at the bottom-half of the rearing container. These positively phototactic larvae were prevented from crawling away from the food by excluding light from the empty space above the food. With these conditions the majority of the larvae would feed in their first-instar and forego diapause. It was possible to rear about 7.5 generations/year with the diapause-free colony.

It was, therefore, possible to rear the spruce budworm and to break its natural diapausing behaviour on artificial diet. These have been useful findings. They permit rapid adjustments in colony size and allow for efficient utilization of the insects in the laboratory.

2.1.3. Chemical Control

Aerial application of insecticides against the feeding larval stages has been used in spruce budworm control operations in North America since the 1940s, especially during extensive outbreaks. Several insecticides have been used over the years. Effective control with DDT was reported (Carolin and Coulter 1971; Eaton *et al*. 1949; Fettes 1960; Johnson and Denton 1975; MacDonald and Webb 1963). DDT was highly toxic to the larval instars (Brown *et al* . 1947; Secrest and Thornton 1959). Aerial spraying in Atlantic Canada using DDT was begun in 1948 (Markin 1979). However, despite the successes of these programmes, and the finding by Carolin and Coulter (1971) that DDT posed little ecological disturbance in sprayed forest, the use of DDT remained controversial because of public concern about residues and adverse environmental impact (Blais and Parks 1964; Cope 1961; Fettes 1960; Fettes and Randall 1962; Harwood 1975; Ide 1956). The need arose, therefore, for alternative insecticides with the efficacy of DDT but without its allegedly adverse effects on the environment. Such insecticides should be effective in controlling the spruce budworm, but should not persist in the environment or be toxic to other organisms.

Relatively non-persistent OP and carbamate insecticides were investigated extensively (Hopewell 1975; Hopewell and Nigam 1974; Randall 1962). As a result of such studies, several

environmentally more acceptable insecticides have been used to replace DDT; including carbaryl, dimethoate, dichlorvos, malathion, phosphamidon, naled, pyrethrum, and fenitrothion (Anonymous 1974, 1975; Carolin and Honing 1972; Johnson and Denton 1975; Mackenzie et al. 1975).

Acephate is an OP insecticide that has been introduced fairly recently (Spencer 1982). With an oral LD_{50}, to male rats, of 945 mg/kg, acephate appears to be of exceptionally low acute mammalian toxicity while being an excellent insecticide (Magee 1974). As a promising candidate for controlling the spruce budworm, it is important to investigate the basis of its selectivity.

2.2. The Organophosphorus Insecticides

2.2.1. General Mechanism of Action

The ability of OP insecticides to react with and inhibit acetylcholinesterase (AChE) is generally regarded as the biochemical mechanism of action for their insecticidal activity and toxicity (Eto 1974; O'Brien 1967). AChE is a hydrolytic enzyme responsible for terminating the action of acetylcholine (ACh), the cholinergic neurotransmitter.

AChE is present in relatively large quantities in insects (Smallman and Mansingh 1969). Biochemical and histological

studies have shown very high AChE activity in their central nervous systems (CNS) (O'Brien 1976; Pichon 1974). Its activity in the head and nervous system of insects varies with the physiological state of the animals, especially during larval development (Dewhurst et al. 1970; Grzelak et al. 1970; Mansingh and Smallman 1967a, b; Van der Kloot 1955). Exclusive of adults, the brain and nervous system of last instars showed the highest activity (Smallman and Mansingh 1969). No AChE has been found at the neuromuscular junction of insects (Pichon 1974; O'Brien 1967).

In vertebrates, two kinds of ChE are common. True ChE, or AChE, is found in the CNS, neuromuscular junction and within the membranes of red blood cells; it is also called specific or erythrocyte ChE. Pseudocholinesterase, sometimes called butyrylcholinesterase (BuChE), or simply ChE, is found in numerous tissues, including blood plasma, liver, and nervous tissues. It is also called nonspecific or plasma ChE by some authors (Mackenzie et al. 1975; Mayer 1980; O'Brien 1976). Inhibition of AChE at certain strategic locations can cause the death of an animal, whereas inhibition of BuChE at most sites produces no apparent functional derangement.

AChE has two ACh binding sites at each catalytic centre: an anionic and an esteratic site. The anionic site binds the quaternary nitrogen of the cholin moiety of ACh, whereas the catalytic process occurs at the esteratic site by nucleophilic attack on the acyl carbon of the substrate. The esteratic site

contains a serine hydroxyl (OH) group, whose nucleophilicity, i.e., the extent to which it will react with a relatively positive centre, is enhanced by hydrogen bonding to the imidazole group of neighbouring histidine residues (Corbett 1974; Taylor 1980). It is the OH group of this special serine which is acylated by the ACh (O'Brien 1976). During the reaction to enzymatic attack on the substrate an intermediate enzyme-substrate complex is formed, which then acylates the enzyme to acetyl-enzyme conjugate by covalent bonding (Lehninger 1975), with the concomitant release of choline. The acetyl-enzyme conjugate is labile to hydrolysis, which results in the deacetylation, with the formation of acetate and free enzyme. The rate of overall reaction is governed by the rate of deacetylation, this being the slowest step (Corbett 1974; O'Brien 1976). This rate, or the catalytic centre activity, is about 3×10^5 ACh molecules/active centre of AChE/min at 37°C; this is equivalent to a turnover time of 150 µsec (Aldridge 1971; Taylor 1980).

The overall mechanism of inhibition of the AChE by OP insecticides is fairly well understood. It has been demonstrated to be the result of an actual chemical reaction between the enzyme and the phosphorus compound (Fukuto 1957; Heath 1961; O'Brien 1960). The reaction mechanism is analogous to that of the reaction with the natural substrate, ACh. OP insecticides phosphorylate AChE by virtue of an electrophilic attack of the phosphorus atom (P) on the serine hydroxyl of the enzyme. Hence,

a requirement for potency is that an electron-withdrawing substituent be attached to the P in order to give it sufficient electrophilic character, such as the presence of a positive formal charge (δ+) on the P of the oxygen analogue of an OP insecticide, like paraoxon. As P becomes more positive (increased δ+) by a more electrophilic substitution, the reactivity of the compound with the enzyme is increased. The overall process leads to the phosphorylation of the AChE. Dephosphorylation, if occuring at all, is much slower than deacetylation. Dephosphorylation rates, reported for some OP's, may be slower by a factor of 10^5 to 10^6 (Aldridge 1971).

Some phosphorylated AChE can undergo progressive conversion to a form which cannot be reactivated by dephosphorylation. This is known to be due to dealkylation, with the loss of an alkyl group attached to the phosphorus. In this case, the phosphate-enzyme ester bond becomes entirely resistant to hydrolysis under physiological condition, i.e., the enzyme becomes permanently phosphorylated and blocked. This phenomenon is known as ageing and is the basis for the cumulative toxicity of OP poisons. It occurs at different rates with many different phosphorylated AChEs (Coult et al. 1966; Pickering and Malone 1967). The AChE cannot be recovered, and further activity of the enzyme in a biological system must be replenished by new synthesis.

According to O'Brien (1967), OP insecticides are either direct or latent inhibitors of AChE. Direct inhibitors are those

compounds which prove to be potent without metabolic activation. Many OP insecticides, however, are latent inhibitors, which owe their potency *in vitro* to the fact that they are converted or activated in the body to give compounds which are direct inhibitors. They are activated during metabolism in the biological systems. This has been the subject of many reviews (Casida and Lykken 1969; Fukuto and Metcalf 1969; Hodgson 1968; Lykken and Casida 1969; Menzer and Dauterman 1970; O'Brien 1967).

Reactions involving oxidative enzymes of microsomal mixed-function oxidase system (MFO), hydrolases or transferases are among the important classes in the metabolism (Dauterman 1971; Matsumura 1975). However, among these the MFO of the microsomal fraction of cell homogenates have an extremely broad spectrum of substrates and catalyse a wide variety of biotransformations, thus playing a central role in the metabolism of insecticides (Nakatsugawa and Morelli 1976).

MFO-mediated activations of phosphorothionates and phosphoramidates, OP insecticides which are poor inhibitors of AChE *in vitro*, have been studied thoroughly. The desulfuration of parathion and the N-methyl hydroxylation of Schradan to potent AChE inhibitors are classical cases. The activation products are the oxygen analogues (P=O) of phosphorothionates and the hydroxy alkyl derivatives of phosphoramidates (O'Brien 1960). Other examples include the desulfuration of malathion (O'Brien 1957), fenitrothion (Hollingworth *et al*. 1967), and the

hydroxylation of dimefox (Arthur and Casida 1958). Another mechanism of activation of OP insecticides by the MFO involves the oxidation of thioethers to sulfoxides and sulfones (Metcalf et al. 1957). All activation processes lead to increased polarity by shifting the distribution of electrons so that the P becomes more positive (increased $\delta+$) and thus more reactive as well as more strongly attracted to the esteratic site of the AChE.

Much evidence supports the hypothesis that the MFO of insects and mammals catalyze similar reactions (Arias and Terriere 1962; Bull 1965; Hayaishi 1969; Holtzman et al. 1967; Menzer and Casida 1965; Morello et al. 1971; O'Brien and Wolfe 1959; Tsukamoto and Casida 1967). In insects, the MFO activity has been demonstrated in fat body, malpighian tubules, and midgut, whereas in mammals it is found mainly in liver, kidneys, lungs, small intestine, adrenal glands, and testes (Hodgson and Tate 1976).

2.2.2. Mechanisms of Selectivity

Selectivity of insecticidal activity is generally indicated by differences in response between various organisms to a toxicant; it may exist between populations of different taxa, between strains of one species, or even between individuals of one species.

Various mechanisms can cause insecticides to be selectively toxic. One or several mechanisms may operate simultaneously, depending on the organisms and insecticide(s) involved in any particular situation.

Most mechanisms accounting for selectivity are physiological or biochemical in nature, but non-physiological mechanisms are known also. The non-physiological mechanisms normally depend on whether or not organisms come into contact with an insecticide, whereas physiological mechanisms involve events taking place after organisms have come into contact with a toxicant.

Selective toxicity of pesticides, if exploited appropriately, can reduce hazards, or injury, associated with the use of such chemicals in pest control. Significant progress has been achieved in this respect during the past decade. For instance, pesticides for the control of harmful tetranychid mites are used now which spare beneficial phytoseiid mites. The euphemism Integrated Pest Management is often used to adumbrate progress of this nature made in pest control practices in recent years.

In order to be able to improve pest control practices further through the use of insecticide selectivity, it is essential to have a thorough knowledge of the mechanisms that function in selectivity.

2.2.2.1. Behaviour Pattern and Life Stages

An insecticide can be selective to organisms only by virtue of differences in behaviour and habitat. Susceptible organisms may possess behaviour patterns that bring them into contact with an insecticide which the unsusceptible organisms avoid. In such a case, it is the ethological factors that cause the selectivity, not physiological or biochemical differences. Knowledge of the behaviour of the organisms is necessary to predict and attain this kind of selectivity. Insecticides are so applied as to result in maximum exposure of the target organisms, and at the same time to avoid or minimize the exposure of non-target organisms. Therefore, it involves manipulating insecticide applications to take advantage of the overall situations. Metcalf et al. (1957) used specific behaviour patterns to spare pollinators by spraying the fields when they were not active. Anderson and Atkins (1958) and Johansen (1977) supplemented this technique by using insecticides with short residual life, so that toxic residues would have disappeared by the time the non-target species became active. In addition, differential susceptibility in the life stages can also be exploited by manipulating the insecticide applications. Pest species can be treated at their most susceptible stage, or when non-target species are most tolerant. Differential susceptibility of insect life stages were reported for heterometabolous predators (Bartlett 1964) and

holometabolous insects in general (Bartlett 1958; van den Bosch and Stern 1962).

2.2.2.2. Penetration into Organisms

Mechanisms of transport of insecticides across insect integuments are still not fully understood, nor is it known insecticide selectivity is caused by penetration differences. O'Brien (1967) has attributed this lack of understanding to difficulties in measuring penetration rates, which may vary with such factors as application methods, insect species or sites of application. Given such uncertainties surrounding the uptake of insecticides, it is not surprising that attempts to correlate rates of uptake with chemical structure and polarity, have not proven to be consistent and satisfactory. O'Brien (1967) stated that there is no evidence that insects, reptiles, amphibia, or mammals have integuments whose permeabilities differ in any consistent way from one another with respect to the role of polarity in permeability.

Although details of mechanisms and factors influencing penetration of insecticides through the integuments are still not well understood, comparative <u>in-vivo</u> studies have shown considerable differences to exist between insect species. Buerger and O'Brien (1965) found the penetration rates of a number of insecticides through the integuments of the American cockroach and adult yellow mealworm as measured by toxicities of

topically applied insecticides to be proportional to the polarity of a given chemical, but no such correlation was noted for the integument of the house cricket. Krueger and O'Brien (1959) attributed the 15-fold difference in the toxicity of topically applied malathion to German and American cockroaches to differences in penetration. They found that the rate of metabolism and the LD_{50} values of malathion were the same in both insects when the insecticide was applied by direct injection into the body. These differences, therefore, could have some toxicological significance and be a considerable factor in selectivity among species.

Injected/contact toxicity ratio is often used as an indicator of insecticide selectivity, especially between insects and mammals (O'Brien 1961). Generally, such ratios are lower for insects than for mammals, indicating that mammals are relatively less susceptible to poisoning by contact insecticides than are insects (Corbett 1974; Winteringham 1969). Although the ratio may indicate the degree of penetration of insecticides through the integuments to some extent, the selectivity may also be due in part to the relatively high surface-to-volume ratio of insects, and the apparent high accessibility of their CNS to contact, lipophilic insecticide(Winteringham 1969).

Insecticide penetration through the integument has also been implicated as a factor for resistance in insect species (Ebeling 1974). Reduced penetration has been reported as a possible resistance mechanism for a number of species against

several insecticides (Plapp and Hoyer 1968). Decreased penetration rates of malathion were shown in resistant strains of mosquito larvae (Matsumura and Brown 1963), and of diazinon on flies (El Basheir 1967; Forgash *et al*. 1962; Krueger *et al*. 1960).

2.2.2.3. Metabolism

After entry into an organism, most organic xenobiotics, including insecticides, are metabolized, usually becoming more polar and amenable to excretion. At the same time, such enzyme-catalyzed transformations can alter significantly the biological activities of xenobiotics, rendering insecticides more toxic, less toxic, or non-toxic. Differences in rates and kinds (e.g., activating *versus* detoxifying) of metabolic reactions between organisms are, therefore, most important in the selectivity of insecticides (Hollingworth 1976; O'Brien 1967). Undoubtedly, the MFO is the most general and important of the metabolic enzyme systems, catalyzing a wide range of chemical reactions. Although some OP compounds may become more toxic (section 2.2.1), MFO-mediated metabolism more typically serves animals as a detoxification mechanism (Nakatsugawa and Morelli 1976). In addition, several other enzymes are known to operate in biological systems, aiding detoxification of insecticides mainly. These include the phosphatases, carboxyesterases, amidases, and glutathione-S-transferases.

Evaluating several OP insecticides, O'Brien (1961) found that mammals possess much more efficient detoxifying enzymes than insects. For instance, levels of carboxyesterases and amidases are proportionately higher in mammals than in insects. Therefore, insecticides that can be hydrolyzed rapidly by these enzymes would be selectively toxic to insects (Dauterman _et al_. 1959; Krueger and O'Brien 1959; O'Brien _et al_. 1958).

Enzymes catalyzing activation and detoxification reactions can operate simultaneously on different chemical groups within the same molecule of the toxicant. In this case, the net result of activation and detoxification determines the toxicity of a particular insecticide. This remarkable mechanism is the basis for the selective toxicity of malathion. Malathion is highly toxic to most insects but of low toxicity to mammals, its selectivity being caused largely by much higher carboxyesterase activities in the mammals (O'Brien 1967). Malathion is activated to malaoxon in animals of both phyla by the MFO. However, the activation product, malaoxon, is also rapidly degraded in mammals, but not in susceptible insects. Krueger and O'Brien (1959) showed that the net operation of the activation and detoxification processes led to an accumulation of the activation product, malaoxon, in insects, but not in mammals, and that the detoxification in mammals was due almost entirely to cleavage at the carboxy-ester group. Typically, the mouse degraded 68% by carboxyesterase, whereas insects (houseflies, German and American cockroaches) degraded only about 30% by this

route.

It is also important to note that the mechanisms of resistance frequently entail increased activities of such detoxifying enzymes in insects (Hodgson and Plapp 1970). Oppenoorth and Welling (1976) stated that detoxifying hydrolytic enzymes are important causes of resistance to OP compounds. Increased activities of hydrolases (phosphatases and carboxyesterases), glutathione-S-transferases, and MFO in resistant strains of several insect species have been reported by several workers (Collins and Forgash 1970; ElBashir and Oppenoorth 1969; Motoyama and Dauterman 1972; Welling and Blaakmeer 1971; Yang *et al*. 1971).

2.2.2.4. Excretion

Excretion of toxic insecticides by insects and mammals is often considered to play a minor role in insecticide selectivity. This is because most insecticides are relatively apolar and not readily excreted by insects and mammals. Metabolism, rendering the compounds more apolar (e.g., hydrolysis and glucoside formation, often following hydroxylation), is often prerequisite to excretion. Since such preliminary reactions are usually detoxifying in themselves, the primary focus of discussion often centers around metabolism as the determinant factor in selectivity.

Urinary excretion can be important when the parent material is hydrophilic. Although polar anti-AChEs are relatively few, it is an important factor when considering the properties of most phosphoramidates, including acephate and methamidophos. Acephate and its metabolic product, methamidophos, are highly polar compounds, with octanol-water partition coefficients of 0.043 and 0.029, respectively (Larson 1975). By comparison, paraoxon, a lipophilic compound, has a value of 38.84 (Hussain et al. 1974). In view of the fact that mammals have advanced and efficient excretory systems compared with that of an insects, the hydrophilicity of acephate can be of significance for its selectivity to mammals. Larson (1975) concluded that the tolerance of mice to intoxication by acephate was partly related to the rapid excretion of parent material as well as the metabolically formed methamidophos through the urinary system. The lower toxicity of the propionyl analogue of acephate to the mouse when compared with that of its hexanoyl analogue was also attributed to the more hydrophilic nature of the former compound (Kao and Fukuto 1977). A large proportion of the applied dose of propionyl analogue was evidently found in the urine of mice, which passed through unchanged after 6 h of exposure. Substantially less of the more lipophilic hexanoyl analogue was found in the urine of the mouse after exposing for the same period.

2.2.2.5. Penetration to Target Site

AChE is the common target of OP insecticides. As discussed earlier (section 2.2.1), in insects the enzyme is entirely ganglionic, i.e., in the CNS, whereas in mammals it is found in the CNS as well as at other peripheral sites such as the neuromuscular junctions and blood. It is known that ion barriers exclude or slow the penetration of polar compounds into the CNS of insects and mammals. AChE in the CNS of both animals, therefore, is well protected from such compounds. However, the peripheral AChE in mammals is exposed to the attack by such polar anti-AChE compounds. In addition, factors such as lipid solubility, metabolism, and size of molecules could also influence the penetration of the barriers. Brooks (1976) stated that passive diffusion through the barriers is related to lipid solubility, and that lipophilic substances pass through these barriers readily. O'Brien (1967) noted that metabolism could influence the influx of a diffusing species by eliminating back-diffusion through incorporation of metabolic products into non-diffusible or slowly diffusing compounds, and that the influx rate decreases with increasing molecule size. Finding that the toxicities of several nerve poisons to the mouse and five insect species were indicative of an ion-impermeable barrier protecting the nervous systems of the insects but not that of the mammal led O'Brien (1967) to conclude that the above selectivity is caused by the existence of peripheral,

ion-sensitive AChE in the vertebrate but not in the insects.

According to the above discussion, compounds like methamidophos and acephate could well penetrate the ion barrier despite their polarity. In addition, methamidophos is relatively stable at physiological pH, allowing it to persist at high concentrations in insects (Khasawinah et al. 1978), and acephate is converted to methamidophos to some extent in biological systems (Kao and Fukuto 1977; Bull 1979). Slow penetration of the ion barrier by methamidophos to reach the target enzyme is supported by the finding of Khasawinah et al. (1978) that, compared to other effective OP insecticides like methyl paraoxon, methamidophos was slow in producing acute symptoms of poisoning and AChE inhibition in housefly. This is because of the requirement for sufficient internal concentration for a period long enough to permit the development of its slowly expressed toxicity. Rojakovick and March (1972) found a significantly slower reaction with acephate than that observed for methamidophos by Khasawinah et al. (1978). This result, therefore, is consistent with a delay factor that would result from a slow conversion of acephate to methamidophos.

2.2.2.6. Affinity to the Target Enzyme

A further selective mechanism can be traced to the target enzyme itself, as expressed by differences in the affinity of a toxicant to homologous enzymes from different organisms. Van

Asperen and Dekhuijzen (1958) observed that AChE from fly heads was a hundred times more susceptible to inhibition by dichlorvos than was AChE from mouse brain. The inhibition in mouse brain was slowly reversible, and the concentration of the toxicant was reduced by binding to non-active sites. Potter and O'Brien (1963) traced lower toxicity of injected paraoxon in the frog than the mouse, to differences in affinities to their respective AChEs. Since these animals did not differ widely in the metabolism and distribution of injected paraoxon, target sensitivity seems to be the major determinant in this case of selectivity. Dauterman and O'Brien (1964) investigated the selective inhibition of AChE in bees and houseflies by diisopropyl phosphorothionate. The chemical was more toxic to the houseflies than to the bees, with greater AChE inhibition in vitro. These differences in sensitivity suggest that there are differences in the nature of AChE between species which can confer selectivity on the inhibitors (O'Brien 1961); e.g., the distance between the anionic and esteratic centres of an insect was found to be as much as 0.1 nm greater than that for a mammalian AChE (Corbett 1974). Since most OP insecticides do not possess a positively charged site in their molecules at the correct distance from the phosphorus atom attaching to the esteratic site of the AChE, other factors must account for differences in reacting with different AChE's.

According to Fukuto (1971), reactivity with the esteratic site is by far the most important factor determining the

anti-AChE activity of OP's. It has been demonstrated that the ability of an OP to inhibit AChE can be enhanced by hydrophobic interactions of non-polar groups on the OP with hydrophobic patches on the enzyme (Hansch and Deutsch 1966; O'Brien 1976). It was later shown to be a factor in the activity of fenitrothion (Fukuto 1971), a broad-spectrum insecticide whose activity is comparable with that of methyl-parathion, but with very much lower toxicity to mammals. Hollingworth et al. (1967) investigated a series of P=O analogues of fenitrothion. They found that introduction of alkyl substituents at the 3-position of the phenyl ring renders the compound more inhibitory to housefly-head AChE, with inhibition increasing in the order H < CH_3 < iso-C_3H_7, while the opposite effect is observed with bovine erythrocyte AChE. Further studies suggested that the formation of an enzyme-inhibitor complex with housefly AChE is aided by interaction of the 3-alkyl substituent with the anionic site, but is hindered in the case of bovine erythrocyte AChE. A similar explanation was given for the more than 200-fold greater toxicity of the diisopropyl homologue of parathion to houseflies than to honey bees (Fukuto 1971).

In insects, mutants have been found whose AChE has an enormously reduced susceptibility to OP insecticides. This results from altered AChE, reducing its sensitivity to inhibition by the compounds, while still binding the natural substrate, ACh, almost normally. The phenomenon often constitutes a major factor in the development of resistant

species (Oppenoorth and Welling 1976). Altered AChE was demonstrated in several resistant strains of spider mites and ticks (Smissaert 1964; Smissaert *et al*. 1970; Wharton and Roulston 1970). Indication of altered AChE was observed in a carbamate-resistant strain of green rice leafhopper (Iwata and Hama 1972). Their AChE was less susceptible to inhibition by carbamates than AChE from non-resistant populations. Tripathi and O'Brien (1973) found a strain of houseflies which appeared to be resistant to tetrachlorvinphos due to the presence of an altered AChE. The enzyme showed a considerably decreased rate of inhibition, which was found to be mainly due to a greatly reduced affinity for the enzyme-insecticide complex. The authors concluded that a change in enzyme structure affects binding sites for tetrachlorvinphos. Large differences in inhibition rates of AChE compared with normal strains were also found in houseflies resistant to dimethoate (Devonshire and Sawicki 1974) and *Anopheles albinanus* resistant to parathion and propoxur (Ayad and Georghiou 1975).

2.2.2.7. Polyfactorial Selectivity

Hollingworth (1976) and O'Brien (1967) used the term polyfactorial selectivity to describe the situation where more than one of the processes discussed above contributed significantly to the selectivity of a chemical on different organisms. It involves the interactions between these factors,

and is probably the dominant feature determining selectivity. For instance, slow penetration will give low toxicity if there is a degradative system present (O'Brien 1961). O'Brien (1967) described the selective nature of dimethoate. He claims that among mammalian species the selectivity is monofactorial, due exclusively to the rate of degradation in the liver. In insects, variations in penetration, activation, and target selectivity are all profound, indicating polyfactorial selectivity.

Hollingworth (1976) stated that selectivity is generally polyfactorial. O'Brien *et al.* (1965) described famphur as equally toxic to milkweed bugs and mice. Desulfuration of the molecule to produce the toxic metabolite, famoxon, occurred to the same extent in both species. However, detoxification was five times slower in the milkweed bug than it was in the mouse. It was discovered that the milkweed bug was compensating for this difference through the mechanism of AChE insensitivity. *In vitro*, its AChE was 15 times less sensitive to inhibition by famoxon than was that of the mouse AChE.

The selective toxicity of the thiono analogue of mevinphos between the mouse and housefly was studied by Morello *et al.* (1968). Its lower toxicity to the mouse was attributed to the overall detoxification being much more effective in the mouse; moreover, mouse brain AChE was four- to five-fold less sensitive to inhibition by mevinphos than was fly head AChE, and the phosphorylated mouse AChE recovered much more rapidly from inhibition than the fly AChE.

2.2.3. Acephate

Acephate is the N-acetylated derivative of methamidophos, another excellent broad-spectrum insecticide and acaricide. Methamidophos was marketed in 1970 under the trade name Monitor[R] in the United States by Chevron Chemical Company and Chemagro, and under the trade name Tamaron[R] in Europe by Farbenfabriken Bayer (Magee 1974). It is relatively toxic to higher animals, having an oral LD_{50} to rats of 20 mg/kg. The discovery by Magee in 1968 at Chevron Chemical Company that N-acetylation of methamidophos greatly reduced the mammalian toxicity led to the development of acephate as a new insecticide. It was marketed by the company in 1972 under the trade name Orthene[R] (Magee 1974). It combines the good insecticidal and acaricidal activity of its parent compound, methamidophos, with low toxicity to higher animals (rat, oral LD_{50}: 945 mg/kg; mouse, oral LD_{50}: 361 mg/kg; dark-eyed junco, LD_{50}: 106 mg/kg; bluegills, 96 h LC_{50}: 2050 ppm; cutthroat trout, LC_{50}: > 100 ppm) (Magee 1974; Spencer 1982; Woodward and Mauck 1980; Zinkl *et al*. 1981).

2.2.3.1. As an Insecticide

Effective against many pest insects, acephate has many registered uses in Canada and the U. S. A. Significant reduction of mines by the leaf-miner, *Liriomyza sativae* Blanchard, and by

the pinworm, Keiferia lycopersicella (Walsingham), was reported in tomatoes for up to 21 days after spraying (Lindquist and Krueger 1975; Poe and Everett 1974). Control of green peach aphids, Myzus persicae (Sulzer), on cabbage and spinach was demonstrated by Harding (1973). A similar result was obtained on sweet pepper by Burbutis et al. (1972), who also reported effective control of European corn borer, Ostrinia nubilalis (Hubner). Trichoplusia ni (Hubner), the cabbage looper, was controlled with acephate (Chalfant et al. 1973). Mistric and Smith (1973) and Tappan et al. (1974) showed that acephate protected tobacco plants against damage by tobacco budworm, Heliothis virescens (Fabricius), tobacco hornworm, Manduca sexta (Linnaeus), tobacco flea beetle, Epitrix hirtipennis (Melsheimer), and green peach aphids. In cotton, acephate is recommended for use against the tobacco budworm, the bollworm, Heliothis zea (Boddie), and boll weevils, Anthonomous grandis grandis Boheman (Bull 1979; Plapp 1972). Other agricultural insect pests, susceptible to acephate, include the leaf roller, Tischeria malifoliella Clemens, in apples (Bobb 1972), citrus blackfly, Aleurocanthus woglumi Ashby, in citrus (Nigg et al. 1979), scale insects, Cerococcus deklei Kotsztarak and Vest, on ornamental plants (Reinert 1976), and palm aphids, Cerataphis variabilis H.R.L., on "Malayan Dwarf" coconut palm (Reinert and Woodiel 1974).

Acephate also showed good control of stored product and household pests. Watters (1977) reported that treatment of

stored wheat with 32 ppm of acephate provided from 90 to 100% mortality of rusty grain beetle, *Cryptolestes ferrugineus* (Stephens), and red flour beetle, *Tribolium castaneum* (Herbst), for 270 to 277 days. Wright and Hillmann (1975) found that it was at least equal in activity to commonly used household sprays against the German cockroach, *Blattella germanica* (Linnaeus).

The effectiveness of acephate in controlling many forest insect pests has been extensively studied. Against the Douglas-fir tussock moth, *Orgyia pseudotsugata* (McDunnough), acephate at 1.12 kg/ha was about as effective as DDT at 0.85 kg/ha with 91.7% and 99.9% reduction of the larval population density, respectively, after seven days (Neisess *et al*. 1976). Similar results were obtained by Brewer and Markin (1978). Doane and Dunbar (1973) showed that acephate gave excellent control of the gypsy moth, *Lymantria dispar* (Linnaeus), and elm spanworm, *Ennomos subsignarius* (Hubner). Acephate has also been effective against several other forest insects including elm leaf beetle, *Pyrrhalta luteola* (Muller) (Brewer 1973), hemlock looper, *Lambdina fiscellaria fiscellaria* (Guenee) (Cameron and Mastro 1975), lodgepole needleminer, *Eucordylea milleri* (Busck) (Brown *et al*. 1979), and Douglas-fir needle midge, *Contarinia pseudotsugae* Condrashoff (Mitchell and Neisess 1979).

Acephate has been effective in controlling the strains of spruce budworms at rates of 1.12 and 0.56 kg/ha, giving respectively 98.8 and 93% mortality of the fourth- and fifth-instars larvae at 15 days after the spraying (Markin

1979). The fourth- and fifth-instar larval stages are those recommended for insecticide spraying by Carolin and Honing (1972), because the insects are then feeding externally on the needles. However, such exact timing is not necessary for acephate, since Brewer and O'Neal (1977) demonstrated that spraying done at the same rates when the larvae were in the second- and third-instars gave good protection to Douglas-fir foliage, with only 6 and 28% defoliation, respectively, compared with a 92% defoliation for the untreated areas. The finding was substantiated by the laboratory studies of Robertson (1980) where the toxicity of acephate was tested on all the six larval instars by feeding and spraying. Feeding tests were done by incorporating the toxicant in liquefied artificial diet, whereas spray treatments were done by spraying the insects directly in Petri-dishes held in a glass spray chamber. The author concluded that the differences in susceptibility dependent on age and instars appeared to be qualitatively small, and the practical implication was that the stage of larval development in the field population at the time of spray application may not be a significant factor.

Evidence indicates that acephate is somewhat plant-systemic. By definition, systemic insecticides, when applied to seeds, roots, stems or leaves of plants, are absorbed and translocated to various plant parts in amounts lethal to insects feeding thereon (Metcalf and Flint 1962). Werner (1974a) reported the systemic activity of acephate in seeds and

seedlings of southern pines after treatment with 10.4 mg/mL for 24 h. It caused a 95% mortality of pales weevils, Hylobius pales (Herbst), fed on one-week-old seedlings or three weeks after the seed treatments, with the detection of 13.6% of the acephate.

Rapid uptake and distribution of acephate by loblolly pine seedlings were shown when the roots were placed in nutrient solution containing 1 mL of a mixture of 7 mg ^{14}C-acephate and 11.6 mg analytical acephate (Werner 1974b). A considerable amount of the radioactive material (8.9, 8.5, and 36.5%) was taken up, respectively, by the roots and translocated to the stem and needles within 24 h. After that the translocation from the roots to the needles proceeded faster than it was absorbed by the roots. At 120 h the concentration in the root tissues declined to 2.9%, whereas those in the needles increased rapidly, reaching 77.6%. Highly toxic levels of the insecticide remained in the needles at 120 h after the treatment, giving > 80% mortality when bioassayed with adult pales weevils. Reinert and Woodiel (1974) also reported the systemic activity of acephate through root absorption. They recorded 100% control of palm aphids on "Malayan Dwarf" coconut four months after a soil drench with acephate at 15 g/tree. An in-furrow treatment of sorghum at planting time with 1.12 kg/ha also provided effective control of chinch bug, Blissus leucopterus leucopterus (Say), and greenbug, Schizaphis graminum (Rondani) on the sorghum seedlings up to 11 days (Mize et al. 1980).

Brewer and O'Neal (1977) and Richmond _et al_. (1978) suggested that the systemic activity of acephate accounted for its ability to kill spruce budworm larvae even though the spraying was done after the larvae had entered the Douglas-fir needles. Mortality of 98 and 99% was reported inside the needles and buds, respectively, one day after the treatment with about 0.45 kg/ha. No translocation to other parts of the tree was shown. The toxicity, therefore, may have been caused only by the penetration or absorption of the insecticide into the needles. In another study, Bull (1979) reported that within 24 h, > 50% of acephate applied to cotton leaves was absorbed and translocated throughout the plants, including the fruits. However, no bioassay study was conducted to support the systemic action.

Despite the general effectiveness of acephate in controlling many insect pests in the field, laboratory experiments indicate that it is comparatively less toxic than most other insecticides, especially to the spruce budworm larvae, when treated topically (Robertson _et al_. 1976, 1978). They reported LD_{50} values at seven days after treatments to sixth-instar larvae as 29.7 and 27 μg/g body weight, which were higher than those of most insecticides tested under the same laboratory conditions, e.g., dichlorvos 3.9 μg/g; fenitrothion 3.4 μg/g; malathion 24.5 μg/g; methamidophos 10 μg/g; naled 5.3 μg/g; phosphamidon 7.9 μg/g; carbaryl 20 μg/g; pyrethrum 1 μg/g.

The fact that acephate showed systemic activity also indicates that it acts as a stomach poison, in addition to its contact effect. Data reported by Robertson (1980) and Robertson et al. (1976) demonstrated this. Acephate was less toxic than carbaryl to sixth-instar larvae of spruce budworm by contact action (LD_{50} equivalent of 122.9 g/ha for acephate and 82.5 g/ha for carbaryl), but its toxicity by feeding was much higher with an LC_{50} of 8.1 ppm compared with 80.2 ppm for carbaryl.

2.2.3.2. Persistence in the Environment

Because the use of persistent and toxic pesticides has been restricted severely, much emphasis has been placed upon finding insecticides of low mammalian toxicity and low persistence. According to available evidence, acephate meets these criteria.

Studies of acephate residues after spraying of forest trees have confirmed its low persistence, although the reported results vary. Such differences may have been caused by differing application methods and climates in which the experiments were conducted. Sundaram and Hopewell (1976) reported a residual half-life of < one day for acephate on spruce foliage applied by simulated aerial spray at 0.28 kg/ha, with a 95% loss of the initial deposit of 55.15 ppm within five days, and complete disappearance after 32 days. Szeto et al. (1978) found that aerial application at 1.12 kg/ha, gave a residue of 3.62 ppm in Douglas-fir needles with a half-life of six days and became

non-detectable after 60 days. Richmond et al. (1978) found the residue level of as high as 45 ppm persisted on Douglas-fir needles for 10 days, and dropped to 7 ppm within 35 days, after application by ground spraying, giving an initial concentration of 135 ppm. Szeto et al. (1978) also studied the persistence of acephate in forest litter. It persisted for 30 and 10 days, respectively, in litter collected from areas densely covered by tree canopy and from open areas at least 10 to 15 m away from any tree cover, although the initial concentrations were much lower in litter from densely covered than from open areas of forest floor, namely 0.62 $\mu g/cm^2$ and 1.16 $\mu g/cm^2$, respectively. In all of these cases, methamidophos was either detected in trace or very low concentrations as an environmental metabolite of acephate. It disappeared within the same time as the parent compound.

Nigg et al. (1981) found no build-up of acephate residues in citrus foliage after three treatments, applied at three-week intervals with 22.8 g/tree using a handgun or with 1.92 g/tree using a mist blower. They considered that it was not dangerous to come in contact with the foliage after seven days, and the estimated half-life was 12.6 and 6.8 days, respectively. These results were comparable with earlier findings by Nigg et al. (1979) and Fitzpatrick and Bogan (1980), reporting a half-life of 10.3 and 15 days, respectively, in rind and pulp of fruits of different citrus varieties, and 8.9 days for that on the foliage. Significant levels of methamidophos, with about the

same half-life, were also detected in these studies.

Spraying and dusting of field tobacco with acephate left no detectable residue on cured and fermented cigar-wrapper or flue-cured tobacco regardless of time intervals from last application to harvest (Tappan _et al_. 1974). Lindquist and Krueger (1975) reported the disappearance of nearly 50% of acephate seven days after spraying greenhouse tomatoes, and a further decline to about 20% was observed after 21 days. A half-life of three days in an agricultural soil was reported by Spencer (1982).

Szeto _et al_. (1979) found that 80% of added acephate was recovered from pond water, held at 9°C for 42 days in the laboratory, but only 45% from creek water after 50 days. Incubation of the waters with their respective bottom sediments, without autoclaving them prior to the treatments, greatly increased the rates of acephate degradation, with a recovery of about 20% from pond water _plus_ sediment after 42 days and 28% from creek water _plus_ sediment after 50 days. There was no detectable loss of acephate to the atmosphere. Applied to the water of a stream at about 1,000 ppb, acephate became non-detectable, within 96 h of application, in both the stream's water and sediment at and below (150 to 2,000 m) the point of release (Geen _et al_. 1981).

2.2.3.3. Impact on Non-target Organisms

Acephate produces only minor effects on some water organisms exposed under laboratory conditions. Among several insecticides tested on cutthroat trout, acephate was the least toxic, with a 96-h LC_{50} of > 100 mg/L (Woodward and Mauck 1980). The LC_{50} values for the other insecticides were trichlorfon 1.7 mg/L, fenitrothion 2.9 mg/L, carbaryl 4 mg/L and aminocarb 28 mg/L. Acephate was also the least toxic to amphipods and stonefly naiads, although these were considerably more sensitive than the cutthroat trout. The 96-h LC_{50} was > 25 mg/L for the amphipods, and the range was from 5.2-28 mg/L, depending on the pH of the water, for the stonefly naiads. Studies of physiological effects on adult rainbow trout exposed to acephate at the approximate 48-h LC_{50} value of 1,660 mg/L caused a decreased heart rate, and increased respiration rate and buccal amplitude (Duangsawasdi and Klaverkamp 1979). There was no difference in the frequency of coughing between control and exposed fish.

The impact of acephate on stream ecosystems appears to be localized and temporary. Acephate added to a stream at about 1,000 ppb was rapidly taken up by fish, insect nymphs and larvae, but the residues declined to trace or non-detectable levels within 24 h, and no mortalities were noted (Geen _et al_. 1981). The brain AChE activity of longnose and common suckers was depressed temporarily by 29%, when they were caged in

streams during aerial spraying of acephate for spruce budworm suppression, and returned to normal within eight days (Rabeni and Stanley 1979). The concentrations of acephate in each stream were 140 ppb and 113 ppb at 1 h after spraying and 41 ppb and 9 ppb after two days, respectively. No significant effect was recorded in brook trout and landlocked salmon, exposed to the same treatments.

Studies of the impact of acephate on forest animals showed inconsistent results, depending on the procedures for collecting the data. Buckner and MacLeod (1975) were not able to detect any adverse effect on forest-inhabiting birds, small mammals, and amphibia, using population census and field observation, begining five days after aerial application of acephate at 0.56 kg/ha. Zinkl et al. (1979, 1980) observed no sign of OP poisoning or mortality in birds and ground squirrels after aerial application of acephate at the same level, but conversely, by monitoring the activity of brain AChE, they found that it caused marked and widespread depression of the AChE activity in these organisms. A 30 to 50% depression was recorded, begining one day after the spraying, and lasting 33 days in the birds, but only six days in the ground squirrels. However, in a controlled laboratory study, Zinkl et al. (1981) demonstrated that dark-eyed juncoes fed with a single, sub-lethal dose of 26.52 mg/kg had depressed brain AChE activity by 44.2% within 3 h after the treatment, but no prolongation, recovering after three days, and the symptoms of ataxia,

tremors, dyspnea, and sternal prostration were seen only during the first 4 h after dosing.

The mortality of ants at specific locations during aerial spraying of acephate to forest areas, was related to the concentrations of spray deposits reaching the ground (Szeto *et al*. 1979). Higher concentrations caused higher mortality within a shorter period. The LT_{50} values were 11.6 h, 11.2 h, and 47.5 h, respectively, for locations with spray deposits of 1.95, 1.59, and 0.55 $\mu g/cm^2$. The effect on honey bees appeared to be temporary(Buckner and MacLeod 1975). Mortalities of nurse bees within the hives occurred for two days, and pollen collections were curtailed for as long as five days after the spraying, but the production of honey crops by the colonies was said not to be reduced. Hydorn *et al*. (1979) observed that caged spiders from forest areas sprayed with acephate showed abnormal behaviour and low survival. Examination of the stomach contents of brook trout from these areas indicated increased consumption of spiders and other arthropods after the spraying, suggesting the susceptibility of the arthropods.

Acephate was more toxic to a predaceous mite, *Phytoseiulus persimilis* Athias-Henriot, than to its prey, *Tetranychus urticae* Koch, regardless of application method (Lindquist and Wolgamott 1980). Significant toxicity occurred in food-chain effects. Acephate applied as soil drenches was more toxic than as foliar sprays. It killed significant numbers of predators feeding on the poisoned hosts until 21 days after the application.

Effects of acephate on microorganisms appeared to be insignificant. Treatment of soil with 20 ppm of acephate or methamidophos did not give any adverse effect upon population levels, nitrifications, ammonifications, sulphur oxidation, or the respiration rate of actinomycetes, bacteria, or fungi tested over 50 days (Fotch and Joseph 1974).

2.2.3.4. Mechanism of Action

The question of how acephate exerts its action in animals, including insects, is not fully settled. Corbett (1974) classified it as an AChE inhibitor. But other workers dispute this, claiming that it is a poor *in vitro* inhibitor of housefly head AChE, and that its *in vivo* toxicity suggests that it is an indirect inhibitor, being converted, enzymatically or chemically, to a biologically more potent anti-AChE compound in the housefly (Rojakovick and March 1972; Suksayretrup and Plapp 1977). Kao and Fukuto (1977) also reported poor *in vitro* inhibition of AChE from several sources by propionyl and hexanoyl analogues of acephate.

Methamidophos is a stronger AChE inhibitor *in vitro* than acephate, although its strength is still much below that of most of the potent inhibitors such as paraoxon. Since some acephate is usually converted to methamidophos in plants, vertebrates, and insects, it is often stated that methamidophos is the active principle of acephate, and that its selectivity depends on this

metabolic activation. Magee (1974) reported this conversion to occur in pinto beans and suggested it as one possible explanation for the bioactivity of acephate. Studies with loblolly pine seedlings, white spruce trees, and Douglas-firs prompted various workers to attribute the insecticidal activity to the conversion of acephate to methamidophos or the combination of methamidophos with acephate (Richmond _et al._ 1978; Sundaram _et al._ 1977; Werner 1974b). Geen _et al._ (1981) reported the conversion of acephate to methamidophos in fish from cages in an acephate treated stream, but no toxicity occurred, and the methamidophos had become non-detectable 24 h after treatment of the stream with acephate.

The toxicity mechanisms of acephate alone and of methamidophos, both alone and as an activation product of acephate, have been studied to some extent in insects. Rojakovick and March (1972) studied the toxicity of acephate in houseflies, and reported an LD_{50} of 1.8 μg/g of body weight. They observed that the first knockdown of the flies occurred 24 h after the treatments with LD_{25} and LD_{50}, and 10 h after treatment with LD_{90}. At these times the AChE activity of the heads and thoraxes dropped to between 30 and 40% of normal.

Later studies by Khasawinah _et al._ (1978) reported on the activity of methamidophos in houseflies, with an LD_{50} of 1.2 μg/g. They found that methamidophos was not activated or degraded in the insects, and that it was slow in producing the acute symptoms of poisoning when compared with other effective

OP insecticides like methyl parathion, as shown by Hollingworth *et al*. (1967). Houseflies treated with 0.04 μg of methyl parathion/fly produced 0.002 and 0.004 μg of methyl paraoxon/fly, respectively, for the susceptible and resistant strains, after 2 h, which was the time for the appearance of major symptoms. Treatment with methamidophos at comparable doses (0.048 to 0.05 μg/fly) produced the internal levels of methamidophos of 0.032 and 0.02 μg/fly, respectively, for the susceptible and resistant strains, after 2 h, and these levels remained unchanged at 4 h and 8 h, when major symptoms appeared. Khasawinah *et al*. (1978) argued that such relatively stable and high levels of internal methamidophos is necessary to compensate for its moderate *in-vitro* anti-AChE activity (I_{50} of 3.9 x 10^{-5} M) in comparison with that of methyl paraoxon (I_{50} of 1 x 10^{-7} M).

Khasawinah *et al*. (1978) also observed the knockdown and mortality of houseflies, which were first evident nearly 8 h after the treatment at LD_{25} and LD_{50}, and 4 h at LD_{90}. At these times the AChE activity dropped drastically to below 10% of normal activity in the heads and to almost no activity in the thoraxes. Rojakovick and March (1972), showed that the slower action and knockdown time of acephate could possibly be accounted for by the time needed for acephate conversion and accumulation of methamidophos.

Studies by Bull (1979) on the metabolism and toxicity of acephate and methamidophos with adult boll weevils and two

strains of tobacco budworm larvae, one susceptible and the other resistant, also suggested the conversion of acephate to methamidophos as an explanation of the toxicity. The susceptible tobacco budworms were only moderately more susceptible than the resistant strain to both acephate and methamidophos by about 2.5- and 0.5-fold, respectively. Internal extracts indicated that the absorbed acephate was metabolized to a small amount of methamidophos in the larvae of both strains, about 1% of the treated dose (1 µg) at 2 h after topical treatment, and 2% at 8 h after the treatment, which accounted for the small difference in toxicity. Methamidophos was 75-fold more toxic than acephate to the boll weevils. Since acephate was rapidly absorbed by the weevils, its low toxicity was attributed to an apparent inability of the insect to metabolize it to methamidophos. No methamidophos was detected from the internal extracts of the boll weevils following topical treatment with acephate.

The *in-vitro* model oxidation system has given invaluable information on the probable pathway of oxidative metabolism of many pesticides by oxidative enzymes. Therefore, in studying the conversion of acephate to methamidophos or to other more potent anti-AChE agents as a possible mechanisms of action, tests were conducted in controlled environments *in vitro*. Commonly, metabolic activation is by MFO-mediated oxidation. However, using *m*-chloroperbenzoic acid (MCPBA) as a chemical oxidant, instead of MFO, Eto *et al*. (1977) produced compounds by the chemical oxidation of acephate and methamidophos, having greater

anti-AChE activities than either possesses alone. Using the methamidophos concentration of 1.4 x 10^{-5} M which produced 18% inhibition of human plasma ChE before the treatment with MCPBA, and the acephate concentration of 1 x 10^{-3} M which produced no inhibition before the treatment, methamidophos oxidized by MCPBA inhibited the ChE almost completely after 15 min, and oxidized acephate inhibited the ChE by about 35% in 60 min. However, incubation of the same concentration of methamidophos with rat liver microsomes did not produce such remarkable results, achieving only a maximum of 58% inhibition after 3.5 h. From certain chemical evidence, these authors concluded that the product formed by the activation of methamidophos was the sulfoxide derivative, O,S-dimethyl phosphoramidothiolate S-oxide. This active intermediate, however, was not identified.

In another study, Klaverkamp and Hobden (1980) reported the activation of acephate (3 x 10^{-3} M) shown by increased inhibition of fish brain AChE when the acephate was incubated for 4 h with fresh and boiled liver homogenates. A small, yet significant, increase in the inhibition (3.3%) produced by acephate in boiled homogenate was presumed to result from non-enzymatic processes. The authors considered that the differences (5.2%) between AChE inhibition by acephate after incubation with fresh and boiled liver honogenates, represented activation by enzymatic processes. They did not identify the activation product, but speculated that it resulted from deacetylation of acephate.

Studies by several other workers were not able to support the findings. Khasawinah *et al.* (1978) observed no change in fly head AChE inhibition by methamidophos (1 x 10^{-3} M) following 6 h of incubation with either cockroach guts or mouse liver slices, and suggested that no major activation or degradative changes occurred in these systems. Kao and Fukuto (1976) also failed to discover activation of methamidophos when they incubated it with a variety of oxidizing agents, including MCPBA, peroxytrifluroacetic acid, mouse liver microsomes, and related chemical oxidation systems. They suggested that if an active product of methamidophos was formed it evidently was highly transitory. In addition, Suksayretrup and Plapp (1977) found methamidophos and acephate to be poor substrates for MFO and glutathione-dependent transferases in strains of houseflies, susceptible and resistant to many OP's.

Although far from being consistent, or free of contradictions, the evidence thus far tends to favour the *in vivo* activation of acephate to methamidophos, which in turn exerts the toxic effects. A poor anti-AChE by itself, acephate is partly converted to methamidophos, a stronger anti-AChE, in many biological systems including plants, higher animals, and insects. There is no complete evidence to confirm the formation of other active intermediate or metabolites *in vivo* or *in vitro* model oxidation systems.

Most of the studies on the activity of acephate that involve insects were mainly on houseflies and cockroaches, but

direct investigation on acephate's range of target insect pests are less common. Not all insects or even strains of an insect responded to acephate treatments by the same behaviour or by the same activation mechanisms. Some interesting reversals in selectivity have been observed (Bull 1979).

It would be invaluable to study some of the toxicological and biochemical responses in target insect species. To this end, the western spruce budworm was chosen as the main species in the study, with the Mediterranean flour moth. Rats and human (erythrocyte AChE) were included to study the effects on non-target organisms.

3. MATERIALS AND METHODS

3.1. Rearing of Western Spruce Budworm

3.1.1. Stock Colony

Pupae of a non-diapausing colony were obtained from Dr. J. L. Robertson of the Pacific Southwest Forest and Range Experimental Station, Forest Service, Berkeley, California. The colony had originated from late-instar larvae collected in eastern Montana and northwest Idaho in 1964 (Lyon *et al.* 1972). The first generations had been reared with normal diapause. Beginning in 1967, a subsample of this colony in the fifth generation was propagated without diapause.

3.1.2. Rearing Environment

The rearing techniques were based on those of Lyon *et al.* (1972) and Robertson (1979). The larvae were reared on ready-mixed artificial diet according to McMorran (1965) and Grisdale (1973) (Table I, BIO-MIX #9769, Bio-Serv, Inc., Frenchtown, New Jersey, 08825, U. S. A.), in 210-cc (7-oz) clear polystyrene specimen containers with fitted paper covers (Lab-Tek Product, Division of Miles Laboratories, Inc.,

Table I : Spruce budworm diet, *BIO-MIX* #9769, and mixing instructions as provided by the commercial supplier[1/].

Part	Ingredient
A	Agar Distilled water
B[2/]	Casein Fiber Salt mix Wesson Wheat germ, toasted Methyl parahydroxybenzoate Aureomycin Ascorbic acid Choline chloride Sucrose Linseed oil
C[2/]	Vitamin mixture #722
D[2/]	4M KOH solution Formaldehyde

Mixing instructions for 1 L of diet

1. Add 25.3 g of Part A (agar) to 835 mL of water.
2. While stirring agar solution constantly, bring to a full boil for 1 min.
3. Transfer agar solution to blender. Cool to 65 to 70°C, add 135.2 g of Part B, 10 g of Part C, and 5.6 mL of Part D.
4. Blend for 60 sec or until mixed thoroughly.
5. Dispense immediately.

1/ Bio-Serv, Inc., Frenchtown, New Jersey.

2/ Premixed by supplier.

Naperville, Illinois). These features allowed easy observation of the insect's development, and prevented moisture from condensing on the container walls by allowing water loss through the cover. Moisture condensation on container walls could easily drown newly-hatched larvae; it also promotes fungal growth on the diet.

All rearing stages were held at 26° ± 1°C and 30 to 40% RH, in a room with 16-h photoperiod provided by two florescent 40-watt tubes. The rearing containers were spaced so as to allow light to reach them.

3.1.3. Preparation of Diet

The diet was prepared according to instructions from the supplier (Table I), and poured immediately into 150 x 25 mm sterilized plastic Petri-dishes, to a depth of about 2 cm, and allowed to gel. After 1 to 2 h, the gelled diet was cut into cubes about 2 x 2 x 2 cm. Each rearing cup was half-filled with these cubes. If the diet was not used immediately, it was kept uncut in the Petri-dishes at 4°C for up to two weeks.

3.1.4. Propagation of Colony

Rearing cups containing larvae destined for propagation, were kept separated from those with larvae for research, and the cups were not opened until pupation had commenced in order to

avoid contamination.

3.1.4.1. Pupal Collection and Egg Production

Pupae were collected from the rearing cups about twice weekly and sexed according to the number of abdominal segments visible on the venter. Five distinct segments were noticeable in the male, four in the female (Figure 1). The pupae were then placed in brown paper bags of about 20 x 13 x 39 cm with six strips of Scotch wax paper loosely tossed in, each strip 2 to 4 cm wide and 30 cm long. The wax paper was provided for newly emerged moths to cling to and for mating and oviposition. Fifty male and 50 female pupae were placed in each bag. The pupae and the emerged adults were held in the same environmental conditions as the larvae.

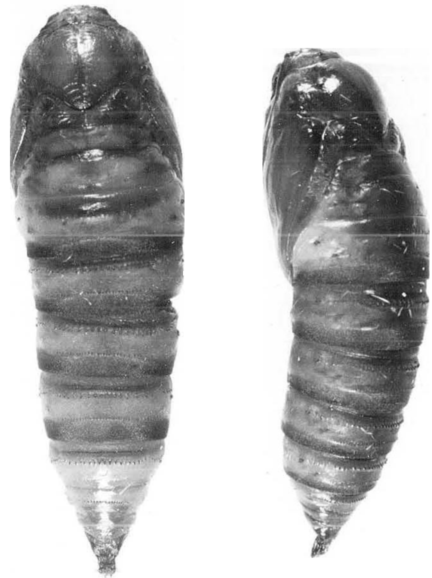
3.1.4.2. Egg Collection and Washing

After 7 to 10 days, depending on the age of the pupae collected, the bags were opened and strips of wax paper with egg masses adhering to them were collected. The adult moths were transferred to new bags, prepared as before, for another oviposition. The eggs were collected from these new bags after three days. The procedure was repeated for one more time before the adults were disposed of by deep freezing at -12°C.

Figure 1 : Western spruce budworm pupae showing the abdominal segments used for separating the sexes.

Left - Female pupa with four.

Right - Male pupa with five.



1 cm

The strips of wax paper were cut into pieces to separate each egg mass. The eggs and the pieces of paper to which they adhered were immediately surface-disinfected to avoid later contamination of the diet, especially by saprophytic fungi. The disinfectant was 10% formaldehyde solution mixed with a drop of wetting agent, polyoxyethylene sorbitan monolaurate (Tween 20^{R}) (Sigma Chemical Company, St. Louis, Missouri). The eggs were stirred gently in the solution for 10 min using a Number-3 camel's hair brush. Disinfection was followed by two ten-minute washes in distilled water. The egg masses were allowed to dry for 1 to 2 h on Whatman filter paper in Petri-dishes.

3.1.4.3. Rearing of Larvae

Each mass of 50 to 100 eggs was placed in a rearing cup with cubes of diet. The cup was covered with the paper cover, and wrapped with aluminium foil around the top-half. Pin-sized holes were punctured through the foil for moisture to escape. The cup was then placed on a shelf for the eggs to hatch and the larvae to develop. The aluminium foil wrapping was necessary in order to keep light from the top-half of the cup. Having the bottom-half of the cup illuminated, attracted the positively phototactic, newly-hatched larvae to the diet. Without the foil the young larvae migrated to the top of the cup, away from the diet, and diapaused.

After about 15 days each cup was opened and three or four fresh cubes of the diet were added to the partially dried old diet. The cover was replaced and the cup returned to the shelf for further development of the larvae. Larvae in suitable stages of development were collected for research as required. The colony size could be adjusted easily, according to the need at any given time.

3.2. Rearing of Mediterranean Flour Moth, *Anagasta kuehniella* (Zeller)

3.2.1. Stock Colony

At the start of this investigation, the numbers of the stock colony in the insectary at Simon Fraser University were increased. This colony had been maintained for seven years, since 1974.

3.2.2. Rearing Environment

Rearing was done according to the technique developed by Mr. A. Syed of Simon Fraser University Insectary (personal communication). The larvae were maintained on a diet comprising whole wheat grains, whole wheat flour, pablum (cereal mix), and water, in the ratio of 10:1:1:1 (w/w/w/v), in 3.6-L glass jars

(Figure 2, left). They were kept at 28° ± 1°C with 30 to 40% RH and 12-h photoperiod.

3.2.3. Propagation and Rearing

For egg oviposition, 5 or 6 cotton balls were put in each glass jar in which the adults had begun to emerge (Figure 2, right). About five days later, the cotton balls, with eggs laid on them, were collected and placed on freshly prepared diet. The new jars were covered with brown paper towels and kept on shelves until another generation had developed. Last-instar larvae were collected for research when required.

3.3. Vertebrates

Male Wistar rats, 2 to 6 months old, were used. They were obtained from Woodlyn Laboratories Limited, Guelph, Ontario. Water and rodent laboratory chow (Ralston Purina Company, St. Louis, Missouri) were given *ad libitum* throughout each study, until the rats were killed.

Figure 2 : Jars for rearing Mediterranean flour moth.

Left - Larvae and their galleries.

Right - Adult moths with cotton balls for oviposition.

3.4. Chemicals

3.4.1. Insecticides and Synergist

Insecticides used were technical and analytical grades of acephate, O,S-dimethyl acetylphosphoramidothioate (A), analytical grade methamidophos, O,S-dimethyl phosphoramidothioate (B), and analytical grade paraoxon, diethyl p-nitrophenyl phosphate (C) (Figure 3). Acephate and methamidophos were obtained from the Chevron Chemical Company, Richmond, California, and paraoxon from the American Cyanamid Company, Princeton, New Jersey. These chemicals were dissolved in acetone, buffer solution, or ethanol, according to the requirement of the study.

Aldrin and Dieldrin, both of analytical grades, obtained from the Shell Chemical Company, New York, were used in the microsomal activation study. These insecticides were dissolved in methyl cellosolve (ethylene glycol monomethyl ether).

Piperonyl butoxide, 80% technical grade, was used to inhibit the MFO in a microsomal activation study. A 1×10^{-2} M stock solution was prepared in absolute ethanol with 1% Triton-X. The solution was diluted to 1×10^{-4} M, when it was added to the incubation medium of the microsomal activation system made up in buffer solution.

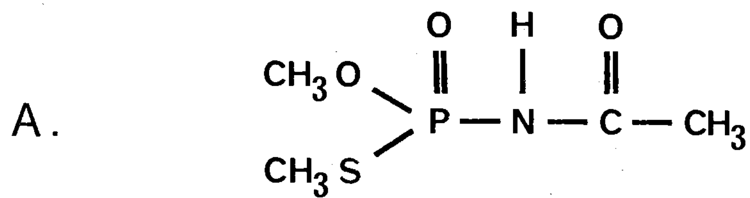
Figure 3 : Chemical structures of the insecticides.

A. Acephate (Orthene[R]), O,S-dimethyl acetyl-phosphoramidothioate.

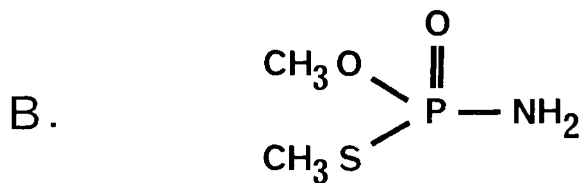
B. Methamidophos (Monitor[R], Tamaron[R]), O,S-dimethyl phosphoramidothioate.

C. Paraoxon, Diethyl *p*-nitrophenyl phosphate.

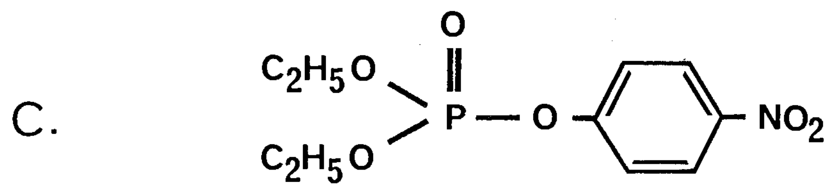
A.



B.



C.



3.4.2. Buffer

Phosphate buffer solutions, 0.1M, pH 7.6, were used in the studies, prepared from 0.2M stock solutions according to Gomori (1955). The solutions of pH 8.0, were prepared when acephate was to be dissolved in buffer solution. The pH of the prepared solutions was checked using a pH meter (pH METER 26, Radiometer Copenhagen).

3.4.3. NADPH-generating System

The solution, making up a component of the incubation medium for the activation study, was prepared in buffer solution. A 1-mL solution of the system contained 2 μmoles of NADPH, 20 μmoles of glucose-6-phosphate, 0.5 units of glucose-6-phosphate dehydrogenase, and 25 μmoles of magnesium chloride.

3.4.4. Reagents

3.4.4.1. Protein Analysis

The method of Lowry et al. (1951) was applied. The reagents used were as follows: 8% (w:v) of sodium carbonate (Na_2CO_3); 1% (w:v) of copper sulphate ($CuSO_4$); 2% (w:v) of sodium potassium tartrate ($NaKC_4H_4O_6$); 1N sodium hydroxide (NaOH); and 1N folin

phenol reagent. These reagents were either dissolved or diluted in deionized distilled water. Bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri), in buffer solution, was used as the standard reference.

3.4.4.2. Enzyme Assays

The AChE was assayed according to Ellman *et al.* (1961). Acetylthiocholine iodide (ACth) or butyrylthiocholine iodide (Bth), 0.075M, were used as the substrate, respectively, for assaying the AChE of all the organisms used in the study or for assaying the plasma ChE of the rats. Dithiobisnitrobenzoic acid (DTNB), 0.01M, was used as the chromogenic reagent. All the reagents were dissolved in buffer solution. They were obtained from Sigma Chemical Company, St. Louis, Missouri.

3.5. Bioassays

3.5.1. Insect Treatments

Last-instar larvae of western spruce budworm were treated topically with either acephate, or methamidophos, or paraoxon. The desired dosage, dissolved in 1 μL acetone, was applied to the dorsal thoracic region of individual larvae with a calibrated 10-μL glass syringe (Hamilton syringe No. 701),

fitted with a hypodermic needle (No. 2 point and 22 style), the stainless steel plunger being driven by a repeating dispenser (PB600-1, Hamilton Company, Reno, Nevada). The hypodermic needle was bent at a 45°-angle near its tip to facilitate proper droplet formation and treatment. Groups of five larvae were anesthesized with CO_2 for 10 sec prior to treatment. Each group of the treated larvae was held in a glass Petri-dish (100 x 20 mm) for observation. Two pieces of thinly sliced diet mix were placed in each dish as food. The larvae were held at a room temperature of 24° ± 1°C. Thirty to 50 larvae were treated at each dose level. Four or five dose levels were used for each chemical. Larvae held for control observations were treated in the same manner with 1 µL of acetone. Mortality counts were made 24 h after the treatments; mortality was determined by probing each larva lightly with a needle; a larva was considered to be dead when there was no observable response or movement.

Last-instar larvae of the Mediterranean flour moth were tested identically, except that only the toxicity of acephate was determined; groups of 10 larvae were treated and held in one Petri-dish for observation and mortality counting, 6 groups/dose; wheat flour was provided as food.

3.5.2. Analysis of Data

The data were analysed according to Swaroop (1966) to compute LD_{50} values and their confidence limits. Using logarithms of doses and probits of the corresponding mortalities, regression equations and correlation coefficients were computed.

3.6. Cholinesterase Inhibition

3.6.1. In-vitro Studies

3.6.1.1. Sources of Acetylcholinesterase

Last-instar larvae of the western spruce budworm and Mediterranean flour moth, rat brains, and human blood were the sources of AChE used for the study.

AChE was obtained from the heads and ventral nervous systems of spruce budworm larvae. Each larva was dissected by cutting the dorsal cuticle longitudinally from the last abdominal segment to the head. The cuticle was then pinned down on both sides of the body to expose the internal organs. The alimentary canal was removed by cutting through it near the anus, lifting and pulling it forward towards the head. The exposed nervous system, adhering to the ventral cuticle, was

cleaned of fat and extraneous cell debris by gently squirting with 0.7% sodium chloride solution using a Pasteur pipette. Large particles were removed with forceps. The head and nervous system were taken with a portion of the ventral cuticle by cutting it along both sides of the nervous system. They were washed in cold buffer solution before extracting the AChE.

Whole larvae were used for the extraction of AChE from the flour moth because they were very much smaller. The larvae were washed in cold buffer solution before the extraction.

Six-month-old rats were decapitated and bled; the brains were removed from the skulls and washed in cold buffer solution. Major blood vessels were separated from the brains by dissection and discarded.

Human AChE was obtained from red blood cells as described by Hussain and Oloffs (1979). A 50-µL blood sample, collected from a finger tip, was used for each preparation. The tip of the finger was wiped with 70% ethanol, and allowed to dry before the skin was pierced with a sterile disposable lancet. The blood was collected in a 50- µL micropipette, and added to 9 mL of cold buffer solution.

3.6.1.2. Extraction and Preparation of Acetylcholinesterase

With the exception of the human blood, the procedure used to prepare the enzyme from each of the other sources was the same. The samples were homogenized in cold buffer solution,

using either 10 heads and ventral nerve cords of spruce budworm larvae, or 20 whole flour moth larvae, or 0.1 g of rat brain per mL. Each batch of the preparations consisted of 5 mL of homogenate of the heads and ventral nerve cords of spruce budworm larvae, or 5 mL of the entire flour moth larvae, or 10 mL of the rat brain. The tissues were homogenized for about 3 min in an all-glass tissue homogenizer (PYREX brand), with the tube immersed in crushed ice during the homogenization. The homogenates were then centrifuged at 10,000 g for 1 h at 0 to 4™C in a Sorvall RC2-B superspeed automatic refrigerated centrifuge with an SS-34 fixed-angle rotor. The supernatants were collected and stored at -20°C until used, within 2 weeks.

AChE from human erythrocytes was prepared by centrifuging the diluted blood samples in a table-top centrifuge at 2,000 g for 15 min, to sediment the erythrocytes. The supernatant containing the plasma was discarded and the sedimented erythrocytes were washed by resuspending in buffer solution, then centrifuged again for another 10 min. The supernatant was again discarded, and the erythrocytes were suspended in 2 mL buffer solution; 0.6 mL of the latter suspension was added to 8.4 mL buffer solution to give a final volume of 9 mL of erythrocyte suspension, the concentration used in the study. The preparation was used immediately for the AChE study.

3.6.1.3. Determination of Inhibitor Activities

AChE preparations from each source were used to determine the I_{50} levels, i.e., the concentrations of each of the insecticides inhibiting 50% of the AChE. Acephate and methamidophos were dissolved in buffer solution, but paraoxon in absolute ethanol. The assays were done according to Ellman *et al*. (1961) at 37°C. The assay depends on the AChE-catalyzed hydrolysis of the substrate (ACth) and the estimation of thiocholine by following the increase of yellow colour produced when it reacts with the DTNB ion. Absorbance was read at 412 nm on a Cary 14 recording spectrophotometer, with the cuvette compartments kept at 37°C using a water bath.

For assaying, 0.1-mL aliquots of thawed rat brain or spruce budworm AChE preparations were mixed with 2.9-mL aliquots of buffer in test-tubes and incubated in a metabolic shaker at 37°C for 10 min; then appropriate doses of one of the insecticides, dissolved in 50 μL of buffer or ethanol, were added to each test-tube. After another 10-min incubation in the metabolic shaker, 50 μL of both the substrate and DTNB were added to each test-tube, except for the reference tube which received 50 μL of buffer instead of substrate. The contents were mixed with a "Vortex" mixer and transferred to silica cuvettes (10 mm path length) for measuring and recording changes in absorbance during 10 min.

The same procedure was used for assaying the inhibition of AChE activity from flour moth larvae. However here, a 50-μL enzyme preparation was incubated in 2.95 mL of buffer solution. Human erythrocyte AChE was assayed after the cells had been lysed by adding 0.1 mg saponin (Sigma Chemical Company, St. Louis, Missouri) to each erythrocyte preparation (Michel 1949). A 3-mL volume of the preparation was pipetted into each test-tube and incubated as above. The absorbance was also read in the same manner as described above.

3.6.1.4. Progressive Inhibition of Acetylcholinesterase

AChE, prepared as described (Section 3.6.1.2), was incubated for 0, 10, 20, 30, 60, 120, 180, and 240 min with acephate, methamidophos, or paraoxon at their sub-I_{50} concentrations respective to the enzyme, derived from the previous experiment using 10-min exposures. The substrate and DTNB were added at the end of each incubation period and absorbances recorded as described. Control samples without the insecticides were incubated for 0, 60, and 240 min.

3.6.1.5. Interaction

Interaction of acephate and methamidophos were investigated by exposure of AChE to acephate and methamidophos, either separately or in combination. The insecticides were added at

their respective sub-I_{50} concentrations at about equivalent to the I_{25}. Then the samples were incubated for 10 min and assayed as described (Section 3.6.1.3).

3.6.2. In-vivo Studies

3.6.2.1. Insects

Individual last-instar larvae of western spruce budworm and Mediterranean flour moth were treated with sublethal doses of 8 μg/g and 15 μg/g of acephate, respectively, as described for the bioassay studies (Section 3.5.1). After the exposure periods of 0, 1, 2, 4, 8, 12, 18, 24, 30, 36, 48, 60, and 72 h, five spruce budworm and 10 flour moth larvae were placed on dry ice for 15 min, then allowed to thaw before the AChE was extracted and assayed as described previously (Section 3.6.1). Each group of spruce budworm heads and nervous systems, and flour moth larvae was homogenized in 1 mL and 1.5 mL buffer solution, respectively. The activity was converted to nmoles of substrate hydrolyzed/mg protein/min. Protein concentration of each enzyme preparation was determined by the technique of Lowry *et al.* (1951). Readings at 660 nm wavelength were taken using a Unicam SP600 series 2 spectrophotometer.

3.6.2.2. Rats

Male Wistar rats were used, 60 to 80 days old and weighing from 100 to 360 g. They were caged individually and maintained at 22° ± 1°C.

Each of 12 rats was treated once with acephate at 270 mg/kg of body weight. The insecticide, at 27 mg/mL in distilled water, was administered by stomach tube, in a dose based on the animal's body weight. Eight control rats received water only.

Groups of three acephate-treated and two control rats were taken, respectively, 0.5, 8, 24, and 72 h after the treatments. A 50-μL blood sample was collected from each rat by tail bleeding.

The blood was collected with 50-μL micropipettes and added to 9 mL of buffer solution and processed further as described for the human erythrocytes in Section 3.6.1.2, but the supernatant containing the plasma was collected and assayed for the ChE activity, also done according to Ellman _et al_. (1961). The plasma ChE was assayed as the difference in activity with and without quinidine sulfate which specifically inhibits plasma ChE (0.15 mg quinidine sulfate added to 3 mL buffer containing plasma (Sabine 1955). The rats were then killed immediately by decapitation. The brains were removed and frozen at -12°C for one week, when the AChE was extracted and assayed as described in Section 3.6.1. The activities of AChE and plasma ChE were converted to nmoles of substrate hydrolyzed/mL blood/min.

3.7. Metabolism

3.7.1. Treatments

3.7.1.1. Insects

Last-instar larvae of the spruce budworm and flour moth were treated topically with acephate at about 10 and 30% of the previously determined LD_{50} values. The procedures were as described in Section 3.5.1. After 0, 1, 2, 4, 8, 12, 18, 24, 30, 48, 60, and 72 h of exposure, groups of five spruce budworm and 10 flour moth larvae were frozen on dry ice for 15 min. The larvae were then thawed for 10 min and weighed before extraction of acephate and methamidophos.

3.7.1.2. Rats

The rats used in this study were those treated for the *in-vivo* AChE inhibition study described in Section 3.6.2.2. The liver was removed from each decapitated rat, washed in cold tap water, blotted with paper towels, weighed, cut into small pieces, and put in 100 mL of acetonitrile in a 235-cc (8 fl. oz.) glass jar. The jars were capped tightly and kept in ice for about 2 h before the extraction was carried out.

3.7.2. Extraction and Clean-up

3.7.2.1. Insects

Each weighed sample was homogenized in 20 mL of pesticide-grade acetonitrile, with 5 g of anhydrous granular sodium sulphate, in a 150 x 25 mm glass tube, using a homogenizer fitted to a Polytron PT20. The supernatant was filtered through a Pyrex glass wool plug, topped with a layer of anhydrous granular sodium sulphate in a filter funnel, and collected in a 250-mL round-bottom flask. The extraction procedure was repeated twice, each time with 20 mL of acetonitrile. After the third extraction, the entire extract was poured into the filtration funnel. The test-tube and the homogenizer were rinsed with 20 mL of acetonitrile which was also filtered into the same flask. The extract was evaporated to dryness with a flash evaporator at 38°C. The residues were picked-up in one to 10 mL of glass-distilled acetone for analysis by gas-liquid chromatography (GLC) without further clean-up.

3.7.2.2. Rats

The procedure of Szeto *et al*. (1982) for fish extraction and clean-up was followed. Anhydrous granular sodium sulphate at 2:1 (w/w) was added to each jar containing the weighed liver and

100 mL of acetonitrile. The liver was homogenized for about 3 min using the homogenizer fitted to the Polytron PT20. The supernatant was filtered as described and collected in a graduated cylinder. The extraction was repeated twice with 75 mL of acetonitrile each. Finally, the extraction jar and homogenizer were rinsed with 50 mL of acetonitrile which was also filtered and collected. The final volume of each crude extract was determined.

An aliquot of the crude extract, equivalent to 2 g wet liver weight, was transferred into a 250-mL round-bottom flask, and evaporated to dryness by flash evaporation at 38°C. The residues were picked up in 1 mL of ethyl acetate (HPLC grade), followed by the addition of 4 mL of glass-distilled hexane. To clean up this solution, a glass column was packed, from the bottom up, with a glass wool plug, followed by 2 cm of Na_2SO_4, 4 cm of a 1:2.5 (w/w) mixture of Nuchar C-190N charcoal (Matheson Coleman & Bell, East Rutherford, New Jersey) and Whatman CF 11 cellulose powder (Whatman Ltd., Kent, England), and 2 cm of Na_2SO_4. The packed column was pre-washed with 10 mL of ethyl acetate, followed by 25 mL of hexane. The extract was then transferred from the round-botton flask to the clean-up column. The resulting eluate was discarded. Finally, acephate and methamidophos residues were eluted from the column with 35 mL of ethyl acetate. The eluate was flash-evaporated at 38°C, and picked-up in volumes of acetone suitable for GLC analysis.

3.7.3. Gas-Liquid Chromatography Analysis

A Tracor 222 gas chromatograph was used. It was equipped with two flame photometric detectors (524 nm phosphorus filter), and U-shaped Pyrex glass column (36 cm x 3 cm I.D.) packed with 1% Carbowax 20M TPA on Chromosorb W "HP", 100-200 mesh. The operating parameters were: detector 160°C; injection port 195°C; column oven programmed from 145 to 190°C at 29°C/min; gas flow rates for N , H , and air were 70, 150, and 180 mL/min, respectively.

The clean extracts were injected directly, or were suitably diluted, for the analysis. If 8 µL of undiluted extract gave no response, the results were reported as non-detectable (N.D.). The limits of quantification of acephate and methamidophos were 4 ppb and 2 ppb, respectively, equivalent to 4% chart deflection. Detectable responses below these limits were reported as trace and computed as one-half of the appropriate quantification limits.

Analytical grades of acephate and of methamidophos were used to prepare standard solutions in glass-distilled acetone. Solutions of 0.6:0.3 and 0.06:0.03 µg/mL of acephate:methamidophos were prepared from stock solution of 60:30 µg/mL, at each time the analysis were to be performed. A standard curve was constructed for each standard solution, and was used to determine the concentrations of acephate and methamidophos residues in the sample extracts. The residues were

identified by the peaks on the chromatograms, and quantified by measuring the peak height.

3.7.4. Recovery Studies

To test recoveries of acephate and methamidophos, samples of residue-free insect larvae and rat livers were fortified with acephate at 1 and 0.01 ppm, and with methamidophos at 0.5 and 0.005 ppm. They were then extracted and processed as described. Recoveries ranged from 93.0 to 108.8%; details are shown in table II.

3.8. Microsomal Activation

3.8.1. Preparation of Microsomes

3.8.1.1. Insect Midguts

The midguts of last-instar larvae of western spruce budworm were isolated by cross-sectioning each larva at the intersegmental membrane between the thorax and abdomen, and immediately behind the last pair of prolegs. The midgut was then extracted with forceps from the resulting middle section. It was sectioned longitudinally with a scalpel, and rinsed in cold buffer solution to remove its contents, and then washed

Table II : Recoveries of acephate and methamidophos from fortified insect larvae and samples of rat liver.

Insecticide	Fortification level (ppm)	Recovery range (%)[1/]		
		Spruce budworm larvae	Flour moth larvae	Rat liver
Acephate	1.00	93.5 - 95.6	98.1 - 98.5	97.5 - 101.5
	0.01	104.8 - 106.3	97.2 - 97.3	100.1 - 108.8
Methamidophos	0.50	93.5 - 94.8	97.2 - 99.5	93.0 - 96.7
	0.005	98.0 - 100.0	99.6 - 100.0	93.6 - 93.7

1/ N = 3 for the spruce budworm and flour moth larvae; N = 2 for the rat liver.

thoroughly in clean, cold buffer.

Samples of 20 midguts were then homogenized in 5 mL of cold buffer with a Potter homogenizer with a motor-driven teflon pestle. The homogenized samples were later combined for centrifugation at 0 to 4°C for 15 min at 10,000 *g*. The supernatant was filtered through glass wool and then centrifuged at 0 to 4°C for 60 min at 105,000 *g* in a Sorvall OTD 75B ultracentrifuge with a Ti60 fixed-angle rotor (Yu and Terriere 1979). The supernatant was decanted and the microsomal pellets were resuspended in cold buffer solution, at the equivalent of 20 midguts/mL, by gentle homogenization. Protein concentration of the suspension was determined by the technique of Lowry *et al*. (1951). The microsomal suspension was either used immediately or stored in ice, in the cold room at 4°C, and used within 3 days, with no loss of the activity.

3.8.1.2. Rat Livers

Microsomes were prepared from perfused livers of male Wistar rats, about six months old. Each rat was killed by decapitation, and the thoracic and abdominal cavities were opened immediately to expose the liver. The liver was perfused with cold 1.15% potassium chloride solution, pH 7.4, by inserting a hypodermic needle into the portal vein, until the liver turned pale yellow. The liver was then removed from the thoraxic cavity, washed thoroughly, and homogenized for 30 sec

in 4 mL of cold 1.15% potassium chloride solution per 1 g of liver weight, using a VirTis blender at full speed (Chan *et al*. 1967). The homogenate was centrifuged for 30 min at 10,000 *g* and 0 to 4°C. Microsomes were prepared from the supernatant as described in the previous section (3.8.1.1), and finally picked up in buffer at the equivalent of 1 g wet liver weight/mL. Protein was determined according to Lowry *et al*. (1951). The microsomal suspension was stored frozen at -12°C, and used within a week without loss of the activity.

3.8.2. Determination of Cytochrome P-450

The carbon monoxide-binding-complex method of Omura and Sato (1964) was applied. A 3-mL microsomal preparation, with or without dilution, was reduced with about 100 mg of sodium dithionite ($Na_2S_2O_4$). CO was then slowly bubbled through the reduced sample for about 1 min, using a Pasteur pipette, and CO difference spectra were immediately determined with a Cary-14 recording spectrophotometer by scanning from 390 to 520 mμ. The concentration was determined by the peak height of the spectrum between 450 and 490 mμ (ΔOD 450 - 490).

3.8.3. Incubation of Microsomal Preparations with Insecticides

3.8.3.1. Aldrin

For each incubation, the following were added to a 50-mL Erlenmyer flask: 0.5 mL microsomal preparation, 1 mL of NADPH-generating solution, and 5.5 mL of buffer. It was then shaken in a Dubnoff metabolic shaker at 37°C for 10 min, before 10 µg of aldrin were added in 50 µL methyl cellusolve. The incubation times were 0, 15, 30, and 60 min. To one of the incubation media, piperonyl butoxide was added to an equivalent of 10^{-4} M, and incubated for 60 min.

3.8.3.1.1. Extraction

The reaction was stopped by shaking and extracting the incubation mixture with 20 mL of a 1:1 mixture of hexane and acetone. Everything was then transferred into a separatory funnel, where the solvent and aqueous phases were allowed to separate. Two additional extractions were made, each with 20 mL of the same solvent mixture routed through the incubation flask in order to remove traces of insecticides which may have adhered to the glass. The combined hexane extracts were washed twice with 2% Na_2SO_4. The hexane phases were then stored over granular anhydrous Na_2SO_4 at -20°C until analysed, within one week.

Extraction efficiency was checked by adding 20 μg of dieldrin and 10 μg of aldrin to an insecticide-free incubation medium and extracting immediately.

3.8.3.1.2. Analysis

A Tracor MT 220 gas chromatograph, equipped with ^{63}Ni electron capture detector was used for the analysis of aldrin and dieldrin residues. The extracts were injected into a U-shaped Pyrex glass column, 183 x 0.3 cm I.D., packed with 2% OV 1 plus 6% OV 210 on Chromosorb W 'H.P.", 80-100 mesh. The carrier gas was nitrogen with a flow rate of 70 mL/min. Other operating parameters were: injector port, 150°C; column oven, 225°C; detector, 245°C.

The extracts were suitably diluted with redistilled hexane before the injection. A standard solution containing aldrin and dieldrin in hexane was used to prepare a standard curve on each day when the extracts were analysed.

3.8.3.2. Activation of Acephate

The incubation medium and conditions used were the same as described in the previous section (3.8.3.1). Acephate was added to the medium in 0.1 mL of buffer and incubated for 1 h. The concentrations of acephate used were 951 μg (0.52 x 10^{-2} M) and 338 μg (0.20 x 10^{-2} M)/mL of incubation medium, respectively, for

assaying with the AChE of spruce budworm larvae and of rat brains. The AChEs were prepared as described in section 3.6.1.2.

Activation of acephate was determined by the increased inhibition of activities of the AChEs, done as described in section 3.6.1.3. After 1 h of incubation a 2.9-mL aliquot of the incubation medium was transferred into each tube, prepared in duplicate. Then 0.1 mL of the appropriate AChE was added to each tube, and incubated for 10 min at 37°C, before assaying.

4. RESULTS AND DISCUSSION

4.1. Rearing of Western Spruce Budworm

A colony of non-diapausing western spruce budworm was established in 1980. Reared as described, the insects reproduced successfully and an ample supply of last-instar larvae was available for this research. Adjustments of the colony size were made according to the need at any particular time during the course of the research.

The time required for the insect to develop and complete its life-cycle was determined. Forty-three developing insects were observed; four of these, or 9.3%, died before reaching the adult stage. The larvae were reared individually in 30-mL clear plastic cups for easy observation.

Results are shown in Table III. The insects required 48 to 50 days for development from oviposition through adult mortality, or 38 to 40 days for a complete generation from egg to egg. The eggs hatched seven days after oviposition. The average times for the development of the other stages, respectively, for male and female were: larvae 21.5 and 22.5 days; pre-pupae 0.5 day for both the sexes; pupae 7.9 and 7.8 days. A further breakdown on the development of the larvae showed that each of the first five instars had a duration of 3

Table III : Development time for non-diapausing colony of western spruce budworm, *C. occidentalis*.

Stage of development	Number of days ($\bar{X} \pm$ S.D.)[1]	
	male[2]	female[3]
Egg[4]	7.00 ± 0	7.00 ± 0
Larva[5]		
1	3.04 ± 0.20	3.40 ± 0.51
2	3.13 ± 0.34	3.13 ± 0.35
3	3.17 ± 0.38	3.20 ± 0.41
4	3.33 ± 0.48	3.33 ± 0.49
5	3.58 ± 0.50	3.60 ± 0.51
6	5.29 ± 0.55	5.87 ± 0.35
Sub-total for the larvae	21.54	22.53
Pre-pupa	0.50 ± 0	0.50 ± 0
Pupa	7.88 ± 0.74	7.80 ± 0.68
Adult	11.79 ± 1.38	11.60 ± 1.06
Total	48.71	49.43

1/ Number of larvae observed was 43, but 3 died as larvae and 1 as pupa (9.3%), and were not included for computing the means.

2/ Mean of 24 observations.

3/ Mean of 15 observations.

4/ Sex not known, n = 43.

5/ Sex determined at pupal stage.

to 3.6 days, but 5 to 6 days for the sixth instar. The adult life-span was 11.8 and 11.6 days, respectively. The female had the longer period of larval development, and thus, the overall period for the completion of the generation. This is consistent with the general observation in the colony, where the female pupae normally occur one or two days later than the male pupae.

Table IV shows the fecundity of the spruce budworm. Each of 12 pairs of newly emerged male and female moths was put in a larval rearing cup (Section 3.1.2) containing strips of wax paper for oviposition, and placed in a brown paper bag. The moths mated within one-half day of their emergence. The first egg mass was laid within about one-half day after mating. Five or six egg masses were laid by each female on successive days. The egg masses contained from 22 to 123 eggs. The total number of eggs laid by each female ranged from 254 to 369, with a mean of 307. Most females tended to lay more eggs in the first three masses than in the last two or three. Two females did not oviposit, although mating of one pair was observed.

The synthetic diet developed by McMorran (1965) also has been used successfully for the rearing of several closely related species such as the eastern spruce budworm, Douglas-fir tussock moth, corn earworm, soybean and cabbage loopers (Grisdale 1973). Lyon et al. (1972) were successful in rearing colonies of diapausing and non-diapausing western spruce budworm on a slightly modified diet. They also indicated the possibility of rearing other species of the genus Choristoneura without

Table IV : Fecundity of 10 female western spruce budworms, *C. occidentalis*: numbers of eggs per egg mass.

Day of oviposition 1/						Total egg production per female
1	2	3	4	5	6	
80	81	81	47	56	-	345
78	78	70	42	46	-	314
44	63	32	22	62	31	254
123	65	40	35	31	31	325
38	37	43	85	28	27	258
61	72	66	39	45	-	283
66	52	41	48	33	34	274
74	104	26	44	44	62	354
53	98	63	74	51	30	369
111	35	32	35	35	47	295
					Total	3,071
					$\bar{X} \pm$ S.D.	307.1 ± 40.67

1/ Egg masses were laid on successive days, beginning about one-half day after mating.

diapause, by a simple adjustment in the laboratory technique.

Comparing this colony of *C. occidentalis* with those reared in the laboratory by Lyon *et al*. (1972) and Robertson (1979), the following general observations were noted: The hatching of the eggs within seven days of oviposition was in complete agreement; larval development in this colony was 7 to 9 days shorter; the period for the pupal stage was longer by about one day; the adult life-span was longer by 5 to 7 days. The shorter larval development period was caused mainly by shorter first and second instars. They lasted 3 to 3.5 days, whereas the reported periods were nine and five days, respectively. The generation time of 38 to 40 days from egg to egg, was shorter here than those reported by Robertson (1979) and Lyon *et al*. (1972), by 3 to 4 days and 9 to 11 days, respectively.

These differences could have been caused by differences in the rearing environment, such as the higher temperature of 26° $\pm$ 1°C and the relative humidity of 30 to 40% used in this work. By contrast, temperatures of 23 to 26°C and relative humidities of 30 to 50% were reported by Lyon *et al*. (1972) and Robertson (1979). In the field, temperature changes have been shown to affect larval development significantly, especially that of first and second instars (Shepherd 1961). According to Peterson (1953) temperature and humidity are probably the most important environmental factors in the habitats where insects breed. Bursell (1964) stated that within the limit of tolerence the velocity of insect development is greatly affected by

temperature. Therefore, the higher temperature used here could be the influential factor, especially in the development of the first- and second-instar larvae.

Lyon et al. (1972) also studied the relationship of adult age to fecundity. Highest fecundity was obtained with mating taking place when the adults were less than one day old. The average number of 174 eggs obtained by Lyon et al. (1972) was less than the average of 307 recorded here with single-pair mating of adults when both were less than one day old. According to Lyon et al. (1972) single-pair matings were not as productive as those of the multiple-pair matings. No definite explanation could be given for the higher fecundity recorded other than the apparent differences in the overall rearing environment. The adult life-span here was longer than that reported by Robertson (1979), but the average periods for mating and oviposition were about the same as those observed by Lyon et al. (1972).

Sanitation is essential to rearing the spruce budworm successfully in the laboratory and on artificial diet. Contamination of the diet by saprophytic fungi and bacteria is a common problem. Although these microorganisms are not pathogenic, insects cannot survive on infected artificial diet. Therefore, any contamination, if detected early enough, must be eradicated at once in order to prevent spreading and total loss of the insect culture. It has been found, in this respect, to be a most valuable safeguard to maintain part of the colony in separate quarters. This allows complete eradication of an

infection in one facility, while breeding can be continued elsewhere.

It should be noted that establishing a colony for the present project failed until the paramountcy of sanitation and the value of a spare culture were recognized and remedial actions implemented. Before that, larvae always died shortly after hatching, until the colony was lost, because the surfaces of the diet cubes, infected with saprophytes, had become slimy and sticky, thus trapping and killing the newly-hatched larvae.

According to Bio-Serv, Inc., the total aerobic plate count of microbial analysis of the diet originally purchased was about 10,000 colonies/g. Mixing the diet ingredients with the agar immediately after boiling, did not reduce the infection. Subsequently, at the time of ordering the diet, it was specified that the diet must yield < 100 colonies/g. Thereafter, rearing of the spruce budworm on the diets complying with these specifications remained largely problem-free. Occasional infections, occurring during processing, were detected early so that such infections could be eradicated.

4.2. Rearing of Mediterranean Flour Moth

The diet mixture described in section 3.2.2 was suitable to rear and maintain the flour moth culture, and to raise enough of the insects for this research.

Only a general observation was made on the development of the flour moth. The insect completed its life-cycle in 30 to 40 days. The eggs hatched within 3 to 4 days of oviposition, and remained in the larval stage for about 20 days. The pupal stage lasted for 7 to 10 days, when the moth emerged and lived for 12 to 20 days.

Boles and Marzke (1966) described an artificial medium for rearing flour moth, consisting of gaines meal, whole wheat flour, corn meal, dried yeast, honey, glycerine, wheat germ, and oat meal. The insect developed from egg to adult emergence within 28 days. Strong et al. (1968) reported that the insect developed within 32 days, when it was reared on diet made up of poultry laying mash mixture, chick starter, rolled barley, raisins, and glycerine. Despite the use of a different diet for the present colony, the insect also required about the same length of times for its development as reported earlier. Therefore, a similar culture of the flour moth was able to be maintained with less ingredients making up the diet mixture. Peterson (1953) suggested the use of whole wheat flour and corn meal separately for rearing the larval stages. The first-instar larvae were to be reared in the whole wheat flour because they were observed to grow better in it, whereas the other instars were to be reared in the corn meal. This procedure is laborious, requiring the transfer of the larvae after their first instar into the corn meal for further development.

The successful rearing and maintainance of the culture through the generations indicated that the diet used here provided a complete source of food for the insect to develop through the entire life cycle. The presence of whole wheat grains seem to provide spaces in the medium, facilitating movement and gallery-making by the larvae, and also for spinning the webs for pupation. The use of cotton balls for oviposition facilitated the collecting of eggs.

4.3. Bioassay

Results of the bioassays are summarized in Table V. The toxicities of the chemicals to last-instar larvae of the western spruce budworm were, in that order: acephate < methamidophos < paraoxon. Acephate was approximately three and 17 times less toxic than methamidophos and paraoxon, respectively; methamidophos was about 5.5 times less toxic than paraoxon. Paraoxon is the oxygen analogue of parathion and of extreme toxicity to animals. Its LD_{50}, rats, oral is 1.8 mg/kg (Fairchild 1978). Therefore, the high toxicity of paraoxon to the spruce budworm could be expected.

Acephate was twice as toxic to the spruce budworm as to the Mediterranean flour moth (Table V). Although this difference is not overwhelming, it may be an indication of acephate's efficacy in controlling spruce budworm under field conditions. Without a doubt, its vertebrate selectivity ratio, VSR (Hollingworth

Table V : Toxicities of acephate, methamidophos, and paraoxon applied topically to last-instar larvae of western spruce budworm, *C. occidentalis*, and Mediterranean flour moth, *A. kuehniella*.

Insect	Insecticide	24-h LD_{50} (μg/g)	95% confidence interval (μg/g)	Regression equation $(y = a + bx)$ [1/]	Correlation coefficient (r)
C. occidentalis	Acephate	23.21	21.05 - 25.59	-1.65 + 4.87x	0.9729
	Methamidophos	7.45	6.52 - 8.52	2.06 + 3.37x	0.9884
	Paraoxon	1.34	1.26 - 1.43	4.10 + 7.05x	0.9958
A. kuehniella	Acephate	48.27	39.89 - 58.40	-0.14 + 3.05x	0.9985

1/ y = Probit mortality; a = y-intercept; b = slope of regression line; x = logarithm of dose.

1976), makes it much more acceptable for use in the field than either methamidophos or paraoxon. The VSR's for these three compounds (LD_{50} rat, oral/LD_{50} spruce budworm, topical) are acephate 41, methamidophos 2.7, and paraoxon 1.3.

Robertson et al. (1976, 1978) reported toxicities of acephate and methamidophos to last-instar larvae of the spruce budworm following topical applications. They reported LD_{50} values of 27.0 and 29.7 μg/g for acephate, and 10.0 μg/g for methamidophos. Although about similar to my results, it is not possible to compare them because Robertson et al. (1976, 1978) determined mortalities seven days after application of the insecticides. Not only is that an unusually long period for the determination of mortality of insects after application of an insecticide, it also raises the question of the longevity of last-instar larvae, which is 5 to 6 days according to Lyon et al. (1972) and according to this study (Table III).

4.4. Cholinesterase Inhibition

4.4.1. In-vitro Studies

4.4.1.1. Determination of Inhibitor Activities

OP insecticides can be ranked according to the strength of their *in-vitro* inhibition of AChE. The concentration of an OP insecticide which inhibits 50% of AChE under specified conditions, called the I_{50}, is often used as a measure of this inhibitory strength. Thus, the lower the I_{50} of an OP insecticide, the stronger an inhibitor of AChE it will be. This study was to determine these values for acephate, methamidophos, and paraoxon, using AChE from different organisms. The method of Dixon and Webb (1964) was used to compute the I_{50} values, by evaluating the linear relationship between 1/v and i, where v is the velocity of the inhibited reaction in percent of the activity of the reaction without the inhibitor, and i is the concentration of the inhibitor.

The results are presented in Table VI. In each case, the strengths of the three insecticides were: acephate < methamidophos < paraoxon, thus reflecting the acute toxicities of these chemicals when applied topically to the spruce budworm (Table V), and their acute oral toxicities to rats, which are, LD_{50}, 24 h, mg/kg: acephate 945, methamidophos 18, and paraoxon

Table VI : The I_{50} of acephate, methamidophos, and paraoxon on AChE from different sources at 10 min and 37°C.

Source of AChE	I_{50} (Molar)			Activity of AChE without inhibitor[1/]
	Acephate	Methamidophos	Paraoxon	
Spruce budworm larvae	5.44×10^{-2}	40.67×10^{-5}	4.41×10^{-7}	28.68
Flour moth larvae	2.24×10^{-2}	7.67×10^{-5}	1.65×10^{-7}	105.88
Human erythrocytes	1.86×10^{-2}	2.33×10^{-5}	0.33×10^{-7}	11.32
Rat brains	0.72×10^{-2}	2.00×10^{-5}	0.23×10^{-7}	61.76

1/ nmoles substrate hydrolyzed/mL enzyme preparation/min.

1.8 (Fairchild 1978; Spencer 1982).

Such data, as shown in Table VI, probably have given rise to the often found statement that acephate is a poor inhibitor of AChE, while methamidophos is of moderate anti-AChE activity. Apparently, the implication has been that paraoxon is the "standard" and that compounds with $I_{50} > I_{50}$ of paraoxon are of "moderate" or "poor" anti-AChE strength. Such interpretation leaves unanswered questions regarding acephate's selectivity, mode of action, activation, etc.

Closer scrutiny of the same data, however, reveals much better information. For instance, how much stronger methamidophos and paraoxon are, than acephate, can be expressed by the fractions I_{50} acephate/I_{50} methamidophos and I_{50} acephate/I_{50} paraoxon, respectively. These fractions are quantitative indices of the _in-vitro_ anti-AChE strength of acephate, relative to those of methamidophos and paraoxon. They show that the _in-vitro_ anti-AChE strength of acephate, relative to that of methamidophos, decreases as both its _in-vitro_ strength, relative to that of paraoxon, and its acute toxicity (LD_{50}) decrease from the house fly to mammals (Table VII). In other words, regardless of whether or not acephate's toxicity _in vivo_ depends, in part or entirely, on some type of activation, its selectivity is reflected by its _in-vitro_ activity _per se_, i.e., by its direct effect on AChE without activation to a more effective molecular species. Relative to methamidophos, it is eight times less effective against human erythrocyte AChE than

Table VII : *In-vitro* anti-AChE strength of acephate (A) relative to methamidophos (M) (I_{50} A/I_{50} M) and paraoxon (P) (I_{50} A/I_{50} P), and the acute toxicities of acephate, methamidophos, and paraoxon.

Organism	I_{50} A/I_{50} M	I_{50} A/I_{50} P	LD_{50} 1/		
			Acephate	Methamidophos	Paraoxon
House fly	1.00×10^2	0.67×10^5	0.9	-	-
Spruce budworm	1.34×10^2	1.23×10^5	23.2	7.5	1.34
Flour moth	2.92×10^2	1.36×10^5	48.3	-	-
Rat	3.60×10^2	3.13×10^5	945.0	18.0	1.8
Human	8.00×10^2	5.64×10^5	-	-	-

1/ 24-h, mg/kg, applied topically to insects, orally in rats (House fly: unpublished data, Spruce budworm and Flour moth: Table V, Rat: Fairchild 1978; Spencer 1982).

against house fly AChE; relative to paraoxon, its anti-AChE effectiveness is 8.4 times lower, Table VII.

The information may be summarized as follows. (i) The acute toxicity of acephate decreases from the house fly to the rat (Table VII). (ii) This is reflected by a decrease in the in-vitro anti-AChE strength of acephate relative to that of methamidophos and by a similar decrease relative to paraoxon. (iii) Acephate per se inhibits AChE in vitro in proportion to its toxicity in vivo. (iv) Paraoxon is about as toxic to the spruce budworm as to the rat (LD_{50} 1.34 and 1.8 mg/kg), methamidophos is slightly more than twice as toxic (LD_{50} 7.5 and 18 mg/kg), but acephate is 41 times more toxic to the insect than the mammal (LD_{50} 23 and 945 mg/kg).

Unquestionably other factors, such as penetration into organisms, transport within organisms, types and rates of metabolic transformations, etc., will have strong effects on the selectivity of acephate in addition to its reaction with AChE in vitro. Nevertheless, the data, as presented in Table VII, are consistent and should allow one to predict susceptibility to acephate and its degree of selectivity on account of in-vitro inhibition of AChE by acephate relative to methamidophos and paraoxon.

This approach also circumvents common problems of enzyme kinetics, and consequent erroneous conclusions. For instance, a direct comparison of results between species (Table VI) is invalid as the enzyme concentrations in AChE preparations were

not known. The activity of each preparation without inhibitor, in terms of substrate transformed/min/mL (Table VI), still does not give the catalytic-centre activity (as an index of the enzyme concentration) as it will vary with the source of the AChE (Aldridge 1971).

4.4.1.2. Progressive Inhibition of Acetylcholinesterase

The inhibition with time of AChE from different sources by acephate, methamidophos, and paraoxon was determined. The enzymes were incubated with concentrations below the known I_{50} of the respective chemicals for up to 240 min, before substrate was added and the activity determined as before.

Data in Table VIII, and graphically depicted in Figures 4 to 7, show the following pattern. Acephate, unlike methamidophos and paraoxon, inhibited 23 to 33% of the insects' AChE instantly (Figure 4 and 5). But the pattern of inhibition of the mammalian AChE was very similar for all three anti-AChE agents (Figure 6 and 7). This acephate-typical phenomenon, namely the instant inhibition of the insects' AChE by acephate, may even be more pronounced than is apparent in these experiments (Figure 4 and 5), if one takes into account that maximal inhibition with time by acephate was higher for mammalian (77% and 82%) than insect (73% and 62%) AChE. For instance, if acephate concentrations that inhibit 80% after 240 min in all AChE preparations could be chosen, the instant effect of acephate could be expected to be

Table VIII : Progressive inhibition of AChE by acephate (A), methamidophos (M), and paraoxon (P) *in vitro*[1/].

Source of AChE	Inhibitor	Mean percent activity at stated incubation times (min)[2/]							
		0	10	20	30	60	120	180	240
Spruce budworm larvae	Acephate	67.24	63.99	57.95	55.59	53.79	38.00	36.99	37.63
	Methamidophos	98.04	73.40	63.94	57.15	51.58	46.28	46.72	47.09
	Paraoxon	95.43	74.29	60.54	55.31	47.30	49.10	45.37	46.83
	Control	100.00	-	-	-	98.48	-	-	97.26
Flour moth larvae	Acephate	77.22	71.06	62.14	54.46	35.92	28.53	27.29	27.34
	Methamidophos	97.40	71.01	54.13	47.76	42.41	41.07	38.77	37.41
	Paraoxon	94.77	69.06	55.90	45.21	37.08	35.78	36.81	36.44
	Control	100.00	-	-	-	98.72	-	-	98.41
Human erythrocytes	Acephate	90.74	67.56	52.92	43.27	31.08	25.33	24.41	23.03
	Methamidophos	89.52	68.57	45.95	35.95	20.00	13.34	12.86	10.71
	Paraoxon	99.52	71.43	51.43	38.10	17.38	7.14	5.71	5.71
	Control	100.00	-	-	-	99.05	-	-	98.59

Table VIII (cont'd) : Progressive inhibition of AChE by acephate (A), methamidophos (M), and paraoxon (P) *in vitro*[1/].

Source of AChE	Inhibitor	Mean percent activity at stated incubation times (min)[2/]							
		0	10	20	30	60	120	180	240
Rat brains	Acephate	85.67	70.38	59.38	49.57	36.72	23.28	19.32	18.34
	Methamidophos	96.41	72.36	56.50	56.39	28.36	23.32	23.56	23.32
	Paraoxon	98.04	74.15	56.31	43.23	24.88	17.56	16.29	16.10
	Control	100.00	-	-	-	99.54	-	-	97.70

1/ Concentrations (Molar): Spruce budworm; A = 2.3×10^{-2}, M = 3.3×10^{-5}, P = 0.9×10^{-7}.
Flour moth; A = 0.9×10^{-2}, M = 3.2×10^{-5}, P = 0.9×10^{-7}.
Human erythrocytes; A = 1.1×10^{-2}, M = 1.3×10^{-5}, P = 0.2×10^{-7}.
Rat brains; A = 0.5×10^{-2}, M = 1.0×10^{-5}, P = 0.1×10^{-7}.

2/ N = 3.

Figure 4 : Progressive inhibition of AChE activity from spruce budworm larvae, when exposed to acephate (2.3×10^{-2}M), methamidophos (3.3×10^{-5}M), and paraoxon (0.9×10^{-7}M) *in vitro*.

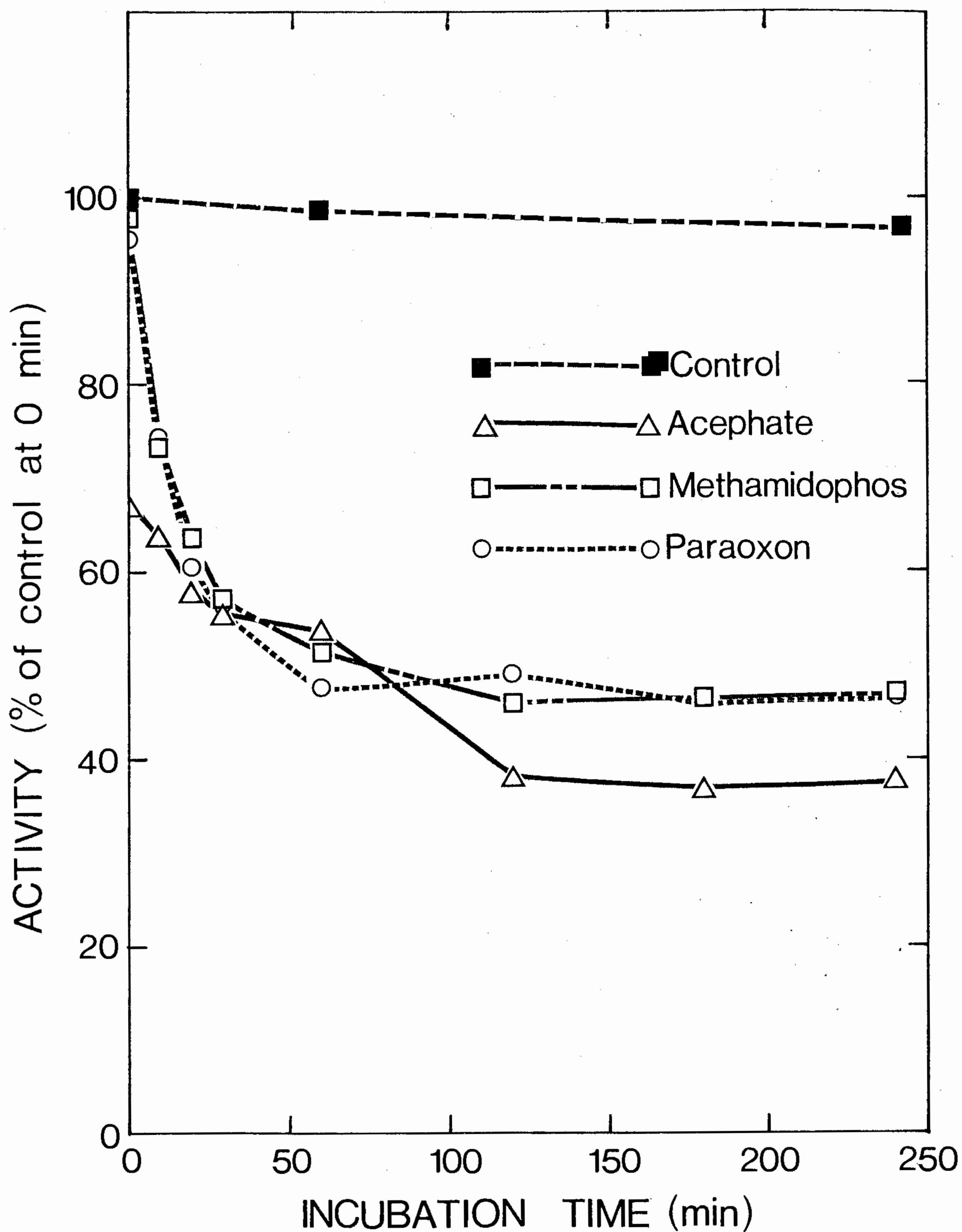
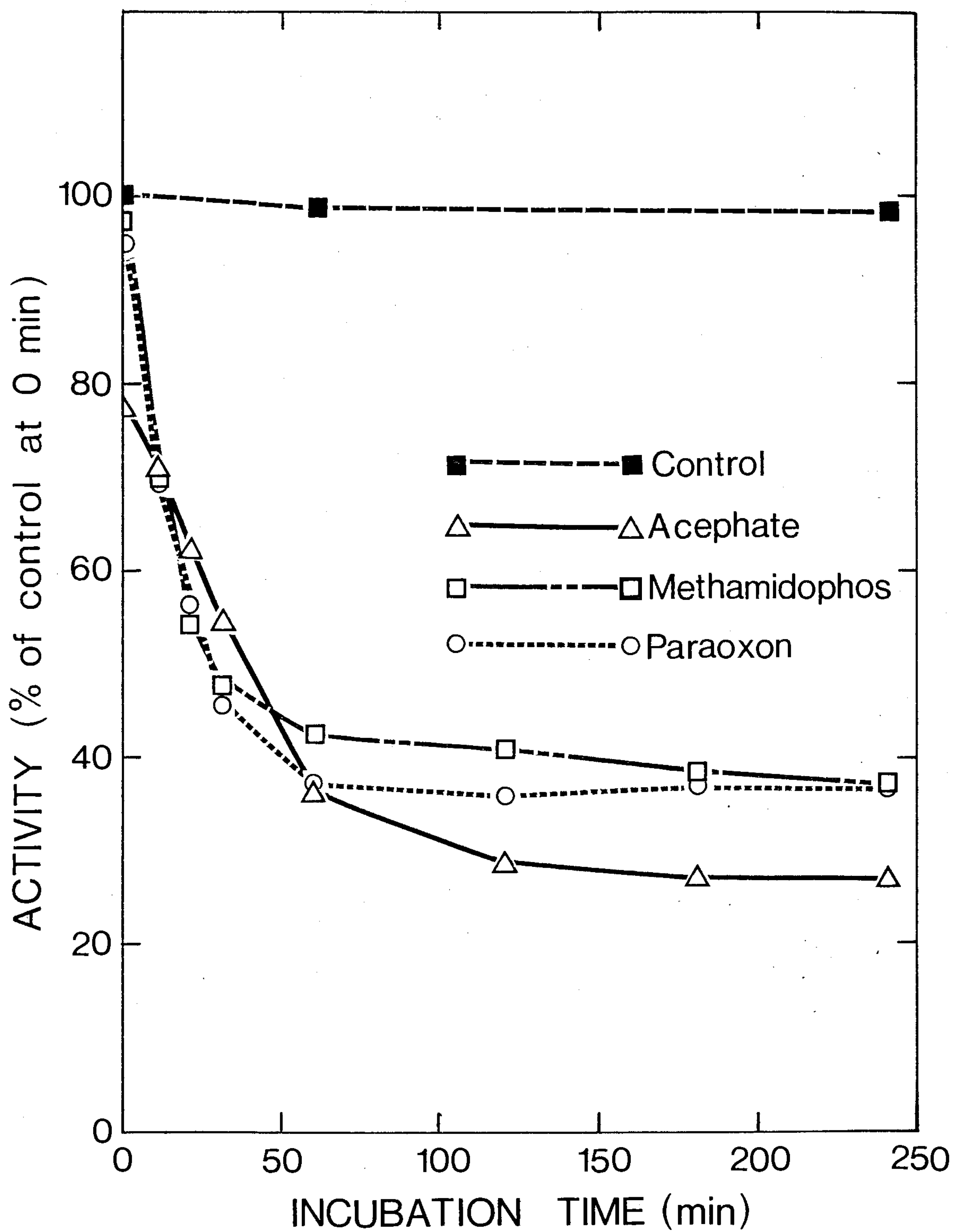

100
80
60
40
20
0
0
50
100
150
200
250
ACTIVITY (% of control at 0 min)
INCUBATION TIME (min)
Control
Acephate
Methamidophos
Paraoxon

Figure 5 : Progressive inhibition of AChE activity from flour moth larvae, when exposed to acephate (0.9×10^{-2}M), methamidophos (3.2×10^{-5}M), and paraoxon (0.9×10^{-7}M) *in vitro*.



100
80
60
40
20
0
0
50
100
150
200
250
ACTIVITY (% of control at 0 min)
INCUBATION TIME (min)
Control
Acephate
Methamidophos
Paraoxon

Figure 6 : Progressive inhibition of AChE activity from human erythrocytes, when exposed to acephate (1.1×10^{-2}M), methamidophos (1.3×10^{-5}M), and paraoxon (0.2×10^{-7}M) *in vitro*.

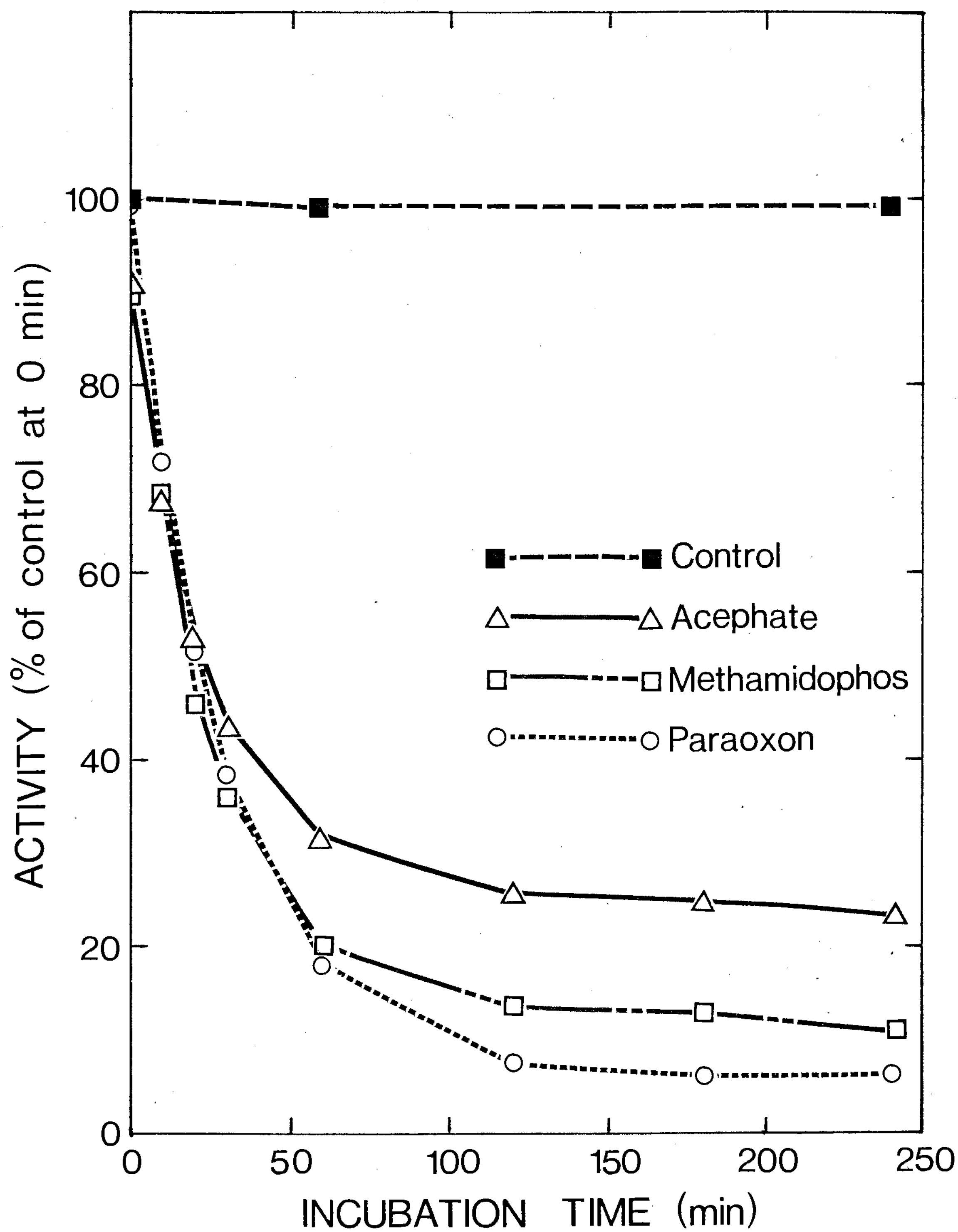
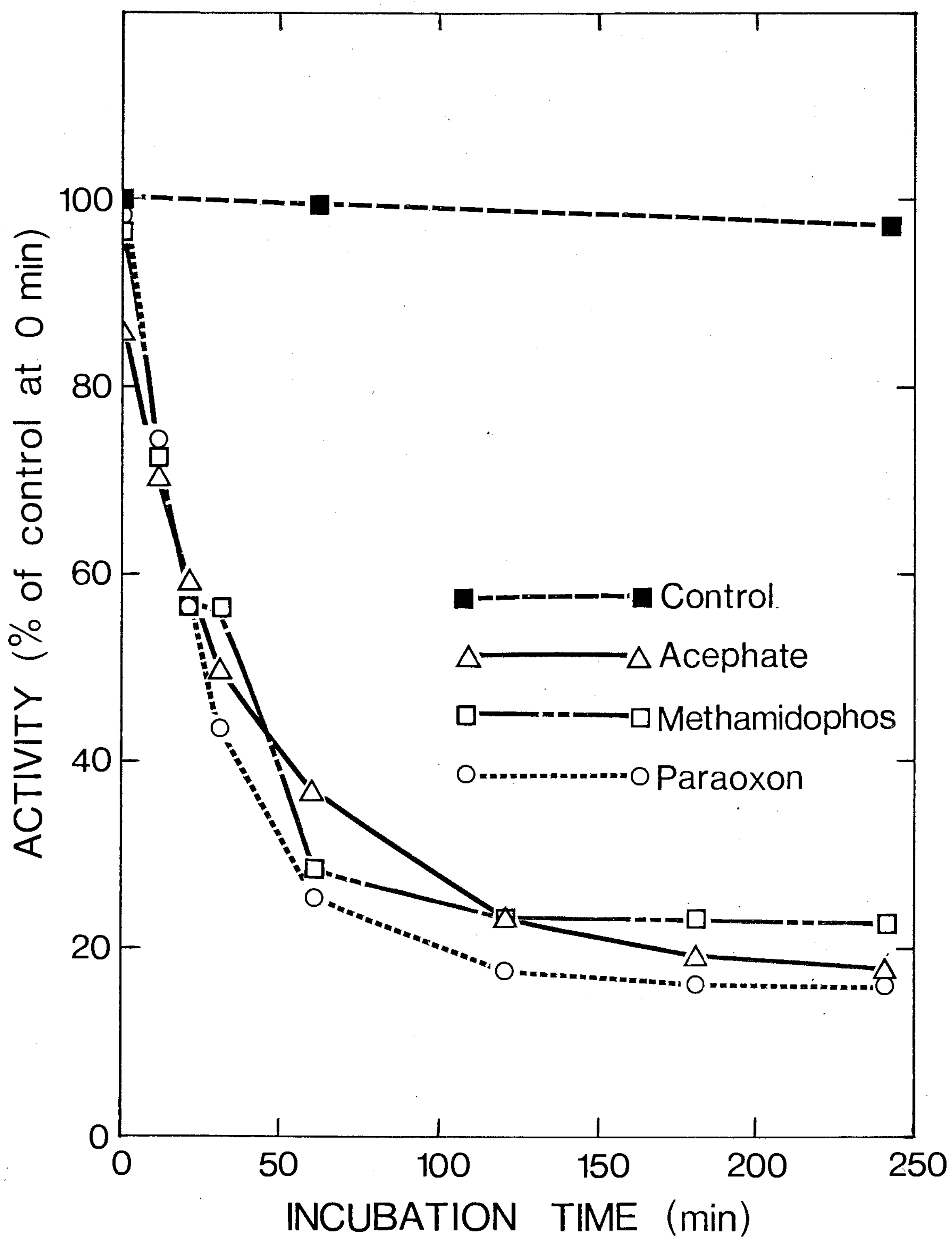

ACTIVITY (% of control at 0 min)
INCUBATION TIME (min)
Control
Acephate
Methamidophos
Paraoxon
0
20
40
60
80
100
0
50
100
150
200
250

Figure 7 : Progressive inhibition of AChE activity from rat brains, when exposed to acephate (0.5×10^{-2}M), methamidophos (1.0×10^{-5}M), and paraoxon (0.1×10^{-7}M) *in vitro*.


ACTIVITY (% of control at 0 min)
100
80
60
40
20
0
0
50
100
150
200
250
INCUBATION TIME (min)
Control
Acephate
Methamidophos
Paraoxon

even more distinguishing.

In general, inhibition of AChE progressed rapidly for 20 to 30 min, slowly from 30 to 60 min, and negligibly, or not at all, thereafter (Figure 4 to 7). This may be interpreted to represent two phases. According to Aldridge and Davison (1952), the initial rapid inhibition in the reaction of OPs with AChE is the reversible step of the reaction mechanism, which is followed by the irreversible step of phosphorylation of the enzyme.

4.4.1.3. Interaction

The combined effects of acephate and methamidophos are shown in Table IX. There were no synergistic effects; in fact, the total inhibition remained between 6.7 and 15.2% below an expected additive impact.

When two biologically active xenobiotics are simultaneously present in living systems, they can interact, causing effects that are synergistic. O'Brien (1966) refers to "synergism" in cases in which two compounds given to an organism, produce an effect which is greater than an additive effect. Apart from the "metabolic" mechanism as the cause of synergism, Karczmar et al. (1963) postulated that other factors may be involved, such as a joint effect upon the neuroeffector in which one compound "sensitizes" AChE to the other. Results obtained in the present study did not show any synergistic effect on the AChE of acephate plus methamidophos, and could, therefore, be considered

Table IX : *In-vitro* inhibition of AChE by acephate (A), methamidophos (M), or acephate *plus* methamidophos (AM).

Source of AChE	Inhibitor	Inhibitor concentration (Molar)	% inhibition ($\bar{X} \pm$ S.D.)[1/]
Spruce budworm larvae	A	1.85×10^{-2}	26.47 ± 2.45
	M	17.00×10^{-5}	21.57 ± 2.45
	AM	2/	37.09 ± 3.75
Flour moth larvae	A	0.70×10^{-2}	22.32 ± 1.50
	M	2.40×10^{-5}	24.40 ± 2.66
	AM	2/	39.99 ± 2.09
Human erythrocytes	A	0.88×10^{-2}	24.66 ± 3.29
	M	1.10×10^{-5}	26.02 ± 2.94
	AM	2/	42.28 ± 1.41
Rat brains	A	0.41×10^{-2}	27.14 ± 1.43
	M	0.80×10^{-5}	19.05 ± 1.65
	AM	2/	30.95 ± 6.60

1/ N = 3

2/ Concentration of each is the same as the concentration when used individually.

only as additive.

4.4.2. In-vivo Acetylcholinesterase Inhibition

OPs inhibit the AChE of vertebrates and insects (O'Brien 1961). The extent of *in-vivo* inhibition of AChE has been correlated with the symptoms of poisoning by OPs. Houseflies and rats treated with parathion and malathion, have AChE inhibition which was closely correlated with the observed symptoms of poisoning (Plapp and Bigley 1961; Seume *et al.* 1960).

Acephate is a weak inhibitor of AChE *in vitro*, but because it has a high insecticidal activity, its effects upon AChE *in vivo* following treatment were examined. Spruce budworm and flour moth larvae were treated topically with acephate at 8 and 15 µg/g, respectively, and rats were treated by direct stomach feeding at 270 mg/kg. These concentrations were approximately equivalent to 30% of the LD_{50} of each.

The activities of AChE up to 72 h following the treatments are listed in Tables X and XI for the spruce budworm and flour moth larvae, respectively, and in Table XII for the ChE activity of brains, erythrocytes, and plasma of rats. The graphical presentation in Figures 8 and 9 shows the pattern of inhibition over 72 h. Treatments with acephate at the respective concentrations partially inhibited the AChE activities in these organisms. In the two insect species, the patterns of inhibition were very similar. The inhibition took place rather quickly,

Table X : Activity of AChE in spruce budworm larvae from 1 to 72 h after topical treatment with acephate at 8 μg/g of body weight.

Exposure time (h)	Protein content (mg/mL) ($\bar{X} \pm$ S.D.)[2/]	Substrate hydrolyzed[1/] ($\bar{X} \pm$ S.D.)[2/]	Percent of control
Control	20.04 ± 3.18	0.67 ± 0.14	100.00
1	22.00 ± 0.87	0.66 ± 0.13	98.15
2	20.17 ± 1.46	0.60 ± 0.06	86.55
4	21.25 ± 1.77	0.48 ± 0.08	71.64
8	21.04 ± 1.48	0.50 ± 0.07	74.63
12	20.58 ± 1.81	0.48 ± 0.03	71.64
18	20.67 ± 1.92	0.50 ± 0.09	74.63
24	20.61 ± 1.82	0.52 ± 0.05	77.61
30	21.25 ± 3.19	0.51 ± 0.06	76.12
36	21.52 ± 1.89	0.49 ± 0.01	73.13
48	21.88 ± 2.42	0.48 ± 0.07	71.64
60	21.72 ± 2.10	0.53 ± 0.03	79.10
72	21.87 ± 1.92	0.52 ± 0.08	77.61

1/ nmoles of substrate/mg of protein/min.

2/ N = 3.

Table XI : Activity of AChE in flour moth larvae from 1 to 72 h after topical treatment with acephate at 15 μg/g of body weight.

Exposure time (h)	Protein content (mg/mL) ($\bar{X} \pm$ S.D.)[2/]	Substrate hydrolyzed[1/] ($\bar{X} \pm$ S.D.)[2/]	Percent of control
Control	11.25 ± 1.05	1.90 ± 0.13	100.00
1	11.87 ± 0.23	1.67 ± 0.05	87.89
2	11.53 ± 0.87	1.59 ± 0.11	83.68
4	11.23 ± 0.67	1.19 ± 0.24	62.63
8	11.20 ± 1.06	1.14 ± 0.27	60.00
12	10.10 ± 1.25	1.15 ± 0.27	60.53
18	11.47 ± 0.93	1.05 ± 0.27	55.26
24	10.70 ± 0.92	0.96 ± 0.28	50.53
30	11.43 ± 1.42	1.12 ± 0.28	58.95
36	10.77 ± 0.35	1.07 ± 0.27	56.32
48	10.97 ± 1.76	1.09 ± 0.27	57.37
60	10.63 ± 1.12	0.86 ± 0.29	45.26
72	11.17 ± 1.20	1.04 ± 0.28	54.74

1/ nmoles of substrate/mg of protein/min.

2/ N = 3.

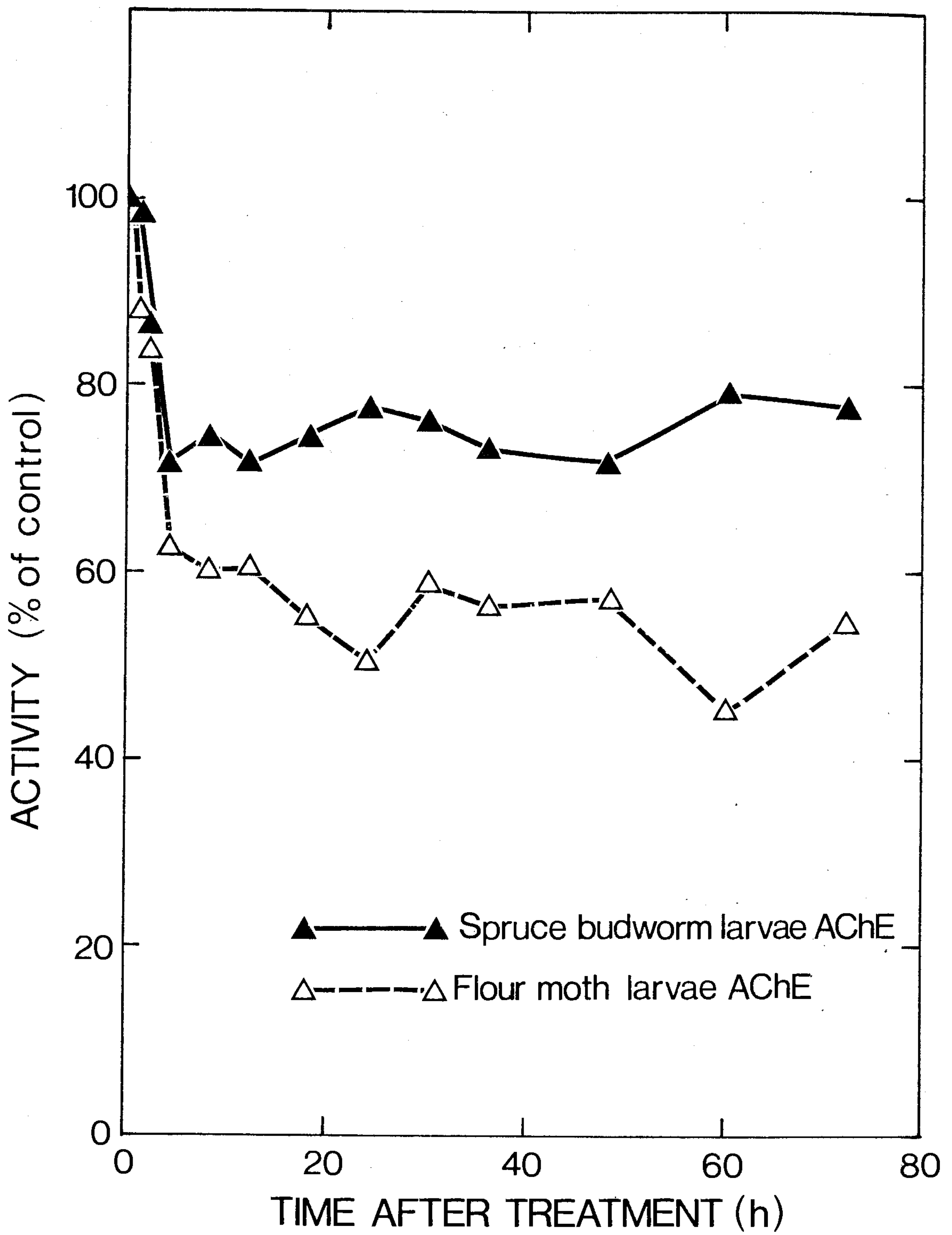
Table XII : ChE activities of erythrocytes, plasma, and brains of rats 0.5 to 72 h after application of acephate at 270 mg/kg of body weight by direct stomach feeding[1/].

Source of ChE	Exposure time (h)	Substrate hydrolyzed[2/]		Percent of control
		Control group ($\bar{X} \pm$ S.D.)	Treated group ($\bar{X} \pm$ S.D.)	
Erythrocytes	0.5	970.6 ± 124.8	514.7 ± 127.4	53.03
	8	1235.3 ± 249.6	441.2 ± 76.4	35.71
	24	926.5 ± 124.8	529.4 ± 44.1	57.14
	72	947.7 ± 95.3	882.4 ± 116.7	93.11
Plasma	0.5	86.0 ± 9.4	70.6 ± 10.1	82.05
	8	79.4 ± 0	31.3 ± 1.5	39.44
	24	76.1 ± 4.7	36.2 ± 5.4	47.55
	72	86.7 ± 14.0	65.3 ± 25.0	78.77
Brains	0.5	18.6 ± 1.8	7.8 ± 1.2	41.93
	8	18.8 ± 0.8	8.7 ± 1.2	46.05
	24	19.3 ± 0.8	9.3 ± 0.2	48.18
	72	19.0 ± 0.4	17.0 ± 0.1	89.48

1/ N = 2 for the control; N = 3 for the treatment.

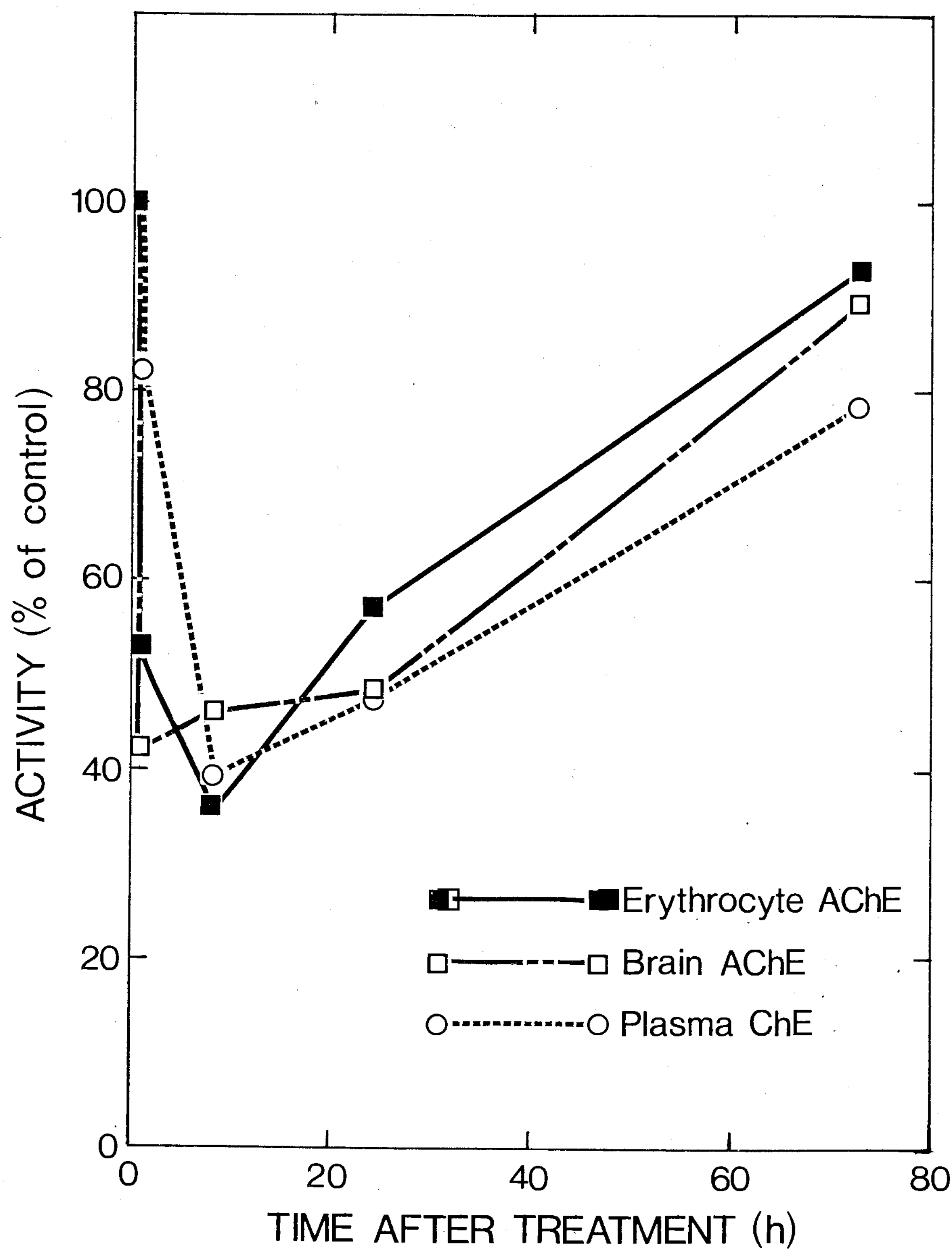
2/ nmoles of substrate hydrolyzed/mL/min for erythrocytes and plasma;
nmoles of substrate hydrolyzed/mg of protein/min for brains.

Figure 8 : AChE activities in spruce budworm and flour moth larvae after topical treatments with acephate at 8 and 15 μg/g of body weight, respectively.



100
80
60
40
20
0
0
20
40
60
80
ACTIVITY (% of control)
TIME AFTER TREATMENT (h)
Spruce budworm larvae AChE
Flour moth larvae AChE

Figure 9 : ChE activities of erythrocytes, brains, and plasma of rats after treatment with acephate at 270 mg/kg of body weight by direct stomach feeding.



100
80
60
40
20
0
0
20
40
60
80
ACTIVITY (% of control)
TIME AFTER TREATMENT (h)
Erythrocyte AChE
Brain AChE
Plasma ChE

with a drastic reduction in AChE activity within 4 h after treatment, decreasing the enzyme activity to approximately 72 and 63%, respectively, for the spruce budworm and flour moth larvae. Thereafter, from 4 to 72 h, AChE activities fluctuated between 70 and 80% for spruce budworm and between approximately 50 to 60% for flour moth. The greater depression in the flour moth evidently reflecting the higher dose applied to this insect. Recovery of AChE in the insects did not occur.

In rats, the inhibition patterns of all three enzymes were quite similar. Activities were reduced strongly a half-hour after the treatment of the rats, reaching maximal inhibition of brain AChE at that time, while activities of the other two enzymes declined further, with maximal inhibitions 8 h after the treatment. After reaching their respective maximal inhibition levels, the activities of all three enzymes began to recover gradually. At the end of the experiment, 72 h after treatments, the AChE activities had returned to 90 and 93%, whereas recovery of plasma ChE had progressed to 79% (Table XII, Figure 9).

Approximately one-third of the median lethal dose (LD_{50}) of acephate was applied to each of the two insect species (8 and 15 mg/kg) and the rats (270 mg/kg); yet, in the mammal, brain and erythrocyte AChE, as well as blood plasma ChE, were initially depressed much more severely than the AChE in the two lepidopterans. But in the latter no recovery of the AChE could be detected, whereas enzyme activities improved remarkably in the rats, begining about 8 h after treatment (Figures 8 and 9).

Again, these are differences indicative of a defence mechanism against acephate insults possessed by mammals, but not insects.

Recovery from the consequences of OP inhibition *in vivo* depends on *de-novo* synthesis of the irreversibly inhibited enzymes or on gradual de-phosphorylation, analogous to the extremely rapid de-acetylation step in ACh hydrolysis. The latter step can be accelerated by certain nucleophilic reagents which have been widely recommended as OP antidotes, although their use in humans has been controversial (Barckow *et al.* 1969).

The pronounced recovery of the severely inhibited rat enzymes (Figure 9) indicates that rapid but reversible inhibition by enzyme phosphorylation is followed by gradual de-phosphorylation. On the other hand, the insects' AChE, although not depressed as severely by acephate at one-third of the LD_{50}, was inhibited irreversibly and activity did not recover. In insects therefore, acephate either leads to irreversible AChE inhibition through ageing, or rates of de-phosphorylation are too slow to become manifest within 70 h. This difference between the rat and the two lepidopterans, may well be important in acephate's selectivity, especially if it were augmented by significant formation of methamidophos in insects but not in mammals.

It is also possible, but very unlikely, that inhibition is irreversible in both the insects and rats but that rats synthesize new enzymes at very high rates whereas insects do

not. There is no evidence known to support this possibility.

Reports by others are contradictory in this regard. Recovery of vertebrate AChE from phosphorylation is common (Hollingworth 1976). Mouse brain AChE inhibited by dichlorvos was slowly reversible, accounting for its lower sensitivity to the inhibitor compared to that of the housefly heads (van Asperen and Dekhuijzen 1958).

Recovery of housefly AChE inhibited by several OPs occurred rapidly *in vivo*, after an initial steady decline in the activity (Mengle and O'Brien 1960; O'Brien 1967). Brady and Sternburg (1967) disputed these findings when they failed to record the recovery themselves. Khoo and Sherman (1973) also failed to find significant recovery of insect AChE *in vivo* after OP poisoning. Therefore, it appears quite common that insect AChE inhibited *in vivo* by OPs, fails to recover. Acephate, applied topically to houseflies, caused inhibition of the AChE which did not recover (Rojakovick and March 1972), in agreement with the results obtained in the present study on spruce budworm and flour moth larvae. According to Rojakovick and March (1972), the degree of inhibition seemed to be a function of the amount of acephate applied: the inhibition was faster and greater at higher doses. A drop in AChE activity to 40 to 55% occurred within 5 to 8 h when the houseflies were treated at LD_{90} and LD_{50}, whereas a gradual drop to 75% occurred 12 h after treatment at LD_{25}.

The metabolic conversion of acephate, at least in part, to methamidophos has now been shown. Studies on the inhibition of

AChE by direct use of methamidophos, indicated a high degree of parallelism to the effect shown in the treatment with acephate. Khasawinah *et al*. (1978) reported a pattern of AChE inhibition in houseflies topically treated with methamidophos, parallel to that when the flies were treated with acephate, as reported by Rojakovick and March (1972). There was little or no recovery of the inhibited AChE with time in the flies treated with methamidophos, and the degree of inhibition was relatively dependent on the dosage level. The AChE inhibition was slowly progressive with time, reaching the maximum level at 64, 16, and 16% of normal for the head AChE, and at 56, 12, and 2% for the thoraxic AChE, respectively, when the treatment was with LD_{25}, LD_{50}, and LD_{90}. The time of maximum inhibitions occurred after about 3 and 25 h, respectively, for the head and thoraxic AChE, at LD_{25}, and after 8 h for both enzyme preparations at LD_{50} and LD_{90}. Discrepancies between some of the results of work done with insects by others and the results shown in Tables X, XI, XII and Figures 8 and 9, are most likely owing to work with OP's other than acephate or with species other than *C. occidentalis* and *A. kuehniella*.

Treatments of rats with methamidophos (Robinson and Beiergrohslein 1980) produced about the same pattern of ChE inhibition as that obtained in the present study when the rats were treated with acephate. The inhibition of plasma ChE proceeded at a slower rate than did the inhibition of brain and erythrocyte AChE. The rates for the latter two were not

significantly different. Inhibited brain AChE reactivated at a faster rate than the inhibited plasma ChE, achieving 88 and 65% of the control activity, respectively, at 25 h after the treatment.

4.5. Metabolism of Acephate

Data on the metabolism of acephate in spruce budworm larvae treated topically at 3.5 and 8 μg/g are listed in Tables XIII and XIV, respectively. There was no mortality of the larvae when they were sampled at each interval. Methamidophos was detected one hour after the treatment and it remained detectable until 72 h later. Figures 10 and 11 present these data graphically, showing the distribution of acephate and methamidophos with time. The percentage residue recovered was calculated from the initial concentration of acephate, which was considered to be 100%. Following the treatment with the lower dose of acephate, there was a rapid decline in residue concentrations for about 12 h. The decline was intermediate from 12 to 24 h (Figure 10) and 12 to 48 h (Figure 11), followed by a gradual phase until 72 h, by which time the loss was almost 100%. Correlating with the initial rapid decline of acephate, there was a small build-up of methamidophos, which reached a peak of about 5% of the initial acephate concentration within 1 h after treatment (Table XIII). Then the methamidophos residue continued a gradual decline to trace amounts at 72 h. Larvae treated at the higher dose showed

Table XIII : Residues of acephate and methamidophos in spruce budworm larvae treated topically with acephate at 3.5 μg/g of body weight.

Time after treatment (h)	Acephate		Methamidophos		
	ppm ± S.D.	%[1/]	ppm ± S.D.	%[1/]	%[2/]
0	3.28 ± 0.41	100.00	N.D.[3/]	-	-
1	2.80 ± 0.01	85.37	0.16 ± 0.02	4.88	5.71
2	2.09 ± 0.01	63.72	0.13 ± 0.02	3.96	6.22
4	1.88 ± 0.15	57.32	0.12 ± 0.04	3.66	6.38
8	1.08 ± 0.08	32.93	0.11 ± 0.01	3.35	10.19
12	0.49 ± 0.20	14.94	0.06 ± 0.01	1.83	12.24
18	0.43 ± 0.13	13.11	0.06 ± 0.01	1.83	13.95
24	0.11 ± 0.01	3.35	0.07 ± 0.03	2.13	63.64
30	0.12 ± 0.05	3.66	0.01 ± 0.34	0.30	8.33
48	0.14 ± 0.05	4.27	0.01 ± 0	0.30	7.14
60	0.04 ± 0.01	1.22	0.01 ± 0	0.30	25.00
72	0.01 ± 0.30	0.30	Trace[4/]	-	-

1/ Percent of initial concentration of acephate (0 h).

2/ As percent of acephate concentration at the time after treatment.

3/ Non-detectable.

4/ < 4 ppb for acephate; < 2 ppb for methamidophos.

Table XIV : Residues of acephate and methamidophos in spruce budworm larvae treated topically with acephate at 8 μg/g of body weight.

Time after treatment (h)	Acephate		Methamidophos		
	ppm ± S.D.	%[1]	ppm ± S.D.	%[1]	%[2]
0	7.87 ± 1.23	100.00	N.D.[3]	-	-
1	7.28 ± 1.53	92.50	0.15 ± 0.05	1.91	2.06
2	6.44 ± 0.28	81.83	0.21 ± 0.04	2.67	3.26
4	6.23 ± 0.95	79.16	0.33 ± 0.36	4.19	5.30
8	3.06 ± 1.02	38.88	0.78 ± 0.72	9.91	25.49
12	1.88 ± 0.78	23.89	0.20 ± 0.02	2.54	10.64
18	1.98 ± 0.49	25.16	0.28 ± 0.33	3.56	14.14
24	1.10 ± 0.51	13.98	0.10 ± 0.01	1.27	9.09
30	0.87 ± 0.48	11.05	0.17 ± 0.06	2.16	19.54
48	0.49 ± 0.49	6.23	0.13 ± 0.07	1.65	26.53
60	0.11 ± 0.03	1.11	0.06 ± 0.02	0.76	54.55
72	0.08 ± 0.05	1.02	0.03 ± 0.02	0.38	37.50

1/, 2/ As in Table XIII.

3/ Non-detectable.

Figure 10 : Acephate and methamidophos residues in spruce budworm larvae treated topically with acephate at 3.5 μg/g of body weight, in % of initial concentration of acephate.

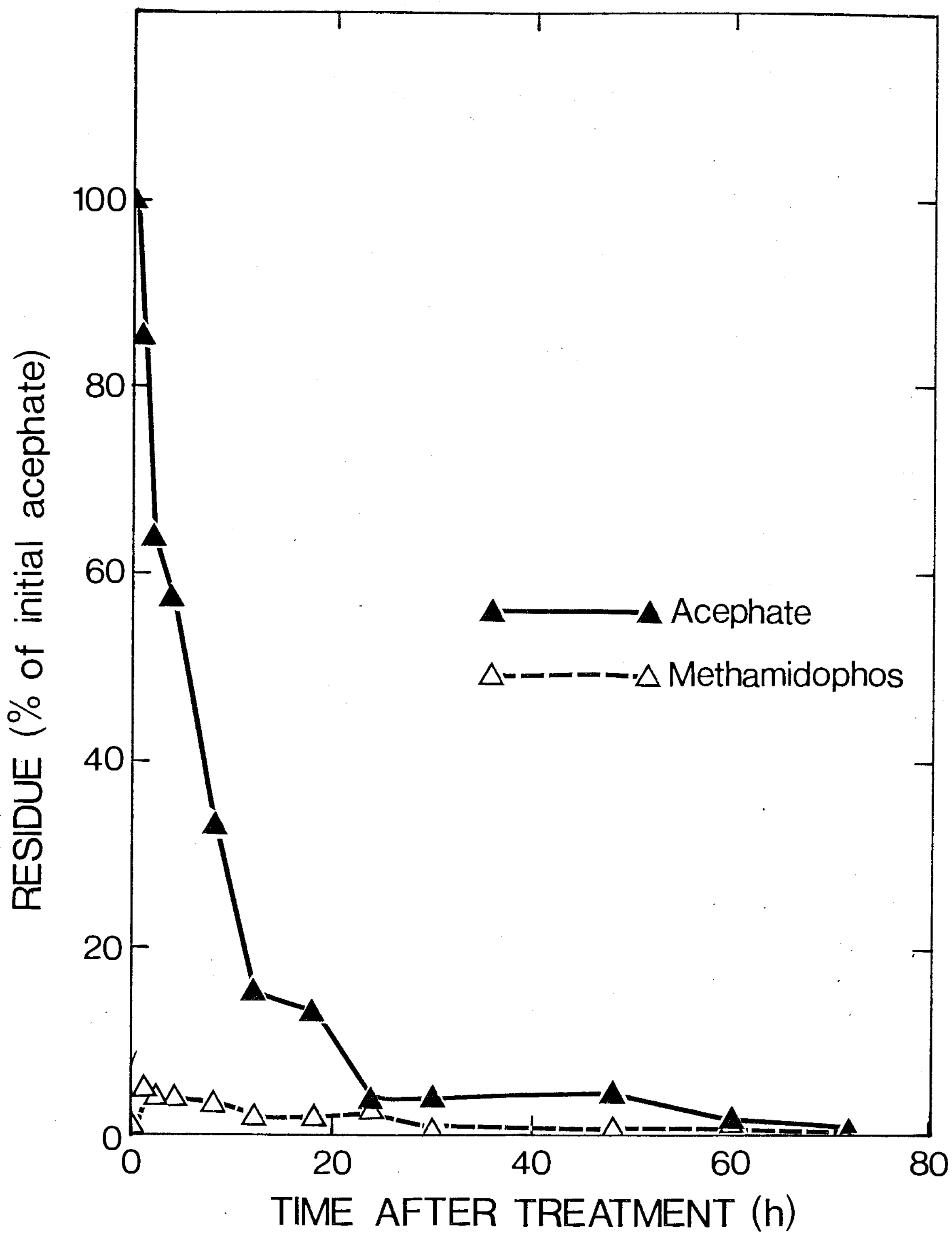
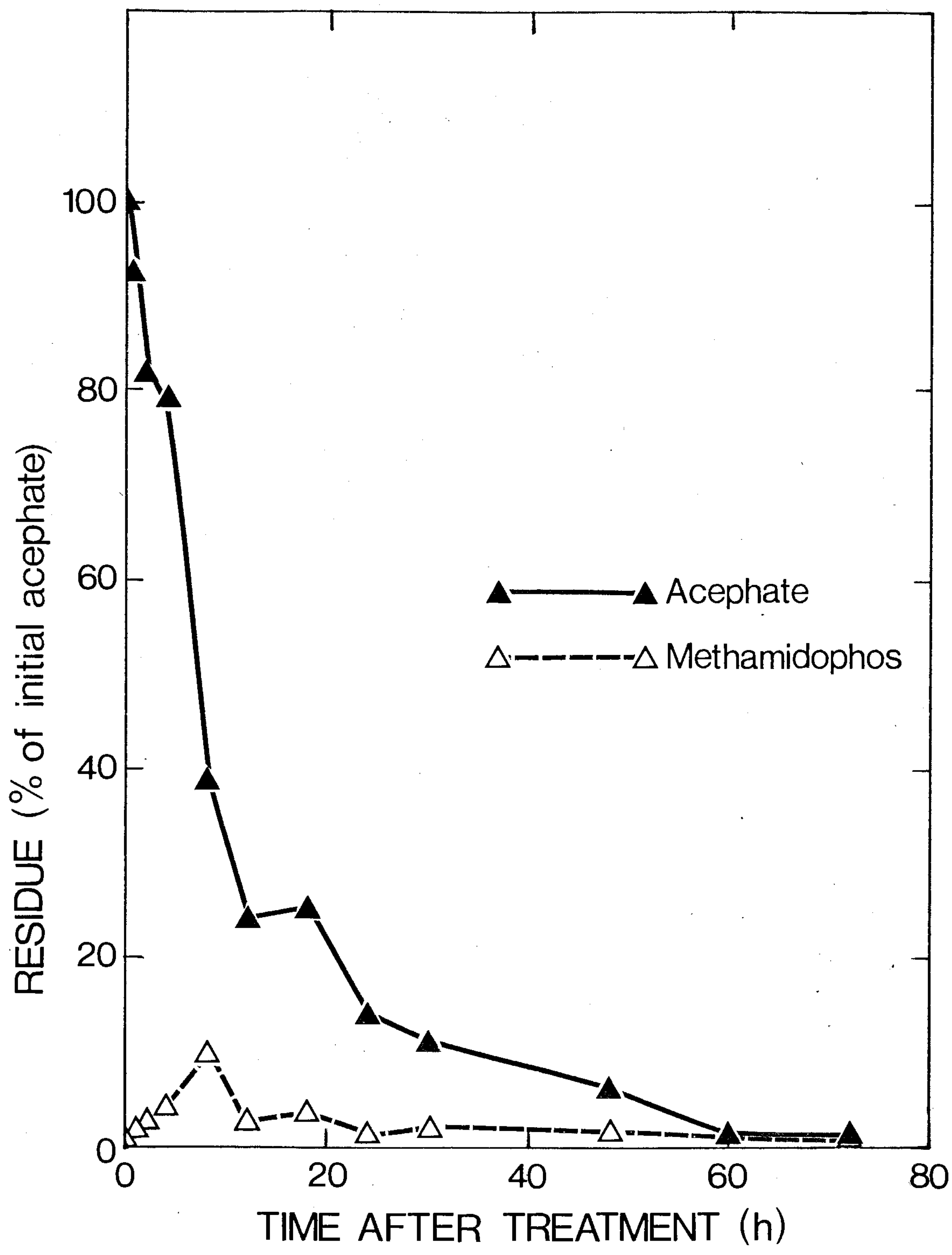

RESIDUE (% of initial acephate)
100
80
60
40
20
0
0
20
40
60
80
TIME AFTER TREATMENT (h)
Acephate
Methamidophos

Figure 11 : Acephate and methamidophos residues in spruce budworm larvae treated topically with acephate at 8 μg/g of body weight, in % of initial concentration of acephate.



RESIDUE (% of initial acephate)
100
80
60
40
20
0
0
20
40
60
80
TIME AFTER TREATMENT (h)
Acephate
Methamidophos

that the pattern of decline of acephate was similar to that for the lower dose. The residue declined to about 39, 14, and 1%, respectively at 8, 24, and 72h after the treatment. The amount of methamidophos detected 1 h after the treatment was about the same as that obtained with the lower dose (0.16 vs. 0.15 ppm), even though the acephate residue was > 2.5 times higher. At this time the methamidophos level was about 2% of the initial higher acephate concentration but almost 5% of the lower (Tables XIII and XIV). The methamidophos residue increased to reach a peak of about 10% of the initial acephate concentration after 8 h, before declining to about 0.4% after 72 h.

Tables XV and XVI present the residues found in flour moth larvae treated with 5 and 15 μg/g, and sampled after the same time intervals. No deaths were observed. The results are graphically depicted in Figures 12 and 13, which show the distribution of acephate and methamidophos residues over 72 h. The decline in acephate residues can be divided into three phases, but the rates were different between the two treatments. At the lower dose, the first phase occurred within 12 h after treatment, when the concentration declined to about 32% of the initial level. The second phase lasted until 24 h, with a drop to about 23%, and the third phase until 72 h, with the considerable residue of 15.5% of the initial concentration still detectable. Simultaneously, there was a build-up of methamidophos, detectable within 1 h after treatment and reaching its peak within 12 to 18 h. The methamidophos residue

Table XV : Residues of acephate and methamidophos in flour moth larvae treated topically with acephate at 5 μg/g of body weight.

Time after treatment (h)	Acephate		Methamidophos		
	ppm ± S.D.	%1/	ppm ± S.D.	%1/	%2/
0	3.36 ± 0.15	100.00	N.D.3/	-	-
1	2.29 ± 0.18	68.15	0.18 ± 0.07	5.36	7.86
2	2.11 ± 0.27	62.80	0.16 ± 0.06	4.76	7.58
4	1.77 ± 0.21	52.68	0.22 ± 0.09	6.55	12.43
8	1.32 ± 0.17	39.29	0.21 ± 0.06	6.25	15.91
12	1.09 ± 0.14	32.44	0.27 ± 0.03	8.04	24.77
18	1.01 ± 0.15	30.06	0.27 ± 0.04	8.04	26.73
24	0.77 ± 0.05	22.92	0.23 ± 0.04	6.85	29.87
30	0.69 ± 0.13	20.54	0.19 ± 0.05	5.65	27.54
48	0.56 ± 0.09	16.67	0.13 ± 0.02	3.87	23.21
60	0.51 ± 0.05	15.18	0.10 ± 0.02	2.98	19.61
72	0.52 ± 0.06	15.48	0.13 ± 0.01	3.87	25.40

1/ Percent of initial concentration of acephate (0 h).

2/ As percent of acephate concentration at the time after treatment.

3/ Non-detectable.

Table XVI : Residues of acephate and methamidophos in flour moth larvae treated topically with acephate at 15 μg/g of body weight.

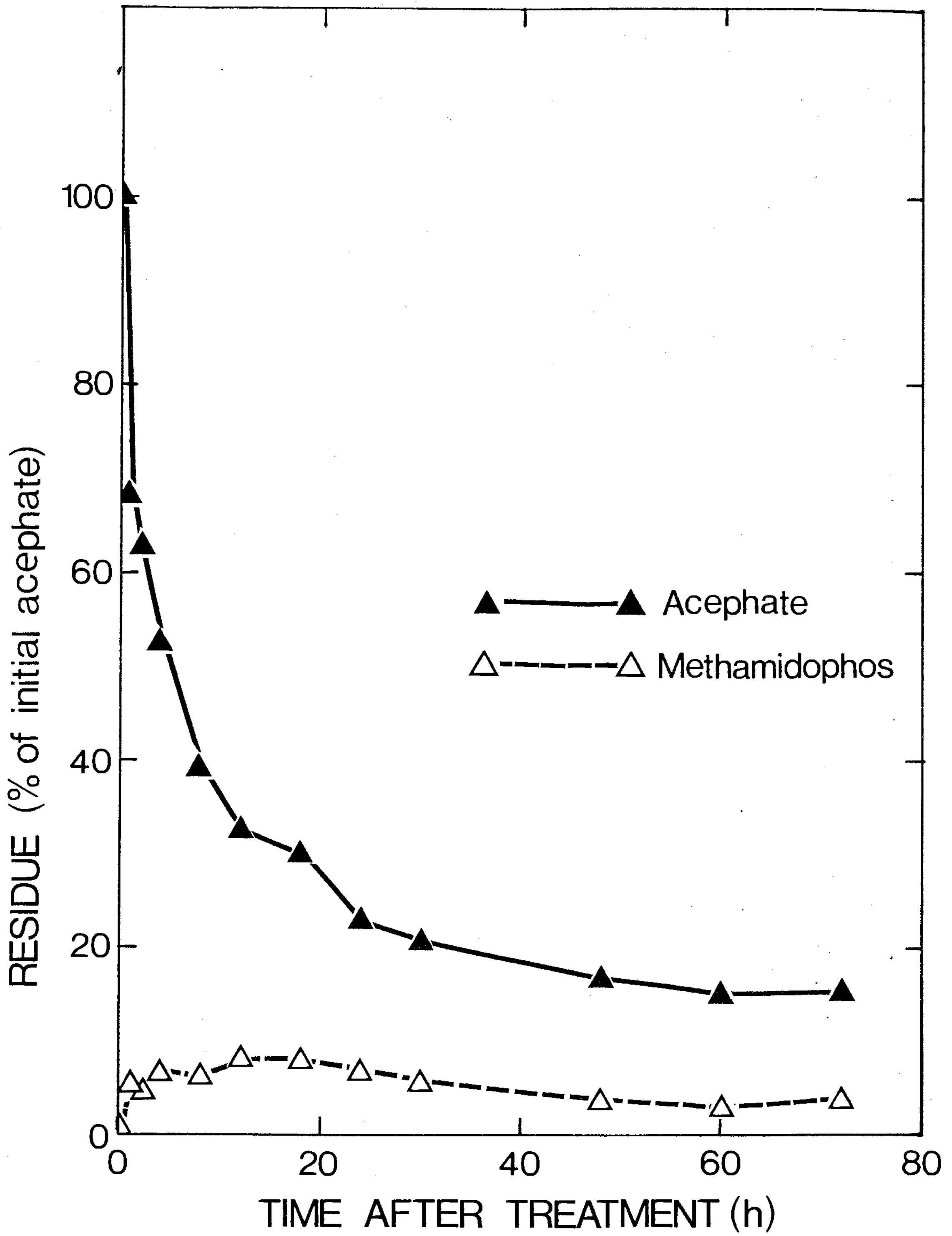
Time after treatment (h)	Acephate		Methamidophos		
	ppm ± S.D.	%1/	ppm ± S.D.	%1/	%2/
0	10.92 ± 1.19	100.00	N.D.3/	-	-
1	6.50 ± 0.38	59.52	0.57 ± 0.13	5.22	8.77
2	5.73 ± 0.57	52.47	0.85 ± 0.17	7.78	14.83
4	4.05 ± 0.16	37.09	1.32 ± 0.12	12.09	32.59
8	3.98 ± 0.69	36.45	1.27 ± 0.14	11.63	31.91
12	3.18 ± 0.39	29.12	1.23 ± 0.09	11.26	38.68
18	2.23 ± 0.28	20.42	1.18 ± 0.18	10.81	52.91
24	2.01 ± 0.14	18.41	1.08 ± 0.04	9.89	53.73
30	1.74 ± 0.12	15.93	0.97 ± 0.14	8.88	55.75
48	1.70 ± 0.19	15.57	0.67 ± 0.14	6.14	39.41
60	1.45 ± 0.26	13.28	0.57 ± 0.02	5.22	39.31
72	1.35 ± 0.26	12.36	0.54 ± 0.13	4.95	40.00

1/ Percent of initial concentration of acephate (0 h).

2/ As percent of acephate concentration at the time after treatment.

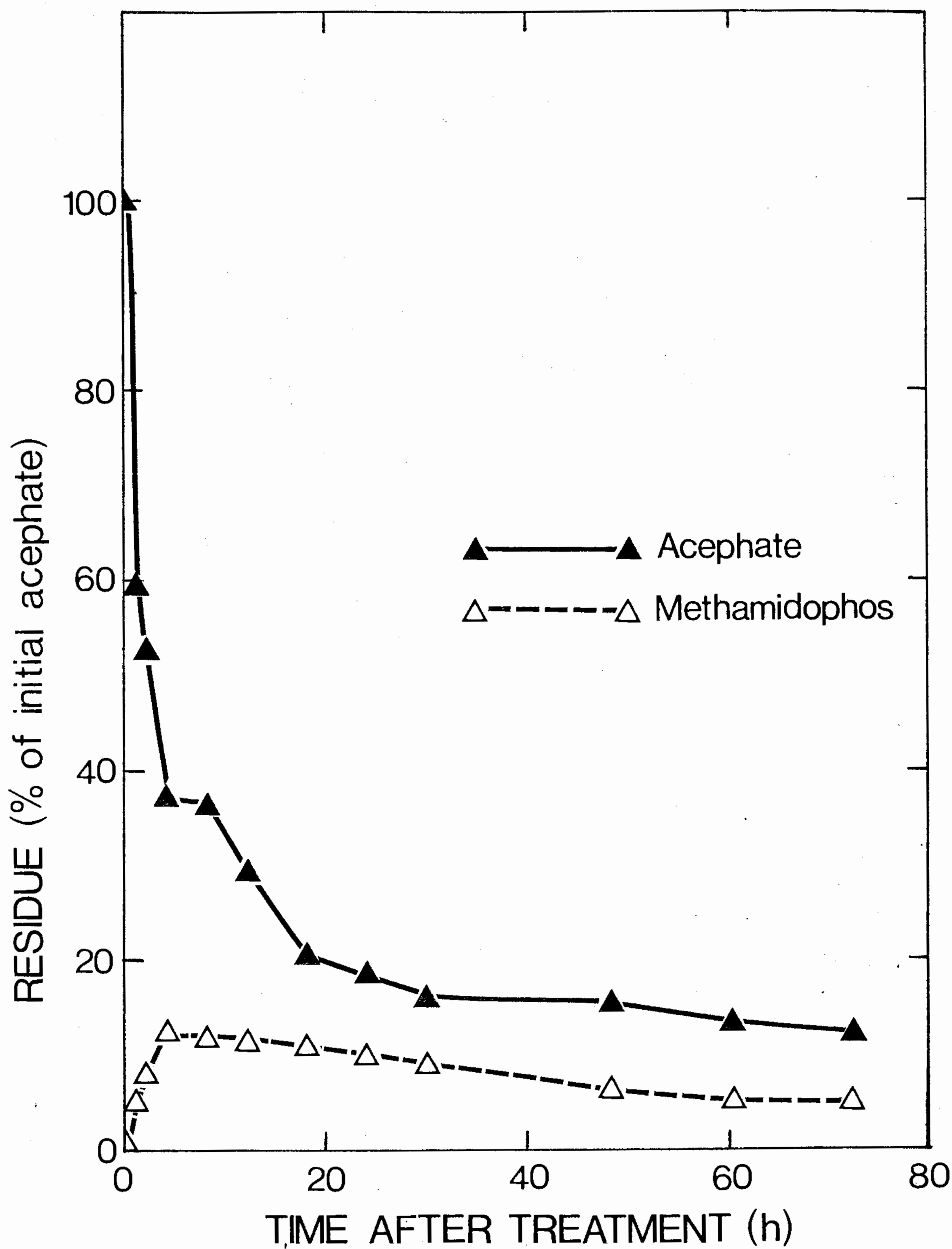
3/ Non-detectable.

Figure 12 : Acephate and methamidophos residues in flour moth larvae treated topically with acephate at 5 µg/g of body weight, in % of initial concentration of acephate.



Acephate
Methamidophos
RESIDUE (% of initial acephate)
TIME AFTER TREATMENT (h)
0
20
40
60
80
100
0
20
40
60
80

Figure 13 : Acephate and methamidophos residues in flour moth larvae treated topically with acephate at 15 μg/g of body weight, in % of initial concentration of acephate.


100
80
60
40
20
0
RESIDUE (% of initial acephate)
0
20
40
60
80
TIME AFTER TREATMENT (h)
Acephate
Methamidophos

at 1 h after the treatment was about 5% of the initial acephate concentration, and at the peak it was about 8%. The gradual decline of the methamidophos concentration began after 18 h, but a substantially high level of nearly 4% still remained at 72 h. In the treatment with the higher dose, the phases of acephate decline were faster. The first phase occurred within 4 h, with a reduction to about 37% of the initial concentration. A drop to 20% in the second phase took place within 18 h, and the third phase declined to about 12% at 72 h. The build-up of the methamidophos residue was faster in the higher than in the lower dose. At 1 h after the high treatment, the concentration of methamidophos was also about 5% of the initial acephate concentration, but at the higher dose methamidophos reached its peak of about 12% within 4 h. The concentration remained > 10% until 18 h after the treatment, which was followed by a very slow declining phase. About 5% still remained at 72 h.

The results obtained for the spruce budworm and flour moth larvae, showed that the pattern of residue loss and metabolite gain and loss were basically the same, but the rates were different. Higher levels of methamidophos were recorded in the flour moth than in the spruce budworm larvae, which correlated with the higher doses of acephate applied. Moreover, the high methamidophos concentrations remained longer in the flour moth larvae, declining only gradually after the peak, whereas in the spruce budworm larvae the loss was faster, so that only very low levels were present after 12 h. This could indicate a less

efficient system in the flour moth larvae than in the spruce budworm larvae in eliminating the chemical. Moreover, after 72 h substantial residues of acephate and methamidophos remained detectable in flour moth larvae, whereas the chemicals were almost completely eliminated from the spruce budworm larvae by this time.

In general, there was a build-up of methamidophos when the rapid first phase of acephate declined. Then the methamidophos either remained high for a while during the second phase of acephate decline or began its own gradual decline until 72 h after treatment.

In summary, distinctively less of the acephate applied to flour moth larvae than of that applied to spruce budworm larvae was metabolized during the 72-h experimental period. While 12.4 and 15.5% of the applied acephate were left unmetabolized in flour moth larvae after 72 h, only 1.0 and 0.3% were recovered from spruce budworm larvae. This may be due to slower percuticular uptake of acephate by flour moth larvae and, therefore, apparently slower metabolism. Such mechanism could also account for the lower toxicity, i.e., for the selectivity, of acephate to flour moth larvae: more acephate remains on the outside of the flour moth larvae and thus unmetabolized.

Acephate, administered to the rats by direct stomach feeding at 270 mg/kg, was quickly transported to the liver, as shown in Table XVII, and graphically in Figure 14. A concentration of acephate (76.11 ppm), equivalent to about 28%

Table XVII : Residues of acephate and methamidophos in livers of rats treated with acephate at 270 mg/kg of body weight by direct stomach feeding.

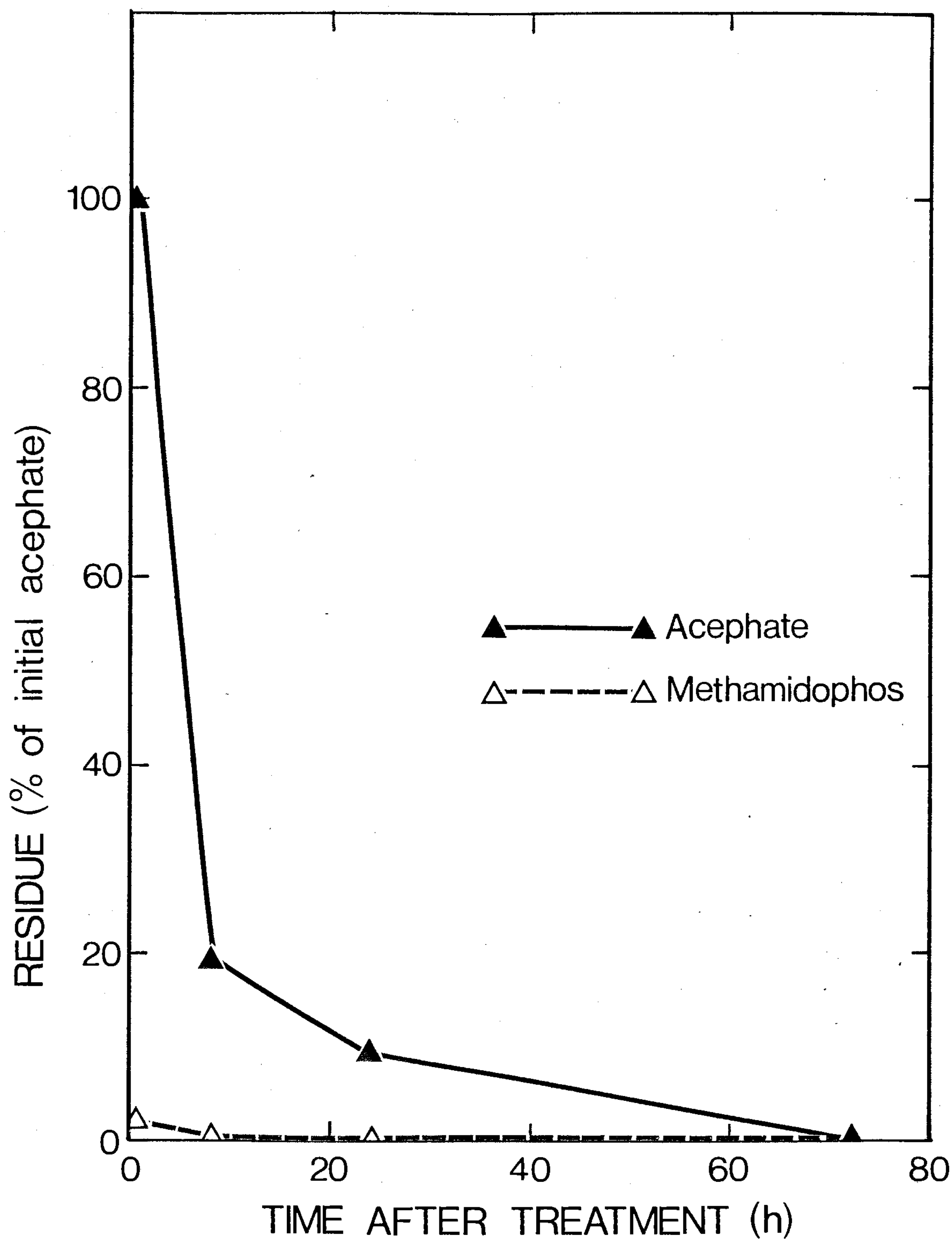
Time after treatment (h)	Acephate		Methamidophos		
	ppm ± S.D.[1/]	%[2/]	ppm ± S.D.[1/]	%[2/]	%[3/]
0.5	76.11 ± 21.42	100.00	1.65 ± 0.49	2.17	2.17
8	14.65 ± 7.10	19.25	0.39 ± 0.22	0.51	2.66
24	7.19 ± 3.19	9.45	0.16 ± 0.08	0.21	2.26
72	0.07 ± 0.02	0.09	0.004± 0.002	0.005	5.71

1/ N = 3.

2/ Percent of concentration of acephate 0.5 h after treatment.

3/ As percent of acephate concentration at the time after treatment.

Figure 14 : Acephate and methamidophos residues in rat livers after treatment of the rats with acephate by direct stomach feeding at 270 mg/kg of body weight, in % of initial concentration of acephate.


RESIDUE (% of initial acephate)
100
80
60
40
20
0
0
20
40
60
80
TIME AFTER TREATMENT (h)
Acephate
Methamidophos

of the applied dose, was detected in the livers within a half-hour after the treatment. Liver samples taken 8 h after treatment indicated a decline to about 19% of the acephate concentration at 0.5 h. The decline continued gradually to about 0.1% at 72 h. Methamidophos was also detected in the liver, but the amounts were comparatively small considering the high concentrations of acephate .

The initial concentration of acephate in the rat livers was higher by 7.5 times than that in the insects, so that a slightly higher level of methamidophos was also detected in the livers at the highest concentration. However, compared with the initial acephate concentration the percent of methamidophos formed was much lower in the rat livers than it was in the spruce budworm and flour moth larvae. The highest in the livers was only 2.17%, compared with 9.91% in spruce budworm and 12.09% in flour moth larvae. It appeared that acephate and methamidophos residues disappeared from the rat liver at much faster rates than they did from the insects. The loss of residues from the livers illustrates the rapid elimination of these from the mammalian body, probably by excretion in urine since they are highly water soluble. Larson (1975) reported rapid elimination by mice of acephate as unchanged parent material and as methamidophos. Most of the applied acephate was virtually excreted before 12 h had elapsed.

4.6. Microsomal Activation

4.6.1. Cytochrome P-450 Contents of Microsomal Preparations

The classical method for preparing microsomal fractions is to centrifuge the mitochondria-free supernatant at about 105,000 g for 1 h. Using this method, the microsomes could be isolated from homogenates of rat livers and of midguts of spruce budworm larvae to make up the microsomal preparations used in this study. Since protein and cytochrome P-450 contents are considered as an index of "activity", at least for those enzymes located in the microsomal fractions of the cells, they were determined (Table XVIII). The main purpose was to test if the microsomal fractions activated insecticides *in vitro* (Section 4.4.1.3), and , therefore, no attempt was made to compare the specific contents of the protein and the cytochrome P-450 in the preparations from the two sources. It was enough to note the consistency of the preparations within each source as each of the preparations will represent a replicate in the activation work done later in the study.

Several workers have reported the isolation of microsomal fractions from the midguts of some other lepidopterous larvae reared on artificial diet. It was interesting to note that the content of cytochrome P-450 based on nmoles/mg protein obtained from the spruce budworm larvae in the present preparations was about the same as that obtained from the southern armyworm by

Table XVIII : Protein and Cytochrome P-450 contents of microsomal preparations from rat livers and midguts of spruce budworm larvae used in aldrin epoxidation and acephate activation studies.

Source of microsomal preparation		Protein (mg/mL)	Cytochrome P-450 nmol/mL	Cytochrome P-450 nmol/mg protein
Rat livers (3 preparations)		10.00	9.56	0.956
		8.00	7.14	0.893
		12.50	9.78	0.782
	$\bar{X} \pm$ S.D.	10.17 ± 2.25	8.83 ± 1.46	0.877 ± 0.088
Midguts of spruce budworm larvae (5 preparations)		2.13	0.27	0.127
		2.03	0.24	0.118
		2.05	0.27	0.132
		1.90	0.23	0.121
		1.95	0.23	0.118
	$\bar{X} \pm$ S.D.	2.01 ± 0.09	0.25 ± 0.02	0.123 ± 0.006

Brattsten and Gunderson (1981), but was 2 or 3 times lower than those obtained from alfalfa looper and cabbage looper larvae, respectively (Farnsworth et al. 1981), and about 2 times higher than that obtained from the variegated cutworm (Yu et al. 1979).

4.6.2. Epoxidation of Aldrin

Epoxidase activity is known to parallel that of other microsomal oxidases of the microsomal fractions in most lepidopterous insects and in rat livers (Brattsten and Wilkinson 1973; Chan et al. 1967; Wong and Terriere 1965). Therefore, aldrin, a model substrate to measure the total enzyme activity of MFO, is often used for comparison. In the present study, aldrin epoxidation would also demonstrate that the reaction took place in the incubation medium used, since a medium of the same nature was to be used in the study of acephate activation later.

The results of in-vitro aldrin epoxidation by microsomal preparations of rat livers and midguts of spruce budworm larvae incubated with the NADPH-generating system are presented in Table XIX. Incubation of up to 60 min did not result in reduced recovery. At least 88% of the added aldrin was recovered, either epoxidized to dieldrin or unchanged. The conversion occurred very rapidly: at least 71% of the total recovery after 15 min of incubation was dieldrin. The conversion was slightly faster in rate, and greater in quantity when aldrin was incubated with rat liver MFO than when it was incubated with MFO from midguts of

Table XIX : *In-vitro* aldrin epoxidation by microsomal preparations at 37°C.

Incubation time (min)	Aldrin added (µg)	Recovery ($\bar{X}$)1/					
		Rat livers			Midguts of spruce budworm larvae		
		Aldrin (µg)	Dieldrin (µg)	%2/	Aldrin (µg)	Dieldrin (µg)	%2/
0	10	9.19	0	0	9.63	0	0
15	10	2.65	7.25	73.23	2.69	6.63	71.14
30	10	2.09	7.50	78.21	1.99	7.50	79.03
60	10	1.69	8.23	82.96	1.99	7.65	79.36
60 3/	10	8.50	1.99	18.97	8.80	0	0

1/ N = 2.

2/ Dieldrin, in % of aldrin + dieldrin.

3/ Incubated with 1 x 10^{-4}M piperonyl butoxide (Section 3.4.1).

spruce budworm larvae. At 15 and 60 min after the incubation, the conversion levels were, respectively, 73.23 and 82.96% of the total recoveries for rat liver MFO, and 71.14 and 79.36% for those incubated with MFO of midguts of spruce budworm larvae. The conversion of aldrin to dieldrin was inhibited when 1 x 10^{-4} M piperonyl butoxide was included in the incubation medium. No dieldrin was detected when piperonyl butoxide was added to the incubation medium containing MFO of midguts of spruce budworm larvae after 60 min. About 19% of the total recovery was detected as dieldrin in the incubation medium containing rat liver MFO after 60 min of incubation with the piperonyl butoxide. This could be due to the following facts: the rate of conversion was very fast in the initial stage of the reaction, and the aldrin was added to the incubation medium a few seconds prior to the addition of piperonyl butoxide; and the concentration of piperonyl butoxide added was not enough to cause a total inhibition of the activity of rat liver MFO. Dahm _et al_. (1962) stated that piperonyl butoxide was one of the most effective insecticide synergists, inhibiting _in-vitro_ microsomal activation of several OPs at 1 x 10^{-3} M. Nevertheless, Shishido _et al_. (1972) found that it only depressed the metabolism of an OP (diazinon) by the microsomes-NADPH enzyme systems prepared from rat liver and American cockroach fat body. However, piperonyl butoxide at 1 x 10^{-4} M was reported to inhibit activation of parathion by microsomes prepared from American cockroach fat body (Nakatsugawa and Dahm 1965).

The results obtained in the present study were comparable with those reported by several workers. Williamson and Schechter (1970) showed that the rate of aldrin epoxidation by MFO obtained from whole larvae of tobacco budworm was rapid for 20 min and reached a maximun after 40 min. Wong and Terriere (1965) obtained similar results with rat liver microsomes. Using diazinon as the substrate, Shishido et al.(1972) found that the rate of the oxidative metabolism was higher with rat liver MFO than with that of American cockroach fat body.

4.6.3. Activation of Acephate

The possibility of oxidative activation by mixed-function oxidases was tested with these enzymes, prepared from rat livers and the midguts of spruce budworm larvae (Sections 3.8.1.1 and 3.8.1.2). Following 60-min incubations of acephate with these oxidases, the activities of AChE from rat brains and spruce budworm larvae were assayed. Results, shown in Table XX, were negative. Neither the insect MFO nor the rat MFO activated acephate.

While acephate alone decreased AChE activities approximately 10%, it inhibited from 13 to 19% when pre-incubated with MFO for one hour (Table XX, A,B,C). However, this apparent, slight increase in activity (Table XX, C *versus* B) cannot be ascribed to acephate activation by MFO. Firstly, inhibited MFO alone, i.e., inhibited MFO without acephate,

Table XX : The inhibition of AChE after incubation of acephate for 60 min with mixed-function oxidases (MFO).

Source of MFO	Treatment	60-min pre-incubation of[1]	AChE assayed with substrate plus:[1]	AChE activity (%) Rat brain ($\bar{X} \pm$ S.D.)	AChE activity (%) Spruce budworm larvae ($\bar{X} \pm$ S.D.)
Rat liver	A	MFO	-	100	100
	B	-	Ac	91.1 ± 1.0	90.7 ± 1.0
	C	MFO + Ac	-	83.1 ± 3.7	81.2 ± 0.1
	D	MFO_I	-	82.3 ± 6.8	85.7 ± 5.0
	E	MFO_I + Ac	-	68.8 ± 3.7	65.7 ± 1.0
	F	-	MFO_I + Ac	68.8 ± 6.1	72.0 ± 4.4
Spruce budworm midgut	A	MFO	-	100	100
	B	-	Ac	91.1 ± 1.0	90.7 ± 1.0
	C	MFO + Ac	-	86.9 ± 3.8	86.6 ± 2.8
	D	MFO_I	-	87.3 ± 2.7	84.3 ± 2.6
	E	MFO_I + Ac	-	78.6 ± 3.8	65.8 ± 4.2
	F	-	MFO_I + Ac	73.4 ± 2.3	73.7 ± 4.0

1/ MFO: Mixed-Function Oxidase (Section 3.8.1). MFO_I: Mixed-Function Oxidase inhibited with 1×10^{-4}M Piperonyl Butoxide (Section 3.8.3.1). Ac: 1.9×10^{-3}M and 5.2×10^{-3}M for Rat Brain and Spruce Budworm AChE, respectively.

reduced AChE activities as much (Table XX, D). Secondly, both pre-incubation of acephate with inhibited MFO, as well as acephate and inhibited MFO present during the assay only (Table XX, E and F), had more severe anti-AChE effects than the other treatments, namely, B, C, and D, Table XX.

If oxidative activation of acephate, as suggested by Rojakovick and March (1972), were to be a major factor, it must either be mediated by a different mechanism in vivo or the MFO, as prepared for these experiments, did not activate acephate in vitro, although effectively metabolizing aldrin (Section 4.6.2).

The concentrations of acephate used were approximately one-quarter and one-tenth of the respective I_{50} values for acephate, but were 12 and 100 times higher than the I_{50} of methamidophos. If MFO-mediated activation of acephate, to anything approaching anti-AChE activities of methamidophos, were to occur, Treatments C, Table XX, should have produced almost complete AChE inhibition.

5. CONCLUSIONS

1. A laboratory colony of the western spruce budworm, *C. occidentalis* Freeman, was established and maintained indefinitely on an artificial diet, without the larvae undergoing the normal second instar diapause. About 39 days were required for a complete generation, from egg to egg. Sanitation was of prime importance to successful rearing. The adults did not feed.

2. The Mediterranean flour moth, *A. kuehniella* (Zeller), was cultured easily in the laboratory on a diet of wheat flour, whole wheat grain, a patented cereal mix, and water. Thirty to 40 days were required for a complete generation. The adults did not feed.

3. Acephate has been found to possess several "acephate-typical" properties which may explain, at least in part, its selective toxicity without the need to invoke selective metabolic activation to more potent AChE blockers, such as methamidophos or some other, perhaps transient molecule.

i. Applied topically, acephate was about twice as toxic to last-instar larvae of western spruce budworm (LD_{50},

23.2 µg/g) as to last-instar larvae of Mediterranean flour moth (LD_{50}, 48.3 µg/g), suggesting some selective activity towards spruce budworm and supporting its efficacy in the field. Together with its high VSR ratio of 41, this was deemed to make acephate safer for field use than methamidophos with a VSR of 2.7.

ii. Relative to methamidophos and paraoxon, acephate inhibits AChE from susceptible species more effectively _in vitro_ than AChE from less susceptible species, i.e., its relative anti-AChE strength reflects its toxicity _in vivo_ and therefore, may be a useful indicator of its selective toxicity. This relative anti-AChE property is exerted by acephate _per se_, i.e., without activation.

iii. Acephate, but not methamidophos and paraoxon, inhibited up to 33% of insect AChE instantly. This acephate-typical phenomenon was not detected with mammalian AChE.

iv. After dosing insects and rats with acephate, AChE and ChE titres initially declined to about 60% in the insects and to < 40% in the rats. After 3 days, activities had recovered to 80 to 90% in rats, but recovery did not occur in the two insect species.

v. The more susceptible spruce budworm metabolized topically applied acephate more rapidly than the flour moth, but corresponding differences in the metabolic formation of methamidophos were not obvious.

vi. MFO-mediated activation of acephate *in vitro* to a more potent anti-AChE agent could not be demonstrated, nor could indirect evidence be found in support of a hypothetical substance that could arise from acephate or methamidophos and be the toxic principle of these two insecticides.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
ACth	Acetylthiocholine iodide
Bth	Butyrylthiocholine iodide
BuChE	Butyrylcholinesterase
ChE	Cholinesterase
CNS	Central nervous system
DTNB	Dithiobisnitrobenzoic acid
MCPBA	*m*-chloroperbenzoic acid
MFO	Microsomal mixed-function oxidase system
OP	Organophosphorus